HIGH-THROUGHPUT SCREENING OF MEMBRANE ADSORBERS FOR BACTERIOPHAGE PURIFICATION: ADDRESSING VARIABILITY IN STRAIN-SPECIFIC PROCESS DEVELOPMENT

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PAGES: xx, 115

Lay Abstract

Bacteriophages are a promising antimicrobial alternative to modern antibiotics as they can rapidly overcome bacterial resistance. However, the propagation of therapeutic phages in gram-negative bacteria results in proinflammatory contaminants, notably endotoxins (lipopolysaccharides), which face strict regulations regarding the level present in therapeutic preparations. Current methods of bacteriophage purification are labor-intensive and produce small quantities of purified phage products. Membrane adsorbers in the context of anion-exchange chromatography pose as a suitable method of generating low endotoxin preparations of therapeutic phage. Unfortunately, the variability in physical properties of bacteriophages has been found to necessitate strain-specific process development. Thus, there is a strong need for a high-throughput screening approach to develop chromatography recipes rapidly. To address this need, a high-throughput screening methodology was developed using a multi-well device to study membrane, bacteriophage, and endotoxin binding in solution. The results of this thesis identified critical factors that need to be controlled to study phage binding, compared the productivities of membranes, and explored the ability of the device to screen and optimize solution conditions for translation to larger-scale purification work.

Abstract

Bacteriophages, a promising antimicrobial alternative to modern antibiotics, can rapidly overcome bacterial resistance. However, the propagation of therapeutic phages in gram-negative bacteria leads to proinflammatory contaminants, notably endotoxins (lipopolysaccharides). To meet strict regulations on endotoxin levels, the widely accepted method for phage purification is based on polyethylene glycol precipitation followed by cesium chloride density gradient ultracentrifugation. This method, while effective, is labor-intensive and produces only small quantities of purified phage products. Moreover, the current studies on using membrane adsorbers in this application have primarily focused on removing endotoxins or recovering phages, without addressing the variability in physical properties of bacteriophages that necessitates strain-specific process development. In contrast, this thesis introduces a novel high-throughput flat sheet membrane screening approach. This approach not only aims to evaluate the effect of varying solution conditions on bacteriophage purification but also provides a comprehensive solution to the limitations of the current method, guiding larger-scale purification work.

Using two clinically derived bacteriophage, this work demonstrates that the multi well device used in this work is appropriate for this application, with little background binding of bacteriophage to the device itself. Bacteriophage binding to the membrane was found to be both sensitive to the dilution of the preparation and contact time with a given membrane. The device was used to evaluate the overall binding productivity of the membranes, finding that the Natrix and Sartobind Q membranes possess superior productivity to that of Mustang Q. Furthermore, this work demonstrated that the method could be used to rapidly screen the effects of varying sodium chloride molarity on both bacteriophage and endotoxin binding, with the effect of greater NaCl molarity on both bacteriophage and endotoxin binding being generally comparable to expectations.

Finally, this work demonstrates that improvements in the translatability of screened conditions to larger-scale techniques (*e.g.*, Syringe Filtration) require a better understanding of the underlying interactions occurring between the biomolecules and membranes.

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List of Tables

List of Figures

List of Symbol and Acronyms

AEX	Anion Exchange
AMR	Antimicrobial Resistance
CFU	Colony forming unit.
CIM	Convective Interaction Media
CsCl	Cesium Chloride
DBC	Dynamic Binding Capacity
DEAE	Diethylaminoethanol
EMA	European Medicines Agency
EU	Endotoxin unit
FDA	Food and Drug Administration
GFP	Green fluorescent protein
HIM	Helium Ion Microscopy
HTS	High-throughput screening
IEP	Iso electric point
IEX	Ion exchange
LAL	Limulus Amebocyte Lysate
LB	Lysogeny broth
LFMC	Lateral flow membrane chromatography
LPS	Lipopolysaccharide
LRV	Log removal or reduction value
MOI	Multiplicity of infection
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PEG	Polyethylene Glycol
PFU	Plaque forming unit.

QA/Q Quaternary Ammonium

qRT – PCR Quantitative Reverse Transcription Polymerase Chain Reaction

SBC Static Binding Capacity

SEM Scanning Electron Microscopy

TEM Transmission Electron Microscopy

WHO World Health Organization

MVM Minute virus of mice

Contents

1 Introduction

1.1 Bacteriophage Therapy

1.1.1 Antibiotic resistance

Antimicrobial resistance (AMR) is the development of resistance to previously effective antibiotic medications in infectious and pathogenic microbes such as bacteria [1]. These opportunistic bacteria pose high threats to immunocompromised individuals in healthcare settings, significantly increasing the risk associated with once rudimentary medical procedures [2]. Most alarming is the rapid spread of AMR microbes globally, causing it to be classified as a "significant public health and economic threat" by the World Health Organization (WHO) [1]. As such, the human and economic cost of antibiotic resistance is enormous, responsible annually for 33,000 deaths and €1.1 billion in additional costs to European Union healthcare systems, while Americans currently face up to $20 billion per year in additional healthcare costs [3], [4]. An estimate by the WHO, predicts that by 2050 AMR will be responsible for 20 million deaths per year, with an associated global economic burden of $ 2.9 trillion [5]. Just as concerning is that few new classes of antibiotics have been discovered, with a significant focus on deriving pharmaceuticals from existing classes because of increasingly lower investment returns for the development of novel antibiotics [6]. As such, greater focus has been placed on developing and applying alternative antimicrobial therapeutics, such as bacteriophages [6], [7].

1.1.2 Introduction to Bacteriophage and Applications in Human Therapy

Bacteriophages or phages are viruses that specifically infect a specific host (or narrow range of host) bacteria, utilizing the host's cellular machinery to replicate [8]. Phages exist in any environment containing appropriate bacterial hosts and occupy a critical niche in biological processes [8]. These infectious particles are usually comprised of a DNA or RNA genome surrounded by a protective protein based capsid, with some possessing an additional membrane layer composed of various assortment of carbohydrates, lipids, and proteins [8]. In the case of phages without a membrane, the functional groups of the capsid proteins determine the net surface charge. Phages generally existing as anionic species, with isoelectric points (IEP) in the range of 3.5 – 7 [9]. Phages exhibit a broad range of morphologies, which includes filamentous phages, icosahedral phages without tails, and icosahedral phages with tails. They also exist in a broad range of sizes, with an individual virion having an average size of 30 – 200 nm, with some species evening reaching sizes of 800 nm [10]. Broadly, phages can be grouped based on their mode of infection, utilizing either a lytic or lysogenic process. A lytic mode of infection utilizes the host cell's protein machinery to generate new infectious particles, weakening the cell in parallel, causing the host to burst, releasing the new virions. The lysogenic cycle involves the integration of the phage genome into the host cell genome, allowing the infectious agent to replicate alongside its host without much metabolic consequence. The phage genes initiate the lytic cycle when specific conditions are met [8]. In both processes, lysis results in the death of the host-bacterium causing the release of new virions alongside cellular components such as macromolecules (host cell proteins, DNA, RNA) and cell wall or membrane debris such as lipopolysaccharides (LPS). An illustration of both processes can be seen in **Figure 1.1.1**. The concentration or titer of phage are typically quantified as plaque forming units (PFU) typically as a unit of volume (PFU/mL) [2].

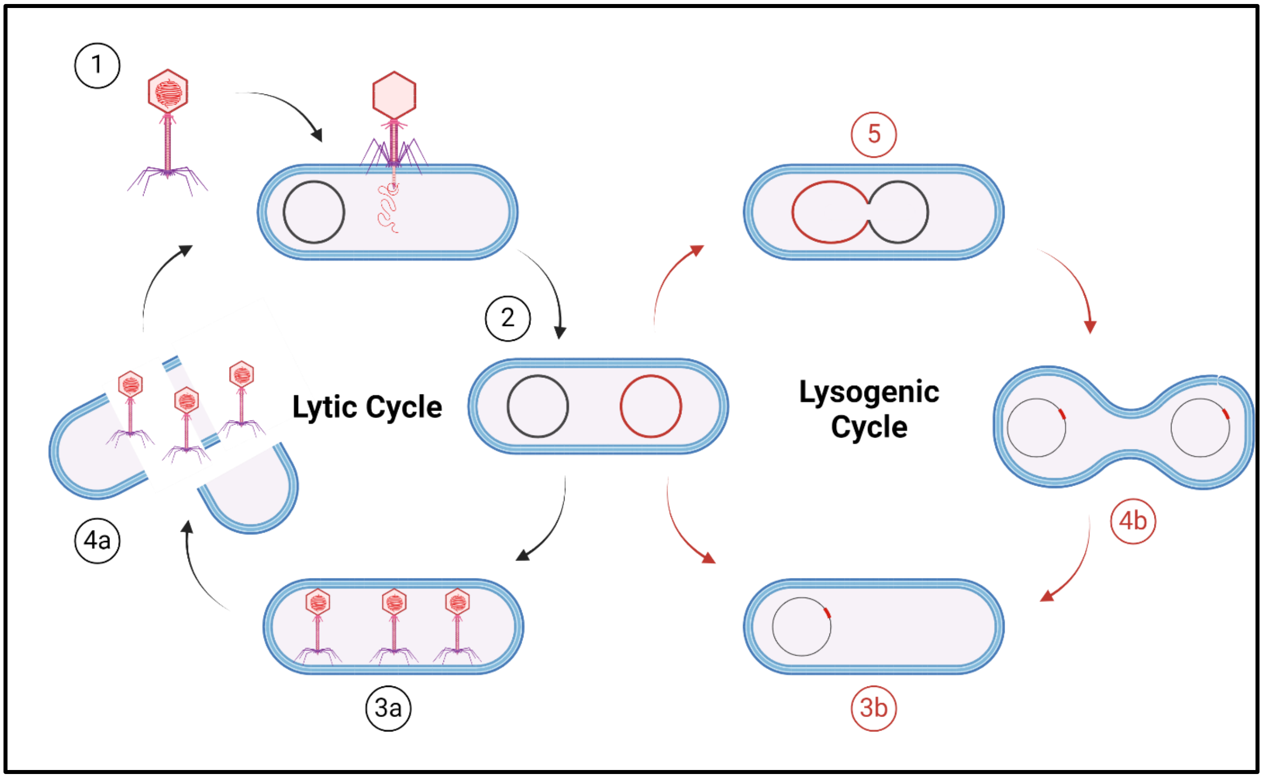

Figure 1.1.1: Two modes of bacteriophage reproduction: 1) Attach of phage to host and injection of genomic material. 2) Bacteriophage genome initiates lytic or lysogenic cycle. 3a) Phage genome is replicated, and phage proteins are produced using host cellular machinery; new virions are assembled. 3/4b) Integration of phage genome into host maintaining normal host function. 4a) Host cell undergoes lysis, releasing virions. 5) Under specific conditions, bacterial genome is excised from host genome, lytic cycle is initiated. Adapted from [8].

As bacteriophages are antimicrobial viruses, they have been used extensively as model systems in molecular biology, vectors for vaccine development, vectors in plant and animal gene delivery, and used in food control and preservation applications [11], [12], [13]. More relevant, is the application of phages as an antimicrobial treatment with the first application nearly coinciding with the discovery of these viruses in 1917 [2]. Unfortunately, from a combination of inconsistent results and the discovery of penicillin (leading to the subsequent development of pharmaceutical antibiotics) the procedure was forgotten by Western countries [2], [14]. But with the onset and exponential growth of antibiotic-resistant strains of bacteria, as well as the success of therapeutic phages in countries of the former Soviet Union, reinvigoration of interest in bacteriophage therapy has begun [2], [15]. This is illustrated through the onset of human-bacteriophage clinical trials in the United States, with the onset of 10 in the last five years alone (clinicaltrials.gov, accessed in April 2024).

Therapeutic bacteriophages have several benefits when compared to traditional antibiotics. The most significant advantage is their ability to target and neutralize specific bacteria while leaving other strains (*i.e.,* gut microflora) and mammalian cells uninfected [16]. As such, phages are deemed safe and tolerable for human applications [16]. Additionally, phages can also be easily isolated from sewage or retrieved from phage banks, providing an adaptable tool to address the rapid proliferation of AMR bacteria [17]. Contrary to traditional antibiotics, lytic bacteriophage preparations do not necessarily require multiple administrations to a patient to treat an infection, as bacteriophage concentration near the site of infection will increase due to the replication of phages after initial administration [16].

Despite the promise of bacteriophage therapy, no phage products have been approved for human use in the European Union and USA [18]. These preparations generally fall under the classification of a "biological drug" by the Food and Drug Administration (FDA), with any preparations needing to be manufactured in accordance with current good manufacturing practice (cGMP) [19], [20]. Bacteriophage therapy is sparingly applied in Western countries, traditionally under the umbrella of Article 37 (Unproven Interventions in Clinical Practice) of the Declaration of Helsinki [18]. Within this context, therapeutic bacteriophage preparations exist as 1) cocktails of multiple phage components, 2) personalized therapeutic preparations, and 3) genetically engineered phage therapeutics [21]. Of these, fixed cocktails are the most popular preparation type as they are the most compatible with existing regulatory pathways for antimicrobials, but they possess limited host range and durability as the strains of phages included in the preparation remain unchanged. As such, periodically modified cocktails have gained greater interest as they can be updated to address evolving bacterial resistance [21].

Reviewing the efficacy of bacteriophage therapy in its current state, a recent systematic review evaluating the safety of phage therapy found that across 52 independent studies, only 7% of patients reported mild adverse events, concluding that phage therapy is safe [22]. Additionally, the same report found that 79% of patients experienced clinical improvement, and 87% experienced complete bacterial eradication. However, the actual value of bacteriophage therapy remains inconclusive because of the heterogeneity between studies and concomitant use of antibiotics in 58% of the patients across the trials reviewed [22]. Furthermore, a comprehensive retrospective review by a Belgium consortium, examined the first 100 consecutive bacteriophage therapy cases of "difficult–to–treat bacterial infections," finding promising results [23]. They found that synergistic application of bacteriophage and antibiotics led to clinical improvements in 77% of all cases and complete eradication of pathogenic bacteria in 61 % of cases, with 7 cases experiencing mild to moderate adverse reactions [23]. The median age of the patients included in this sample was 53 years old, and 56.7% of the population was male [23]. The most common form of infections were lower respiratory tract infections (25.4% of the sample), skin and soft tissue infections (22.8 % of the sample), and bone (14.0% of the sample). *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections were the most common, making up 44% and 35% of total infections [23]. Ultimately, this review concluded that when combined with antibiotics, personalized therapeutic bacteriophage preparations were beneficial, although the efficacy of bacteriophage therapy in isolation is still unclear [23]. Interestingly, intravenous applications in these studies were rare and only applied to 20 patients generally utilizing 50 – 100 mL of phage preparation suspended in saline [23]. Critically, this review compiled the average titer of bacteriophage present in all preparations reviewed and the level of a critical contaminant, endotoxins, which will be discussed later in this section. The formulations described above exhibited an average bacteriophage titer of $8.34 \times 10^9 \pm 1.16 \times 10^{10}$ (standard deviation) PFU/mL with median endotoxin levels of 5.0 ± 89.14 (standard deviation) endotoxin units (EU/mL), giving the field a standard for future applications [23].

1.2 Manufacturing of Therapeutic Bacteriophages

1.2.1 Upstream processing

Any bacteriophage intended for a therapeutic application must be characterized, produced, and purified [2]. Process development for manufacturing suitable bacteriophage preparations for therapeutic applications involves multiple unit operations, separated into upstream (bacteriophage production) and downstream (recovery and purification) processes. Currently, the production of phages for pre-clinical or clinical applications is done through lab-scale techniques such as petri dish cultures and shaker flask liquid cultures, which can produce small volumes of product (~ 1 –

1000 mL) with final titers in the range of $10^9 – 10^{12}$ PFU/mL [2],[14, Ch. 12]. These protocols intended for therapeutic phage propagation have inadequate productivity for commercial-scale needs. As such, larger scale techniques have been explored to increase phage production productivity while operating at low production costs at scale [2], [24]. Technologies such as stirred tank bioreactors and wave bags have been successfully implemented in the production of bacteriophages. Wave bags are disposable bioreactor systems, where cell cultivation is performed in a plastic bag. These bags are usually placed on an incubation platform (*e.g* thermoplate) that rocks at a specified frequency and angle to promote the mixing of culture fluids and oxygen transfer [25]. Bourdin et al. successfully amplified T4 bacteriophage in a wave bag disposable system (Biostat CultiBag RM system; Sartorius) over four hours, producing over 4 L of lysate at clinically relevant titer (>10^9 PFU/mL) [26]. Additionally, work done by Ali et al found that production of T4 and phage K using a batch- fed bioreactor could produce preparations with titer comparable to that produce by stirred flask production, with a final titer > 10^{12} PFU/mL at ~ 3L of product using a 5L stirred tank bioreactor [27]. Within the context of bioreactors, a framework developed by Agboluaje and Sauvageau, established several operating factors that will need to be optimized for each desired phage type for productive operation. Factors include growth media selection, determination of initial infection conditions (initial quantity of host, multiplicity of infection (MOI), and infection procedure), and various operating conditions such as temperature, pH, and agitation rate [28]. These authors also compared different operation modes (batch, semi-continuous, and continuous) finding that the more traditional batch mode of operation produced high titer phage preparations but faced several operational limitations (*e.g.* long preparation time) reducing its overall productivity, which could be overcome through a continuous process [28]. Interest in continuous manufacturing processes have steadily increased in biomanufacturing because of the higher productivity, capacity, and reduced capital costs associated with the process as compared to batch-fed systems, with exploration of continuous modes of bacteriophage production currently well underway [29]. The simplest approach reported is that of a single bioreactor system called a chemostat, in which there is a continuous flow of media in the system, with addition of bacteriophage only after bacteria have achieved a steady state of growth [24]. Unfortunately, the long-term interaction between phages and bacteria result in significant mutations of both, limiting the overall productivity of this system [24]. These limitations would be overcome through the creation of a two-tank bioreactor system developed by Husimi et al, know as "cellstat" [30]. In this two-tank system, bacteria are continuously grown in the first bioreactor, before flowing into the second where a controlled, discrete amount of phage with high dilution rates of media would be introduced to limit the number of mutations [24], [30]. This system has shown promise with more recent work done by Mancuso et al, successfully employing a modified 3-tank cellstat process, achieving stable operation over several days (~ 1 week) and producing high titers of T3 phage (10^{11} PFU/mL), demonstrating the potential of this continuous system [31]. Although increases in upstream development are promising signs towards commercial scale development of therapeutic phages, a significant bottle neck still exists in the downstream space where development is needed to capitalize on the yields produced from these upstream processes.

1.2.2 Downstream Processing

The goal of downstream processing is the isolation, concentration, and purification of target products to meet specific regulatory standards [2]. Therapeutic bacteriophages are considered either a biological medicinal product by the European Medicines Agency (EMA) or a biological drug by the FDA [1]. As such, key impurities need to be removed before the product is deemed safe for therapeutic application. The first step in down-stream processing is the bulk removal of cellular debris, which is usually done through low-speed centrifugation and/or microfiltration (using 0.22 or 0.45 μm membranes) [32] . This is traditionally followed by a concentration step facilitated by tangential flow filtration or precipitation of the phage(s) [32]. Products at this stage will contain the bacteriophage and key impurities that need to be removed from the preparations before the product is deemed safe for therapeutic application. These contaminants include host cell proteins, nucleic acids (RNA and DNA), and, of most concern to this work, lipopolysaccharides (LPS), also referred to as endotoxins [2].

LPS or endotoxins are a significant component of the outer membrane of gram-negative bacteria (*e.g. Esherichia coli (E. coli)*). The structure of endotoxins is consists of a conserved lipid A region, a core variable length oligosaccharide, and a hydrophilic O-antigen [33]. Endotoxins are amphiphilic and possess a negative charge at a physiological pH (Isoelectric point (IEP) = 2) due to the presence of negatively charged phosphate groups embedded in their structure [2], [33]. The structure of a LPS monomer can be seen in **Figure 1.2.1**. The molar mass of endotoxins can range from 10 – 1000 kDa as they tend to form macromolecular micelles in solution, which are often further stabilized by the presence of specific factors such as divalent cations [34]. Concerningly, endotoxins are highly pro-inflammatory when introduced intravenously, thus making them a key target. As such regulatory bodies such as the FDA demand that any treatment to be applied intravenously to patients should introduce no more than 5 EU per kg of patient body weight per hour of administration [35] [36].[37]

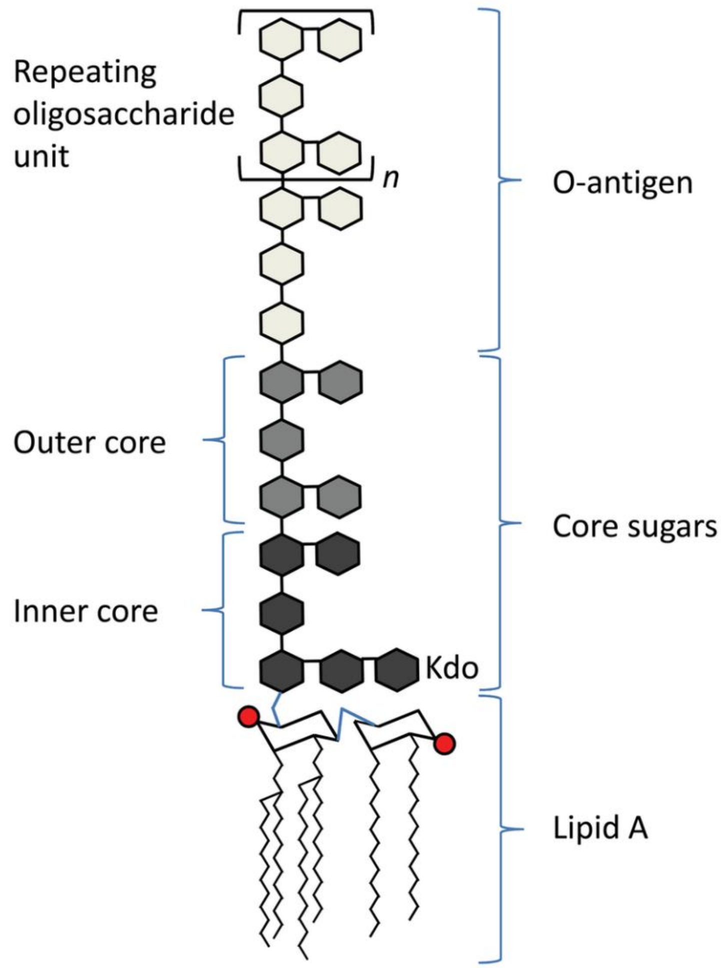

Figure 1.2.1: Chemical structure of endotoxin: from the top, polysaccharide O-antigen, with repeating units denoted by “n”, hydrophilic core oligosaccharides (core sugars), and hydrophobic lipid A tail with red dots indicating phosphate groups. Retrieved from [34].

1.2.2.1 Traditional Methods of Bacteriophage Purification

Traditionally, bacteriophage preparations are purified through cesium chloride (CsCl) density ultracentrifugation and/or polyethylene glycol (PEG) precipitation [2]. These techniques are often used together to increase purification performance, although they can be used independently with great effect [38]. A brief description of both techniques is provided below.

CsCl density gradient ultracentrifugation is a form of isopycnic separation. This form of separation exploits the differential settling of particulates suspended in solution. This technique utilizes aqueous soluble reagents to generate the gradients, cesium chloride (CsCl) being the most common for phage purification [39]. In the context of phage purification, bacteriophage lysate is layered on top of a CsCl gradient, followed by centrifugation, causing the formation of a density gradient by forcing the sedimenting cesium ions to move towards the bottom of the container while the intact solvated CsCl ions diffuse towards the center of the container [40]. When the solution stabilizes, a density gradient forms, with density decreasing when moving from the bottom to the top of a tube/container. Bacteriophages will equilibrate into their appropriate density gradient layer based on molecular weight, while all contaminants with differing densities and molecular weights will aggregate in another respective layer [40]. A CsCl centrifugation purification procedure will generally take anywhere from 1-4 h to complete, generating ~ 1 mL of product, with times varying depending on the procedure used to remove residual CsCl ions [41], [42]. Alternatively, Polyethylene glycol (PEG) precipitation is a separation technique that separates target biological compounds such as proteins or bacteriophages from contaminants by leveraging the differences in their solubility [43]. When added to the solution, PEG will act as a solvent sponge, reducing overall solvent availability and increasing the effective concentration of the compound one seeks to concentrate [43]. Different molecular polymers of PEG will be chosen based on the specific phage type used. PEG precipitation has shown promise within the specific context of endotoxin removal from bacteriophage preparations. For example, Branston et al reduced endotoxin content of M13 bacteriophage lysate by 88% through one cycle of PEG-NaCl (2% w/v PEG 6000 and 500 mM NaCl), reducing endotoxin load by an additional 18% in subsequent cycles while retaining 97% of bacteriophage titer [44]. Both techniques are well characterized, but limitations exist when considering applications on a commercial scale. These techniques are time-consuming, labor-intensive, and possess low scalability and throughput [18], [45]. PEG precipitation can take anywhere from 12 -14 h to complete, while CsCl ultracentrifugation can take anywhere from 1-4 h, both techniques traditionally limited to a productivity of 1-5 mL of purified product for a given batch [42], [46]. As such, much focus has been placed on the exploration of scalable purification technologies.

1.2.2.2 Chromatography

As the previous techniques primarily share the limitation of scalability, chromatography poses as a suitable alternative to traditional methods due to its high purity output in biomanufacturing applications and ease of scale-up [2]. Three primary forms of chromatography are used in biomanufacturing: ion exchange (IEX), affinity, and size exclusion. IEX, specifically anion exchange (AEX) chromatography, has garnered significant interest because of its relative speed, consistency, simplicity, ease of scaling up, and automation compared to the other forms [2]. This is especially relevant when considering bacteriophages are anionic (IEP = 3.5-7), with highly variable capsid properties and, therefore, surface charge between species, necessitating the need for an adaptable modality [8], [47].

Traditional packed bed chromatography is generally deemed inappropriate for bacteriophage purification applications as bacteriophages are generally significantly larger than bead pore diameters (30 – 100 nm) in pack bed resins and possess relatively small diffusion coefficients [2]. As such, the transport process is challenging to optimize, and the overall binding capacity of units is limited. A representative example of this limitation can be seen with plasmids. Imaging of individual resin beads and plasmids has shown they are unable to diffuse inside the tiny pores of traditional resins, limiting binding to the surface of the resin beads, significantly reducing the purification efficiency by reducing the available binding area [48].

Alternative anion exchange chromatography modalities, such as monoliths, offer superior resolution, binding capacity, and increased productivity compared to traditional chromatography [47]. AEX monoliths have shown variable promise in endotoxin removal from bacteriophage preparations. For example, in one study 10 mL lysates of 2 *P. aeruginosa* bacteriophage strains were purified using convective interaction media (CIM) diethylaminoethanol (DEAE) disk columns, resulting in 40 – 98 % endotoxin removal and a reduction in titer from 10^{13} – 10^{11} PFU/mL (~ 2- log reduction) depending on phage type [41]. Another study using *P. aeruginosa* bacteriophage examined the endotoxin removal performance of 3 monoliths: CIMmultus PrimaS, H-bond, and quaternary ammonium (QA). With CIMmultus QA, the group was able to retain 100% of phage titer (1.24 x 10^{12} PFU/mL) while achieving a four-log reduction (4 LRV) in endotoxin concentration (5.29 x 10^{6} to 1600 EU/mL). Other modalities achieved greater endotoxin removal (PrimaS – 5.29 x 10^{6} to 0.24 EU/mL and H-bond - 5.29 x 10^{6} to 0.8 EU/mL) but with inferior phage recovery (60 – 85%). Another group using a CIM QA disk monolithic column purifying T4 phage found that 70% of applied phage infectivity was retained in eluted fractions after purification. However, nearly all endotoxin content was retained (10^{4} EU/mL) [26].

Alternatively, membrane chromatography is another potential alternative to traditional methods. Membrane-based systems possess distinct flow rate advantages as compared to traditional packed bed chromatography, sufficient capacity for binding trace contaminants (such as endotoxin), and exist as single-use disposable units as opposed to monoliths, which can degrade over their lifespan and require costly replacements or cleanings [49], [50]. Membranes also possess large pore sizes, making them ideal for large particles such as viruses [51]. Although studies of membrane-based bacteriophage purification or endotoxin removal have been reported, very little exists regarding endotoxin removal from bacteriophage preparations. Roshankhah et al. demonstrated the use of a lateral flow membrane chromatography (LFMC) device for the purification of a model T7 bacteriophage. The group found that diluting the raw, sterile filtered lysate with binding buffer (10 mM Tris, pH 7.5) improved endotoxin removal performance at higher volumes of lysate of the device, removing 94% of endotoxins from 50 mL of the original lysate but with a 65% bacteriophage recovery [52]. The chromatography process only took 15 min to complete, significantly more rapid than traditional purification techniques, which may take hours, demonstrating the promise of this technique, albeit for only a single phage [52].

Data from a range of bacteriophage purification or endotoxin removal studies are shown in **Tables 1.2.1** and **1.2.2** respectively with the membrane type, sodium chloride molarity (NaCl mM), as well as endotoxin or phage removal represented as log removal or reduction value (LRV), as seen in **Eq 1**. Interestingly, many of these studies employ a flow-through mode of purification although bind and elute methods are still common. Bacteriophage bind most strongly (2-7 LRV) to commercial AEX membranes (Sartobind, Natrix, Mustang) at NaCl molarities < 50 mM with endotoxin removal expected to be proportionally high (generally 3 – 5 LRV). As NaCl concentration is increased to ≥ 50 mM, bacteriophage removal is found to sharply decrease (~ 0 LRV), while endotoxin removal decreases slightly (2-3 LRV). As the salt concentration is increased from ~ 0 mM NaCl, bacteriophage binding to AEX membranes or removal from solution is expected to rapidly decrease, whereby at 50 mM NaCl no binding is expected. Endotoxin binding is expected to be more inelastic in response to increasing NaCl molarity, while a slight decrease in removal is expected at and above 50 mM NaCl. Average endotoxin removal across all studies listed at salt concentrations < 50 mM NaCl was 4.3 LRV, whereas for molarities ≥ 50 mM NaCl average LRV was 3.5. Phage removal decreased significantly, where average removal at salt concentrations < 50 mM NaCl was 3.29 LRV and at ≥ 50 mM NaCl it significantly decreased to an LRV of 0.0375.

Table 1.2.1: Bacteriophage removal achieved using various AEX membranes in Tris-buffer pH 7-8 through a range of ionic strengths (0 – 300 mM). Removal is reported as log removal value (LRV). "n/a" indicates that sample type or initial titer was not explicitly stated in the study.

Membrane	Sample Type	NaCl (mM)	pH	Initial Titer (PFU/mL)	LRV	Ref.
Sartobind Q	Bacteriophage Lysate	0	7	1.50E+07	2	[53]
Mustang Q	Bacteriophage Lysate	0	7.5	1.00E+09	6.7	[54]
Sartobind Q	n/a	10	8	4.00E+07	4	[55]
Sartobind Q	Bacteriophage Lysate	18	7.5	n/a	0.46	[52]
Sartobind Q	Bacteriophage Lysate	50	7	1.50E+07	0	[53]
Sartobind Q	n/a	50	8	4.00E+07	0.1	[55]
Mustang Q	Bacteriophage Lysate	50	7.5	1.00E+09	0.1	[54]
Sartobind Q	Bacteriophage Lysate	100	7	1.50E+07	0	[53]
Mustang Q	Bacteriophage Lysate	150	7.5	1.00E+09	0	[54]
Sartobind Q	n/a	150	8	4.00E+07	0.1	[55]
Sartobind Q	Bacteriophage Lysate	200	7	1.50E+07	0	[53]
Sartobind Q	Bacteriophage Lysate	300	7	1.50E+07	0	[53]

Table 1.2.2: Endotoxin removal achieved using various AEX membranes in Tris-buffer pH 7-8 through a range of NaCl molarities (0 – 300 mM). Removal is reported as log removal value (LRV). "n/a" indicates that sample type or initial endotoxin concentration was not explicitly stated in the study.

Membrane	Sample type	NaCl (mM)	pH	Initial endotoxin (EU/mL)	Reported LRV	Ref.
Sartobind Q	Endotoxin spiked buffer	0	7	500	3.5	[53]
Sartobind Q	Endotoxin spiked water	0	7	10000	5.22	[56]
Sartobind Q	Endotoxin spiked buffer	0	7	10000	4	[56]
Sartobind Q	Endotoxin spiked Antibody Preparation	0	7.5	100	3.2	[56]
Sartobind Q	Endotoxin spiked buffer	0	8	6.00E+04	5	[57]
ChromaSorb	Endotoxin spiked buffer	0	8	6.00E+04	7	[57]
Mustang Q	Endotoxin spiked buffer	0	8	6.00E+04	5	[57]
Sartobind Q	Bacteriophage Lysate	18	7.5	N/A	1.4	[52]
Natrix Q	Endotoxin spiked buffer	40	8	>1000	4	[58]
Sartobind Q	Endotoxin spiked buffer	50	7	500	3.5	[53]
Sartobind Q	Endotoxin spiked buffer	100	7	500	3.0	[53]
Natrix Q	Endotoxin spiked buffer	120	8	>1000	4	[58]
Sartobind Q	Endotoxin spiked buffer	150	8	26900	5.3	[59]
Sartobind Q	Endotoxin spiked buffer	200	7	500	3.0	[53]
Sartobind Q	Endotoxin spiked buffer	250	8	6.00E+04	2	[57]
Mustang Q	Endotoxin spiked buffer	250	8	6.00E+04	2	[57]
ChromaSorb	Endotoxin spiked buffer	250	8	6.00E+04	7	[57]
Sartobind Q	Endotoxin spiked buffer	300	7	500	2.5	[53]

1.2.3 Challenges and Limitations

Traditional methods of bacteriophage purification are unsuited for commercial scale production of phage preparations as they are limited in their scalability. Chromatography poses as a suitable technology but with the highly variable morphology, size, and charge properties of bacteriophages in parallel with the equally broad and overlapping distribution of endotoxin properties, namely charge (IEP ~ 2), chromatography recipe development must be strain-specific [9], [60]. Although the electrostatic properties of both phage and endotoxin overlap, there is clear room to optimize for specific separation of the two products through careful manipulation of solution conditions. As purification recipe development for every new bacteriophage to be used for treatment or inclusion in a therapeutic cocktail would be laboursome, a high-throughput technique to assist in the rapid generation of chromatography recipes is needed. Unfortunately, there exists little literature focused on endotoxin removal from bacteriophage preparations using membrane chromatography. Studies in this space often solely focus on endotoxin removal from spiked buffers or bacteriophage purification without the quantification of endotoxin removal. This highlights a need to better understand how the removal of phages and endotoxin by AEX membranes is impacted using phage lysate.

1.3 Project Motivation and Research Objectives

Given the needs highlighted in the previous section, a high-throughput flat sheet membrane screening approach was developed utilizing a novel multi-well flat sheet membrane screening tool previously developed by the Latulippe lab. The primary goal of this work was to develop a methodology to simultaneously screen bacteriophage and endotoxin binding to AEX membranes using phage lysates in various solution conditions. The specific goals of this project were as follows:

- Use a multi well screening tool to identify key operational parameters to screen bacteriophage binding to commercial AEX membranes.
- Evaluate the ability of the high-throughput methodology to screen the effects of solution conditions on bacteriophage and endotoxin binding.
- Evaluate the translatability of the select screened solution conditions to a larger-scale purification modality such as syringe filtration.

2 Materials and Methods

2.1 Materials

2.1.1 Buffers

All buffers were composed of 10 mM Tris – HCl made using high-purity milliQ water (produced by an EMD Millipore Milli-Q Reference system), NaCl salt, Tris-HCl salt (OmniPur MW 157.60 g/mol), and 1 M NaOH (VWR) for pH correction to 7-7.1. Preparation occurred using 500- or 1000-mL glass bottles, where previously weighed salts (such as NaCl, $MgSO_4$, and $CaCl_2$ (BioShop)) were added to preparations and continuously stirred using a stir rod and plate. All buffers were then pH corrected using a pH meter (ThermoFisher, Orion 8102BNUWP) before being sterile filtered through a 0.2 μm polyether sulfone (PES) membrane (ThermoScientific, 5954520) into a sterile plastic bottle using an air pump.

Two primary buffer compositions were used in this work. Earlier work studying the titer stability of bacteriophage in various storage conditions and high-throughput screening work solely focussed on bacteriophage binding to membranes utilized a bacteriophage storage buffer (phage buffer) provided by Burrows lab, with the composition of 68 mM NaCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7. All dilutions performed for later bacteriophage generation and titer quantification used phage buffer as the dilutant as the presence of divalent cations is deemed critical for the efficient adsorption of bacteriophage to their host strain bacterium [61], [62], [63]. Later, high-throughput and syringe filtration work, which studied the binding of both endotoxin and bacteriophage to membranes, utilized buffers with a composition of X mM NaCl 10 mM Tris-HCl, pH 7.

2.1.2 *Biological Reagents*

Lysogeny broth (LB), 0.6 % (w/v) LB Agar, and 1.5 % (w/v) LB Agar solutions were the primary reagents used in the culturing of bacteria, phage and titer testing, the preparation of each reagent is as follows. Liquid LB was prepared by solubilizing 5 g of LB-Lennox (Sigma–Aldrich, L3022) with 250 mL of milliQ water into an autoclave-safe glass bottle with a stir bar. After visually confirming that the LB has fully solubilized, the preparation was then autoclaved. To prepare 250 mL of 0.6 % or 1.5% (w/v) LB agar, 5 g of LB-Lennox alongside 1.5 g (0.6%) or 3.75 g (1.5%) of Agar (Sigma – Aldrich, A5306) was solubilized in 250 mL of milliQ water into an autoclave safe glass bottle with a stir bar. After complete solubilization was achieved, preparations were autoclaved. As LB Agar preparations will solidify into a gelatinous solid when cooled, these preparations must be heated and stirred on a hot plate before being used so that the gelatinous solid mass fully liquefies and boils. This can be achieved in 30 – 60 minutes (based on the volume of preparation) by heating the bottles at a plate temperature of 300 – 340°C until the solution boils, after which the plate temperature can be set to 80 - 90°C to ensure the LB agar remains liquid until the time of use. The preparations should constantly be stirred during these steps. LB agar should always be boiled after use and before being stored to reduce the chance of contamination.

2.1.3 *Membrane Materials*

Three commercial AEX flat-sheet membrane adsorbers functionalized with QA groups which include Sartobind Q (Sartorius, 94IEXQ42-001), Mustang Q (Pall), Natrix Q (Millipore, NATQ04725) were used in high-throughput screening experiments. The properties of the flat-sheet membrane types used in this work are reported (**Table 2.1.1**). A 25 mm diameter Natrix Q membrane capsule (Millipore, NXF-00) with a membrane volume of 0.2 mL was used for the final syringe filtration experiment. A cross section revealing in inner structure of the membranes can be seen in **Figure 2.1.1**.

Table 2.1.1: Commercially available membrane AEX membrane adsorbers used in this work. Membrane thickness reflects dry values.

Membrane	Nominal pore Size (µm)	Nominal Membrane thickness (µm)	Matrix	Functional Group	Function	Manufacturer
Natrix Q	0.4	500	Polyacrylamide hydrogel	QA	Anion Exchange Adsorption	Millipore [65]
Sartobind Q	>3	275	Regenerated Cellulose	QA	Anion Exchange Adsorption	Sartorius [66]
Mustang Q	0.8	138	Polyether sulfone (PES)	QA	Anion Exchange Adsorption	Cytiva (formerly Pall) [67]

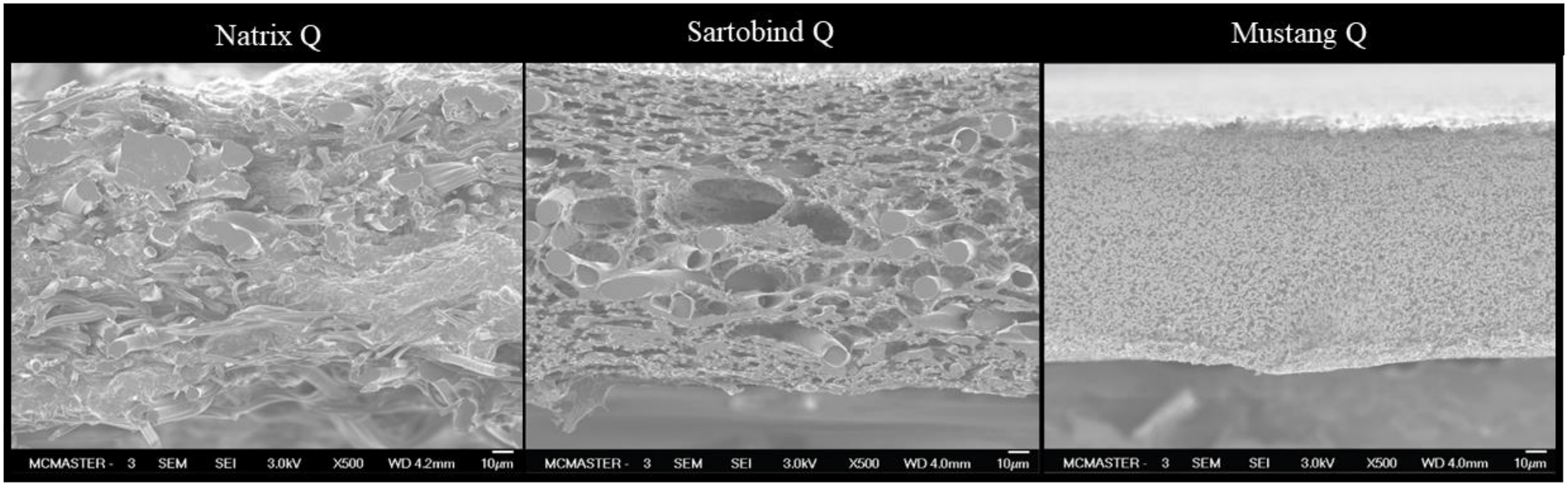

Figure 2.1.1: SEM cross-sections (500×) of Natrix Q, Sartobind Q, and Mustang Q membrane. Retrieved from [60].

2.2 Methods

2.2.1 Bacteriophage production

E. Coli J1062 was the sole host cell line for this work, provided by the Burrows lab. The specimen was isolated from a patient's urinary tract infection and found antibiotic-resistant, making it an ideal candidate for this work. Two phages identified as bacteriophages J1MH1 and SK2 were isolated, sequenced, and propagated using *E. coli* J1062. The primary cell line was stored as an LB glycerol stock (15 % v/v) in a – 80ºC freezer. Cells were streaked onto 1.5 % (w/v) LB Agar every week before incubating at 37 ºC overnight (14-17 h). The next day, single colonies on the streaked plate were suspended in 4 mL of LB to prepare a liquid culture before being placed into a shaking incubator for ~ 2 h. In parallel, a bottle of solidified 0.6% (w/v) LB agar was boiled on a plate heater by setting the temperature to ~ 300 - 340 °C and stirring the solution until the LB agar was fully liquified and began to boil. After boiling, the temperature was set to 80°C and left stirring until the moment of use. To prepare infected cell cultures, previously prepared liquid cultures of *E. coli* J1062 were diluted using LB until an optical density at 600 nm (OD_{600}) of ~ 0.25 (~2.0 x 10^8 CFU/mL) was achieved at a volume of 1 mL. Similarly, a stock of bacteriophage was diluted from ~ 10^{10} PFU/mL to 10^6 PFU/mL using phage buffer for a final volume of 1 mL. To produce a bacteriophage stock at a titer of ~ 10^9-10^{10} PFU/mL, 225 µL of stock phage at a titer of ~ 10^6 PFU/mL would be added to a 50 mL Falcon tube with 225 µL of $O.D_{600}$ ~ 0.25 *E. coli* J1062 culture (~2.0 x 10^8 CFU/mL) (MOI= 0.01), incubated at room temperature for 10 min before 23 - 24 mL of previously prepared liquid LB Agar (0.6% w/v) was added. After briefly mixing, the solution was decanted into a 150 mm petri dish and solidified, then incubated overnight at room temperature. Each plate culture received 7 mL of phage buffer in the morning before being placed on an orbital shaker at the 3-speed setting, rocking for 4 h. Then, 6 – 6.5 mL of solution from the petri dish cultures were aspirated into a separate 20 mL Falcon tube(s) using a 1000 µL pipette (filtered tip) before centrifugation at 3000 x g for 15 min. The solution is then passed through a 0.2 µm sterile filter using a 30 mL syringe (BD), discarding the pellet at the bottom of the Falcon tube(s). These sterile-filtered bacteriophage preparations were stored at 4ºC. This process is illustrated in **Figure 2.2.1**. A Transmission electron microscopy (TEM) image of J1MH1 can be seen in **Figure 2.2.2**

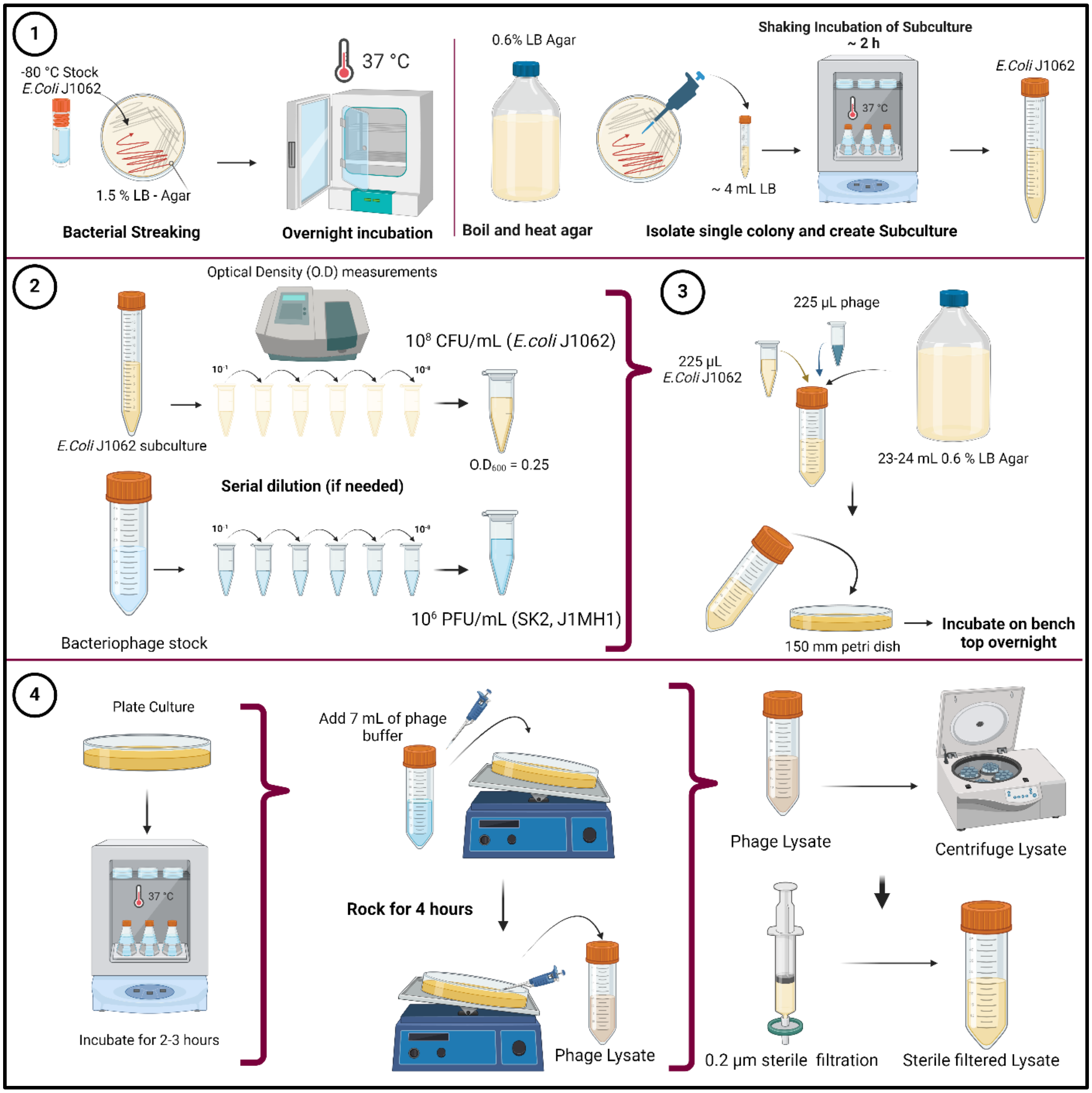

Figure 2.2.1: Methodology of generating SK2 and J1MH1 bacteriophage stock at a titer of ~10^{10} PFU/mL. A summary of the steps is as follows 1) Streak out frozen *E. coli* J1062 culture onto 1.5 % LB agar plate and incubate overnight. Boil then heat agar for later use, isolate single colony of bacterium and create a liquid sub-culture in LB. 2) Generate bacteria and bacteriophage preparations at an appropriate confluence and titer to achieve an MOI of 0.01. 3) Create bacteriophage petri-dish culture by mixing phage and bacterium preparations and liquid 0.6% LB agar, leave cultures to solidify and incubate at room temperature overnight 4) Briefly incubate plate culture(s) in a 37°C incubator before adding phage buffer and rocking for 4 h. Extract the phage buffer using a 1000 µL filtered pipette tip and pipette, centrifuge the lysate to remove large debris from the suspension and sterile filter the resulting lysate before storing in a 4°C refrigerator.

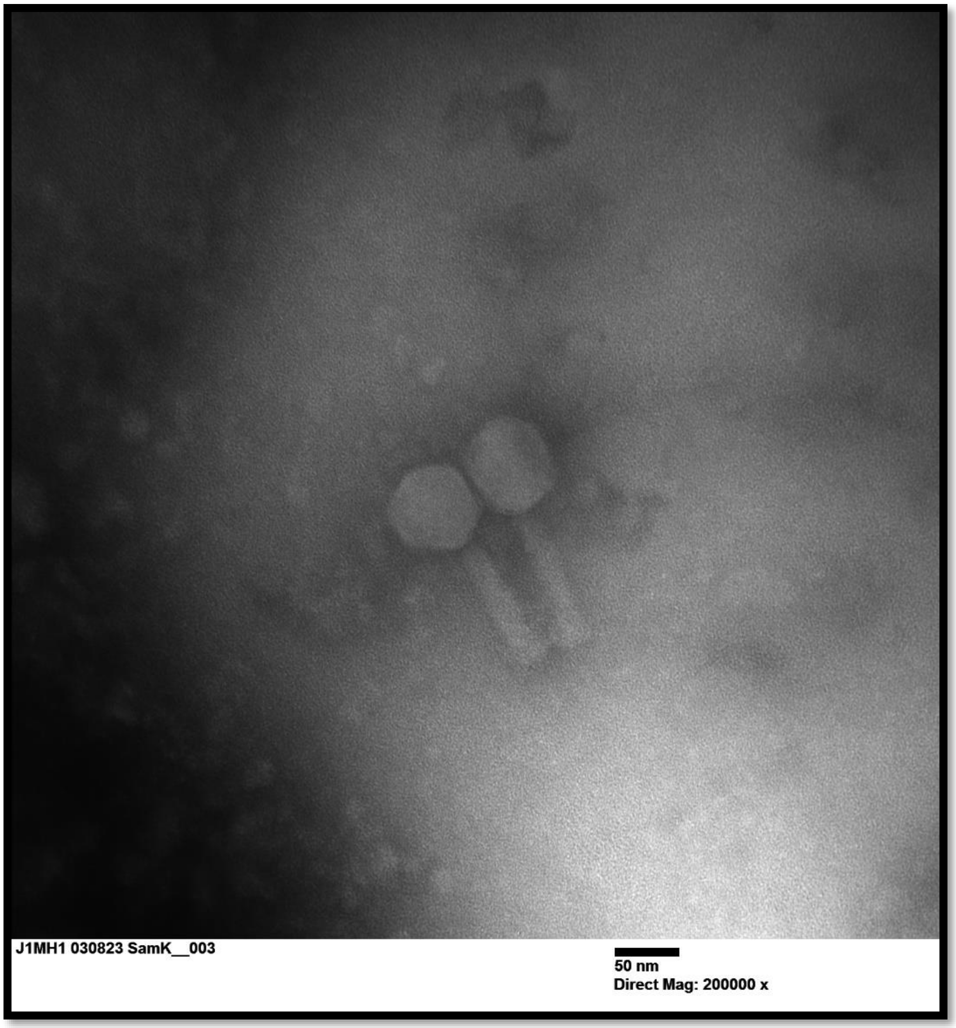

Figure 2.2.2: Transmission electron microscopy (TEM) image of J1MH1 bacteriophage fixed to silicon substrate at 200 000 X magnification. Bacteriophage was suspended in phage buffer (68 mM NaCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM Tris- HCl, pH 7) before TEM preparation.

2.2.2 Stability Analysis of J1MH1 Bacteriophage

Preliminary work focused on a single phage, J1MH1. To better understand how to store phage stocks to ensure consistency in samples used, 4 storage conditions for J1MH1 were evaluated. Phage preparations were left suspended in phage buffer or mixed with stabilizers resulting in final concentrations of 0.8 M sucrose and 15 % (v/v) glycerol. The conditions evaluated were 1) storage in phage buffer at 4ºC, 2) storage in phage with 0.8 M sucrose at -20°C, 3) storage phage buffer with 15% (v/v) glycerol at -20°C, and 4) storage in phage buffer at -20ºC. All conditions evaluated represented common and well-characterized conditions for the adequate stability of bacteriophage titer in storage over short (≤ 1 year) and long periods (> 1 year) [35], [68], [69], [70]. All samples were stored as 1 mL preparations at the start of the study in either 1.5 mL microcentrifuge tubes (4°C conditions) or 1.5 mL cryovials (-20 °C conditions). Bacteriophage titer was quantified monthly, taking 10 µL aliquots for titer quantification. – 20ºC J1MH1 samples were allowed to slowly defrost over ~ 40 min in an ice bath before aliquots were taken, followed by immediate refrigeration. All conditions were run in duplicate, and the study was conducted over a 2-month period from September 29, 2022, to November 23, 2022. This study period reflected the expected turnover of a given stock of phage.

2.2.3 High throughput Flat sheet Membrane Screening Platform

A novel 3D printed, high-throughput platform for the screening of flat sheet membranes was utilized in this work for the rapid screening of bacteriophage and endotoxin binding to AEX membranes [64]. The screening platform, shown in **Figure 2.2.3**, possesses exterior dimensions (131.8 x 89.5 mm) like that of a standard 96-well microplate. The multi-well design of the device consists of 32 individual units, each representing an experimental unit. Each unit contains two square wells interconnected by a circular membrane cell via flow channels, with each well or unit accommodating a liquid volume of 1 mL. These groupings of interconnected wells are also identifiable with a letter (A-H - rows) and number labeling (1-4 - columns) for the device's rows and columns, respectively. A singular 8 mm membrane disc from any single flat-sheet membrane can be enclosed in the membrane cell with a 3D-printed plug secured with a silicone O-ring (McMaster-Carr, 9396K13), which works to eliminate the risk of sample cross-contamination. These plugs are removed using forceps, allowing simple retrieval of membranes from individual units for post-experimental membrane processing (*e.g.* imaging). The device was designed to sit on a benchtop plate rocker for gravity-driven lateral fluid mixing. The flow channels between the circular membrane cell and adjacent square wells in the device allow gravity-driven tangential fluid flow across the membrane (rather than a normal flow pattern). The device is continuously rocked to ensure repeated interactions between the liquid solution and membrane.

8 mm diameter membrane discs used for experiments were generated from a stock of flat-sheet membranes using an 8 mm hole-punch (Allwin, Oregon Lamination Premium). The primary plate rocker used in this work was the Boekel Scientific Rocker II (260350), set to achieve 1 tilt per 5 min. The rocker's tilting action, which was set to tilt to a 17° angle from the horizontal, resulted in an appropriate fluid height difference across the square wells, generating hydrostatic pressure that facilitates fluid flow across the channel.

Before use in an experiment, each device alongside associated plugs were soaked in a 70% (v/v) ethanol bath for 20 min, followed by a 30 min exposure to UV light (280 nm) in a biological safety cabinet (BSC) (Baker SterilGard Class II). Once complete, previously prepared 8 mm membrane discs were added to individual units and secured with plugs. A plastic plate lid covered all plates during experiments. An illustration can be seen in **Figure 2.2.4.**

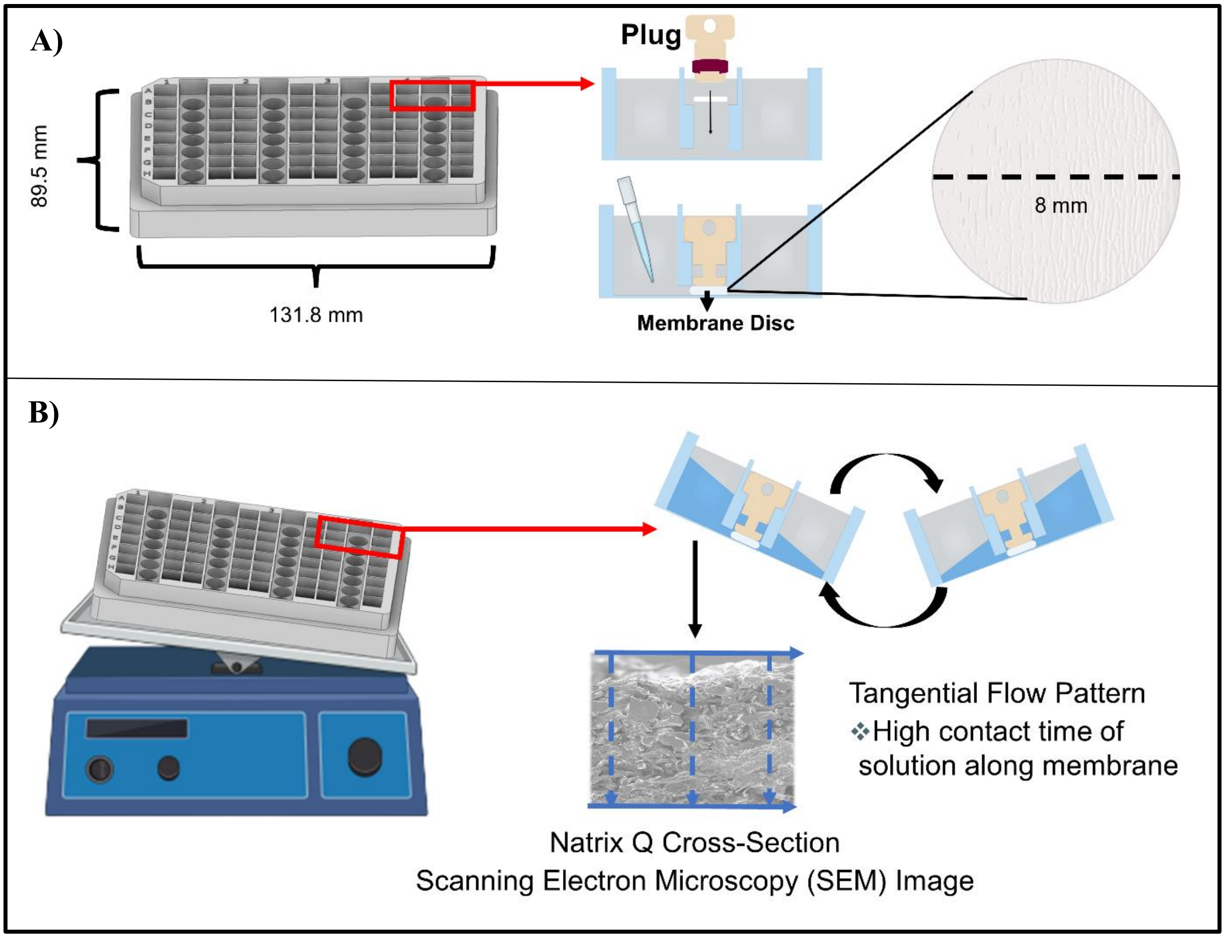

Figure 2.2.3: Illustration of **A)** multi-well device and individual experimental unit (32 total) composed of two vertical, columnar wells, central cylindrical well all connected by an underlying channel. 8 mm diameter membrane discs are inserted into the central channel, secured by a 3D-printed plug. **B)** Gravity driven flow of solution across membrane disc caused by motion of bench top plate rocker.

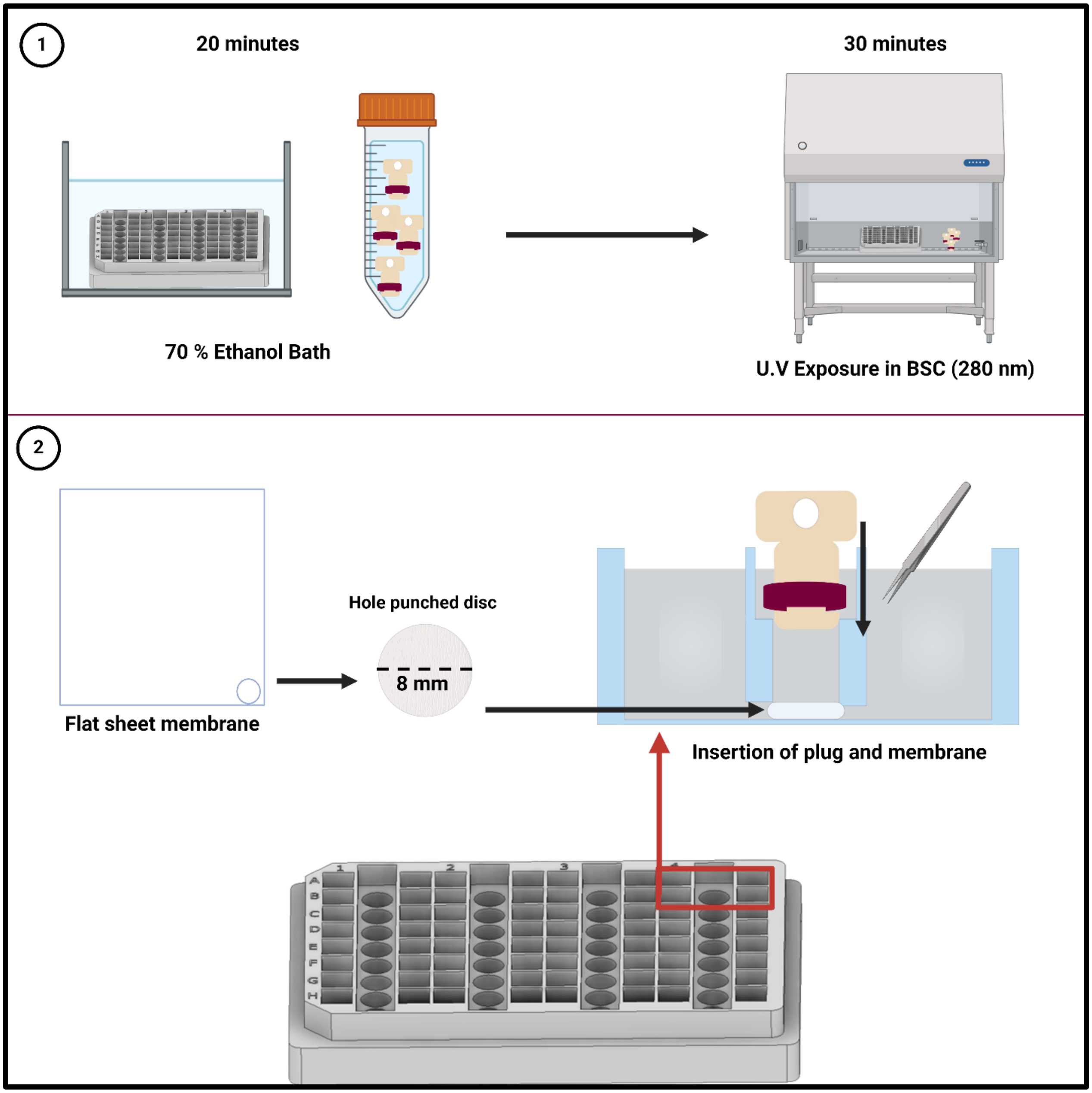

Figure 2.2.4: Preparation of multi-well device prior to high-throughput screening experiments using bacteriophage preparations. **1)** 70% ethanol bath of plugs and multi well device and subsequent drying under U.V light**. 2)** Preparation of individual wells in multi-well device; insertion of 8 mm diameter membrane discs and plugs using forceps.

2.2.4 High-throughput Screening Studies

Prior to any experiments, meticulous preparation of the multi-well devices, as described previously in Section 2.2.1, was undertaken. All experiments using the multi-well device follow a rigorous three-step process; equilibration, loading, and analysis as depicted in **Figure 2.2.5**. During the equilibration phase, 1 mL of appropriate buffer was loaded into relevant wells or units and the device allowed to rock for 1 min. This step was repeated an additional 4 times, with 800 µL of buffer aspirated and replaced with fresh buffer each cycle. This serves to wet the membrane and remove preserving agents as well as any contaminants that may interfere with binding. After the final equilibration step, 800 µL of buffer was discarded and replaced with 800 µL of bacteriophage preparation (SK2 or J1MH1). A 1 mL volume was maintained through all steps. Once experiments were concluded, plugs for each experimental unit are removed and 800 µL of solution as well as associated membrane discs were extracted and stored in the 4°C refrigerator, ensuring the integrity of the samples.

Work discussed in Sections 3.2.1 was conducted in phage buffer with each condition and control (no-membrane with equivalent bacteriophage sample) carried out in triplicates for each dilution. Four different preparations of J1MH1 were generated by diluting a 10^9 ($7.50 \times 10^9 \pm 5.00 \times 10^8$) PFU/mL stock, into 3 separate dilutions with initial titers of 10^8 ($1.50 \times 10^8 \pm 7.00 \times 10^7$), 10^7 (4.00×10^7), and 10^6 ($2.50 \times 10^6 \pm 5.00 \times 10^5$) PFU/mL. Each preparation was in contact with Natrix Q and appropriate controls for 6 h. The titer of samples and controls was quantified using a bacteriophage spot assay, and average titers were calculated for each sample and no membrane control with associated standard error calculations. These values were used to calculate the average bacteriophage LRV for each dilution, as seen in **eq. (1)**. LRV serves as a measure of the log reduction of a certain element (phage, endotoxin) after a purification process.

Work in Section 3.2.2 employed a SK2 sample at an initial titer of 4.00 x 10^6 PFU/mL generated by diluting a stock (3.50 x 10^9 ± 7.07 x 10^8 PFU/mL) by a factor of 1:1000 using phage buffer. At the time of study, genomic sequencing revealed that the J1MH1 preparations consisted of a cocktail of phages, rather than a homogenous strain, potentially introducing confounding factors to this work. As such, work moved towards use of SK2 bacteriophage as there was greater confidence of strain homogeneity. Three membranes (Natrix, Sartobind, and Mustang Q) were evaluated over a range of contact times (0.75, 1.5, 3, 6, 24 h) in phage buffer with associated no membrane controls. Multi-well devices were prepared, and samples and membranes were stored post-experiment as previously described. All samples and controls at each time point for all 3 membrane types were run in triplicates. It is important to note that, an additional experiment was conducted for both Natrix Q and Sartobind Q, only examining 3, 6, and 24 h of contact time. As such, for these conditions a total of 6 replicates exist for both samples and controls. The titer of samples and controls were quantified using a bacteriophage spot assay and average titers were calculated for each sample and control with associated standard error calculations. Average LRVs were calculated to determine virus removal at each time point. Average titer at 24 h of contact time for the no-membrane controls across all experiments was compared to the average titer of samples using each membrane (Natrix, Sartobind, and Mustang Q) to determine the relative productivities of membranes.

$$Log\ Removal\ Value\ (LRV) = Log\left[\frac{Initial\ Titer\ or\ Concentration\ \left(\frac{PFU\ or\ EU}{mL}\right)}{Final\ Titer\ or\ Concentration\left(\frac{PFU\ or\ EU}{mL}\right)}\right] \quad (1)$$

For work discussed in Section 3.2.3, 8 dilutions were prepared for SK2 and J1MH1 bacteriophage at an initial titers in the range of ~10^6 PFU/mL through dilution (1:1000 dilution factor) of respective stocks (SK2 = $2.50 \times 10^9 \pm 2.12 \times 10^9$ PFU/mL, J1MH1 = 1.0×10^{10} PFU/mL) using various buffers with a composition of 10 mM Tris – HCl, pH 7 and a range of NaCl molarities which were 0.068, 10, 20, 30, 50, 100, 200, 300 mM. Two AEX membranes (Natrix and Sartobind Q) were evaluated at 6 h of contact time across all buffers previously described using both phage types. Samples for each condition were tested in triplicate, while controls were run in duplicate. The titer (PFU/mL) of samples and controls were quantified using a bacteriophage spot assay and average titers were calculated for each sample and control with associated standard error calculations. Endotoxin concentration (EU/mL) of samples and controls were quantified using the Pyrochrome Limulus Amebocyte Lysate (LAL) endotoxin quantification assay (Associates of Cape Cod, #C1500-5) and average endotoxin concentration were calculated for each sample and control with associated standard error calculations. The LRV of bacteriophage titer and endotoxin at each condition was determined for both membranes across both phage species.

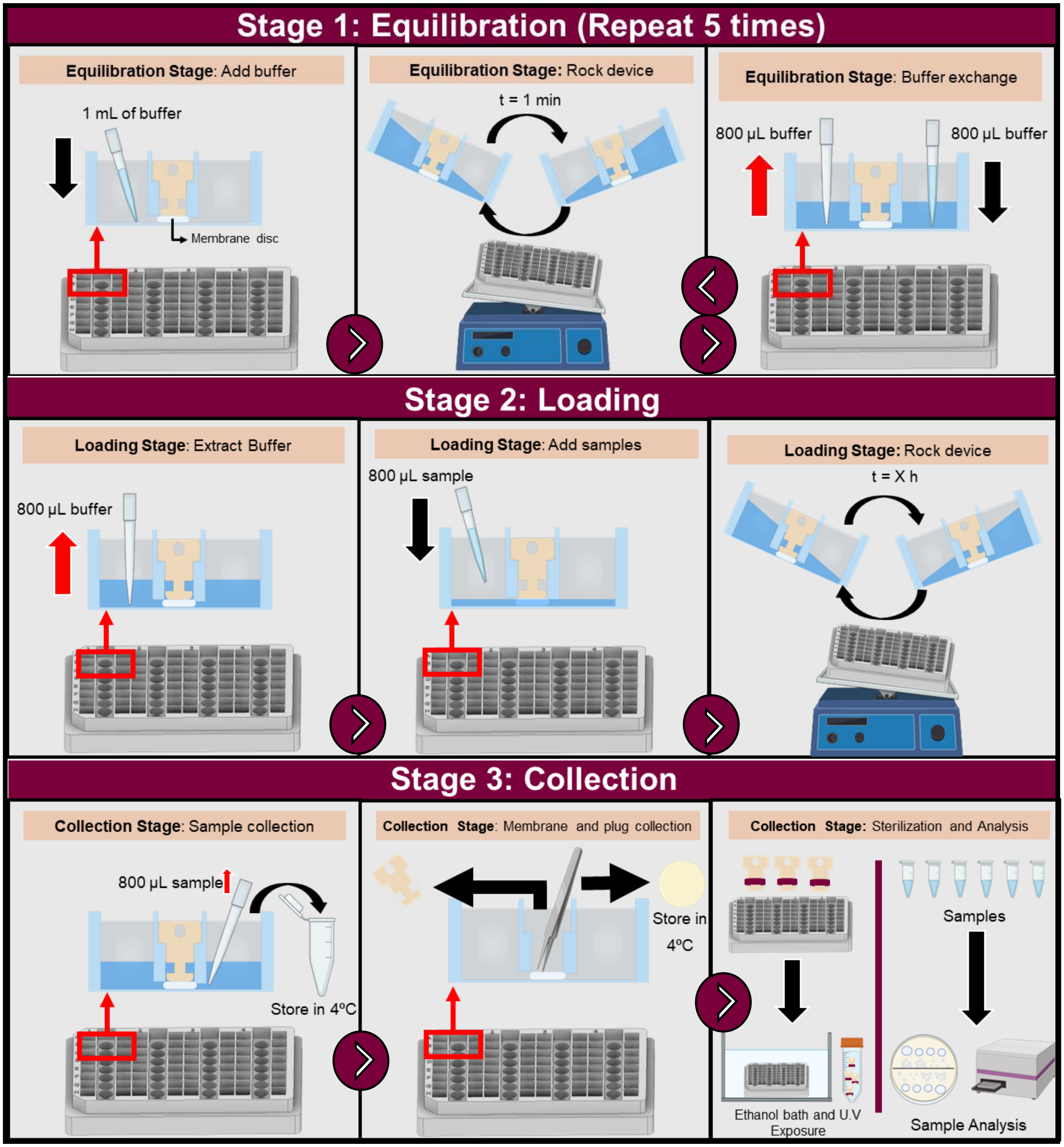

Figure 2.2.5: Illustration of general methodology of high-throughput screening experiments. An initial equilibration stage with desired buffer followed by loading of desired sample into each well before rocking for desired contact time. Finally, samples are aspirated from each well for either or both bacteriophage titer quantification and endotoxin concentration quantification.

2.2.5 *Syringe Filtration*

Natrix Q membrane capsules (25 mm) were first affixed to 30 mL syringes (BD) on a syringe pump (PHD ULTRA Harvard Apparatus). Before filtration, each 25 mm Natrix Q filter was prewet using 20 mL of buffer with a composition of 30 mM NaCl 10 mM Tris-HCl, pH 7 at a 2 mL/min flow rate. After prewetting, 10 mL of previously diluted (factor of 1:1000 from stock) SK2 and J1MH1 bacteriophage preparations (initial titers of $3.33 \times 10^6 \pm 8.82 \times 10^5$ and 1.0×10^6 PFU/mL, respectively) were permeated through the membrane at 2 mL/min. A separate syringe filter was used for each phage preparation. Ten ~ 1 mL fractions of permeate were collected in 1.5 mL Eppendorf tubes throughout the experiment. Note that pre-wetting membranes used in syringe filtration was critical as preliminary experiments demonstrated that the complete removal of bacteriophage occurred without this step.

2.2.6 *Analytical Methods*

2.2.6.1 Bacteriophage Titer quantification assay

Bacteriophage concentration or titer was determined using a titer spot test. To prepare bacterial–agar overlays on Petri dishes, 100 µL of 0.25 O. D_{600} *E. coli* J1062 liquid culture was added to a 15 mL Falcon tube alongside 12-14 mL of liquid LB Agar 0.6% (w/v). The tubes were gently rotated to eliminate bubbles and poured into a standard petri dish. This step was repeated to create as many assays as needed for a given experiment. The plates were then left covered on the bench top to solidify for 6 min before being transferred to a BSC uncovered to dry for 15 min, rotating each plate 180º every 7.5 min to allow even and consistent drying. Once dried, these plates were bagged, stored in a 4º C refrigerator, and generally used within a week; the plates were inverted (lid down) when stored. To conduct the spot titer test and quantify bacteriophage titer, previously prepared bacterial agar overlay Petri dishes were placed under a BSC to dry without a lid for 15 min (rotated 180º every 7.5 min). Once dried, plates were removed and appropriately labeled. Bacteriophage samples were serially diluted using phage buffer and 96-well transparent microplates (Thermofisher) to achieve eight 10-fold dilutions (dilution factor ranging from 10^{-1} to 10^{-8}) for each tested sample. 10 µL of each dilution for a given sample was pipetted onto a specific plate section. After all dilutions for all samples had been added, all assays were left to dry and developed overnight on the benchtop. The following day, plates were moved into a 37ºC incubator for 2-3 h, after which the clearances or "plaques" at each dilution were counted for each given sample, and titer (PFU/mL) calculated given the respective dilution and volume of a given subset of plaques as seen in **Eq. (2)**. An illustration of this procedure can be seen in **Figure 2.2.6**. A sample spot titer assay is shown in **Figure 2.2.7**.

$$Bacteriophage\ Titer\ \left(\frac{PFU}{mL}\right) = \frac{Counted\ Plaques}{Dilution\ Factor(10^{-x})\ \times volume\ of\ aliquot\ (mL\,)} \quad (2)$$

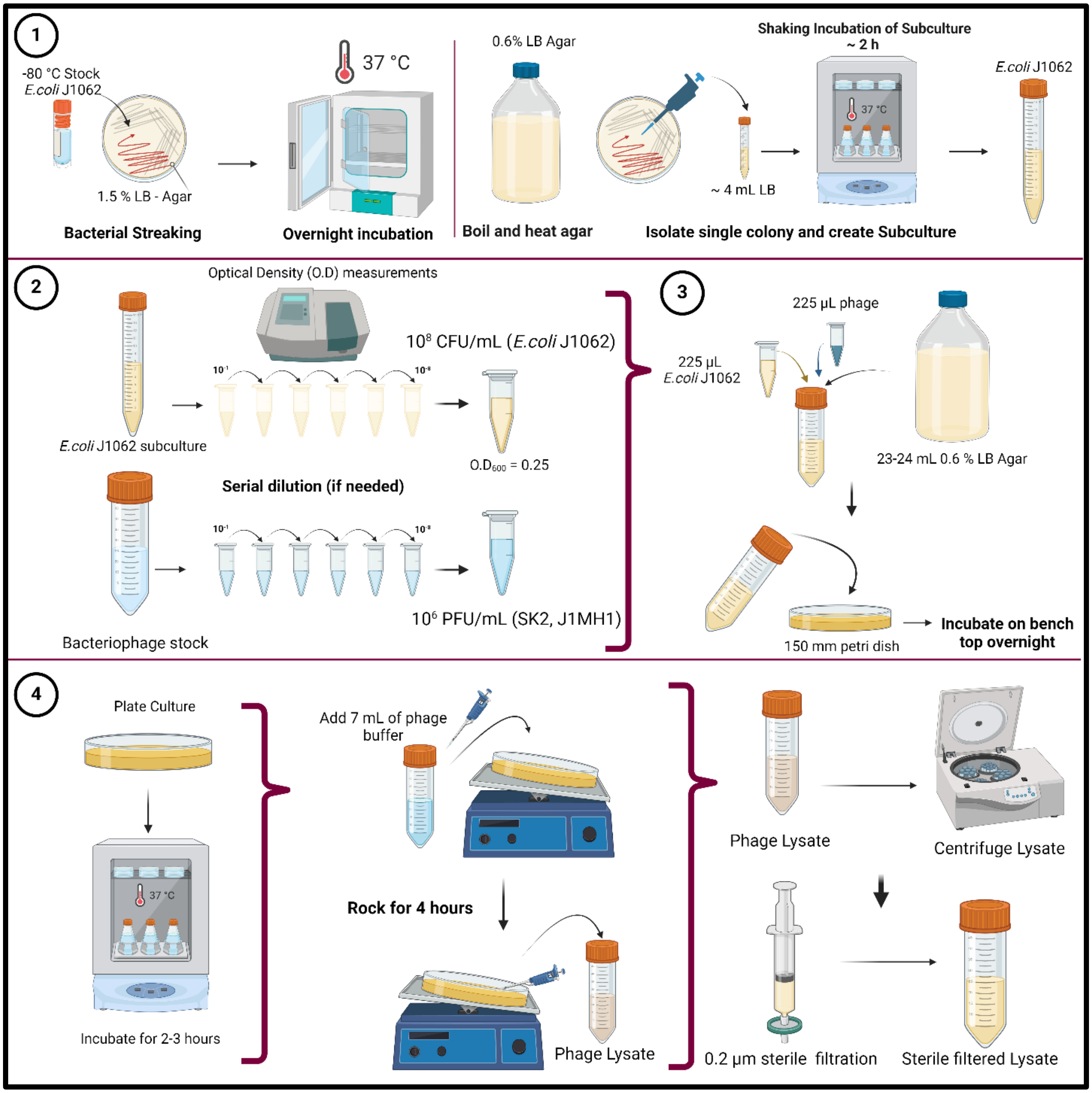

Figure 2.2.6: Methodology of preparing and conducting a bacteriophage spot titer test. **1)** Streak out frozen *E. coli* J1062 culture onto 1.5 % LB agar plate and incubate overnight. Boil then heat agar for later use, isolate single colony of bacterium and create a liquid sub-culture in LB. **2)** Generate bacterial culture at an appropriate confluence through dilution if needed. Pipette appropriate volumes of bacterial culture in 15 mL Falchion tubes before adding 0.6% LB Agar and subsequently pouring the solution into a petri dish. Allow plates to set and dry in a biosafety cabinet before use or before bagging and storing in 4°C fridge. **3)** Prepare serial dilutions of all samples in 96-well plate **4)** Label and dry assays in BSC before use, aliquot dilutions for a given sample onto appropriate sections in plate. Once all dilutions of all samples have been added to assays, allow assays to incubate on benchtop overnight, before incubating at 37°C for 2 h. Titer is determined by counting the plaques formed on the highest dilution to show entirely separated plaques.

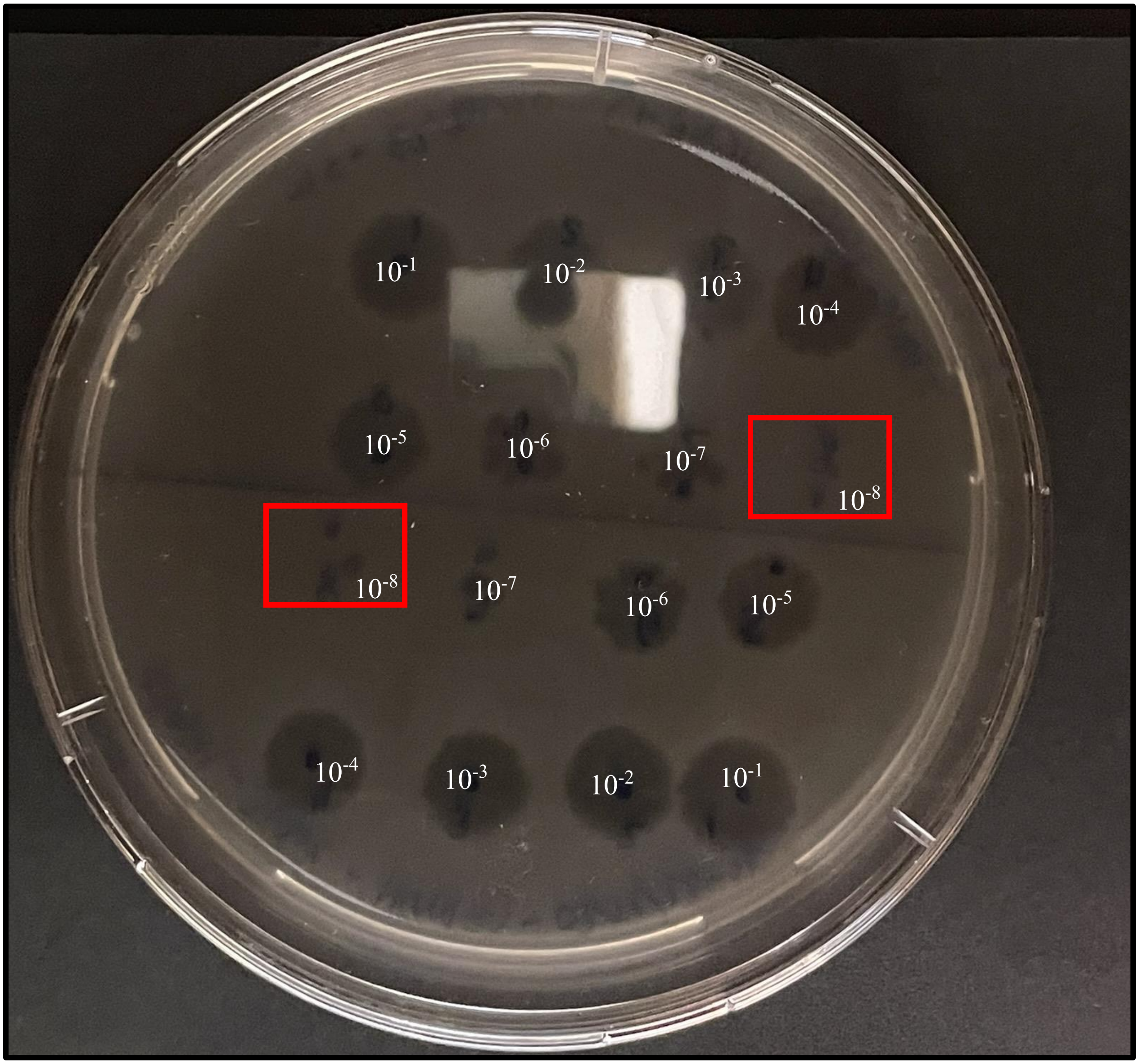

Figure 2.2.7: Image of completed titer test with eight 10-fold serial dilutions of J1MH1 spotted in duplicate. The location of added J1MH1 dilution aliquots is indicated by a dot and subsequent numerical value (10^{-1}-10^{-8}) representing the order of magnitude (factor 10) of dilution. Plaques counted to calculate titer of sample are outlined in red, these represent the highest dilution (factor 10^{-8}) prepared. Dilution factor of original J1MH1 sample for a given labeled section is shown.

2.2.6.2 Endotoxin Quantification Assay

Endotoxin concentration (EU/mL) for samples used in screening experiments and syringe filtration experiments was quantified using the Pyrochrome LAL assay (Associates of Cape Cod, #C1500-5) following manufacturer instructions. All samples to be quantified were diluted 10-fold (100: 900 µL dilution) with endotoxin-free water (Cytivia, SH30529.FS) (due to the narrow top range of assay <10 EU/mL). A 50 EU/mL stock of control endotoxin standard (CSE) (Associates of Cape Cod, #C0005-5) was serially diluted 2-fold (500: 500µL dilution) to generate a set of standard control serial dilutions with concentrations ranging from 10 – 0.625 EU/mL or 5 – 0.125 EU/mL. Then 50 µL aliquots of standard controls, samples, and controls were pipetted into a polystyrene transparent flat-bottom 96-well plate, with the standard and endotoxin-free water controls added in triplicate, before adding any reagent to the 96-well plate a Tecan Spark 10M plate reader was pre-heated to 37°C. Lyophilized LAL reagent was then resolubilized with 3.2 mL of buffer provided by the manufacturer before 50 µL of the reagent was pipetted to each well in the 96-well plate. The 96-well plate was then lightly tapped to induce mixing of reagents and samples, before rapidly being placed into the plate reader with absorbance at 405 nm being measured every minute for 30 minutes. As the manufacturer did not provide a specific incubation time, post-assay analysis of the absorbance data determined the time point at which the linear regression and subsequent quantification of endotoxin content were to be generated. This generally occurred at 14 – 20 min of incubation for a standard dilution control series of both 5 – 0.125 EU/mL and 10 – 0.625 EU/mL. An illustrated outline of this is shown in **Figure 2.2.8**. Standard curves used in each experiment that quantified endotoxins can be seen in **Figure 2.2.9, 2.2.10,** and **2.2.11**.

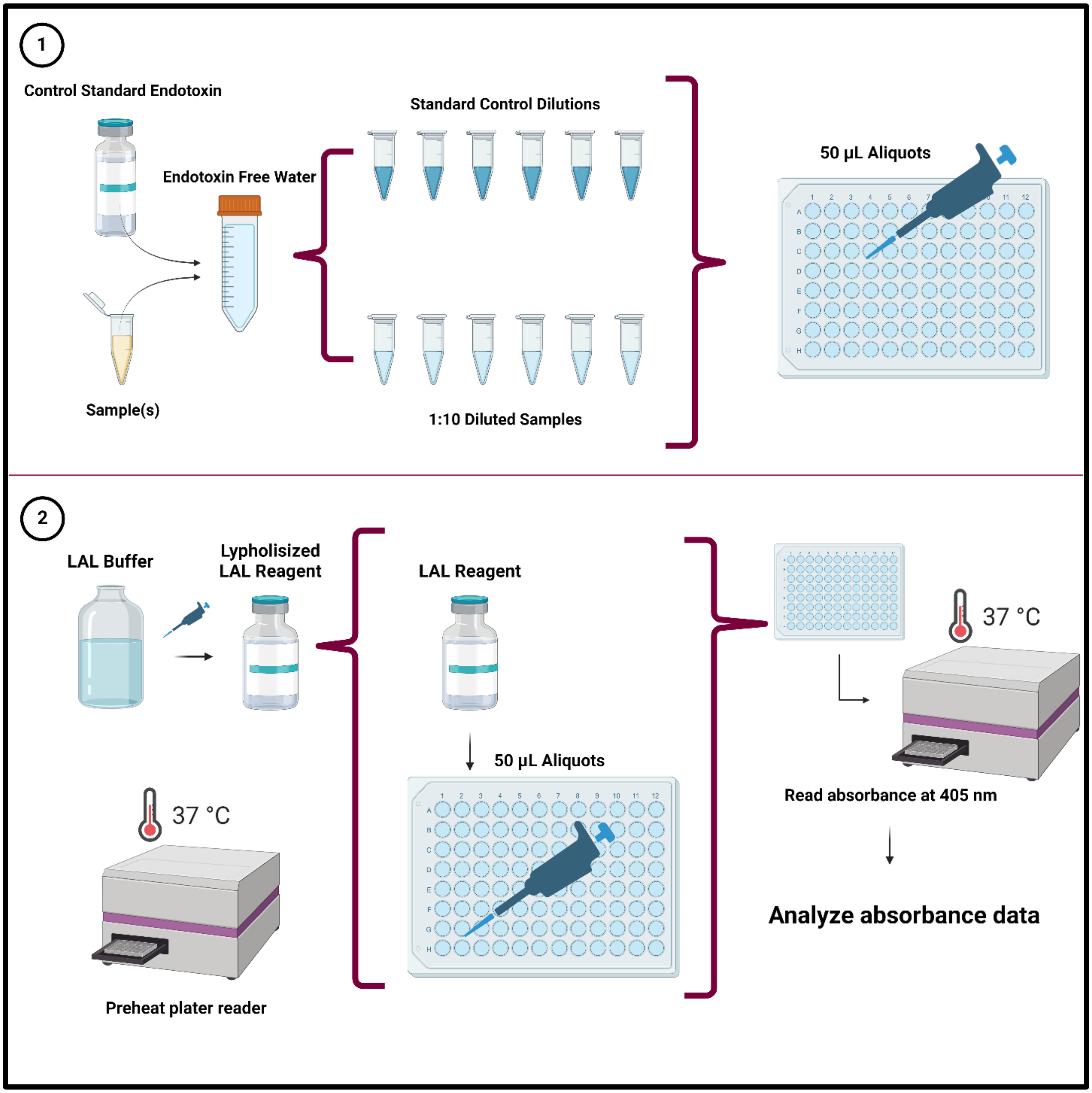

Figure 2.2.8: Procedure used to quantify endotoxin concentration (EU/mL) using the Pyrochrome Limulus Amebocyte Lysate (LAL) assay. **1)** Standard dilutions are prepared, and samples are diluted 10-fold using endotoxin free water before being pipetted into a transparent 96-well plate. **2)** Plate reader is preheated to 37°C, while the LAL reagent is resolubilized and pipetted to each sample containing well in the 96-well plate. Once LAL has been added to each well and the plate reader has reached temperature, absorbance at 405 nm is quantified every minute over a predetermined period.

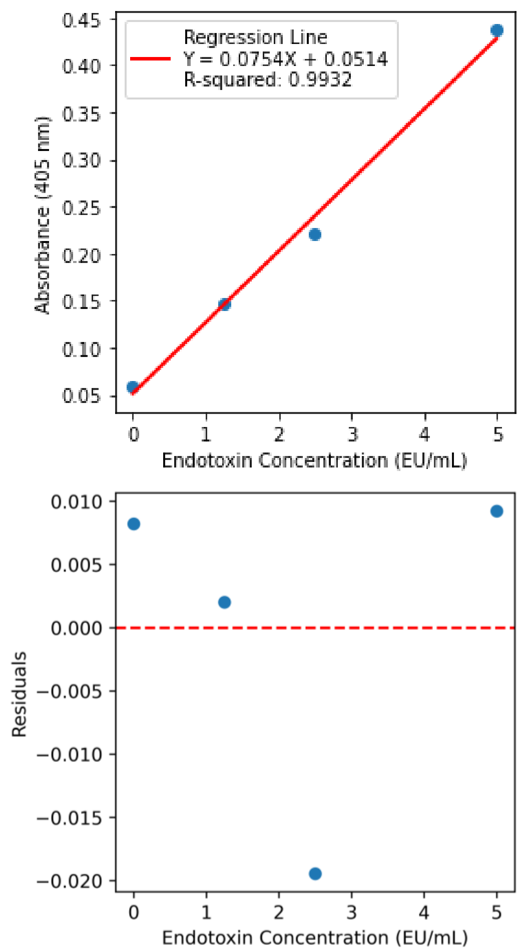

Figure 2.2.9: Calibration plots with averaged residuals for 5 – 0.125 EU/mL loading solution used for endotoxin quantification during the background binding high-throughput screening experiment using SK2 bacteriophage preparation. All controls were run in triplicate.

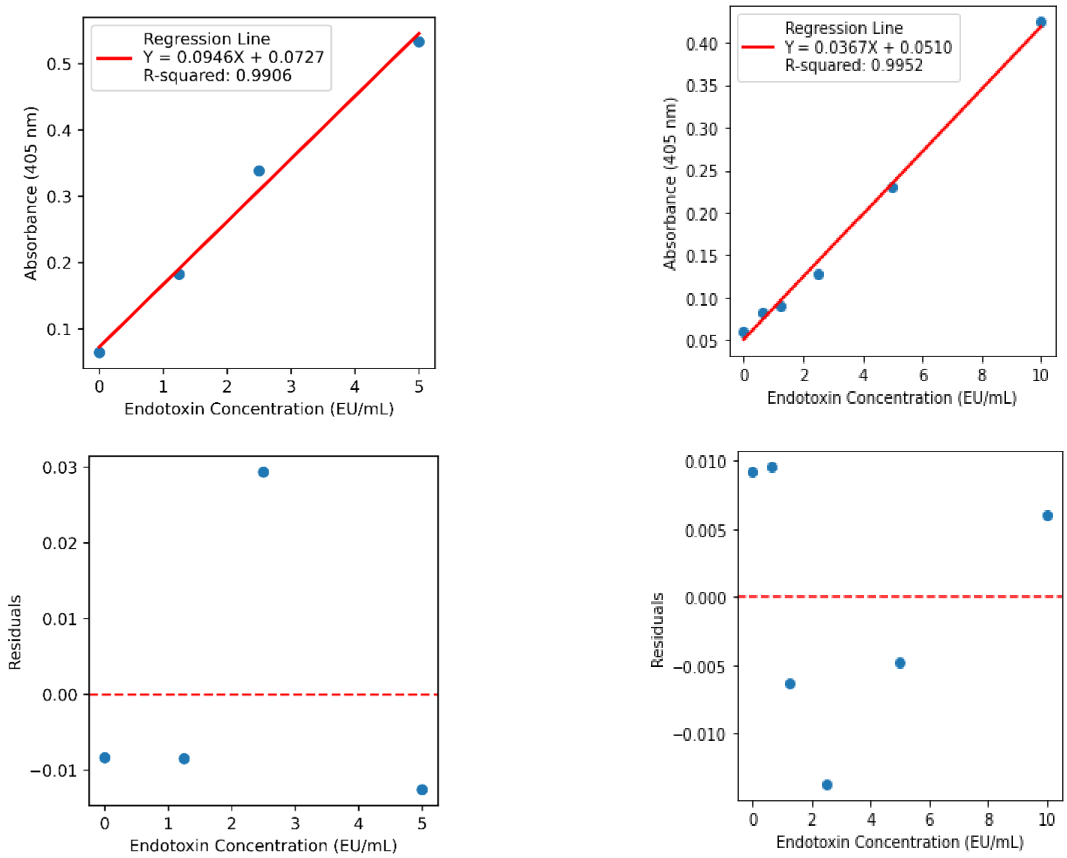

Figure 2.2.10: Calibration plots with averaged residuals for 5 – 1.25 or 10 – 0.625 EU/mL loading solution used in the ionic strength high throughput screening experiments using both SK2 (A) and J1MH1 (B) preparations.

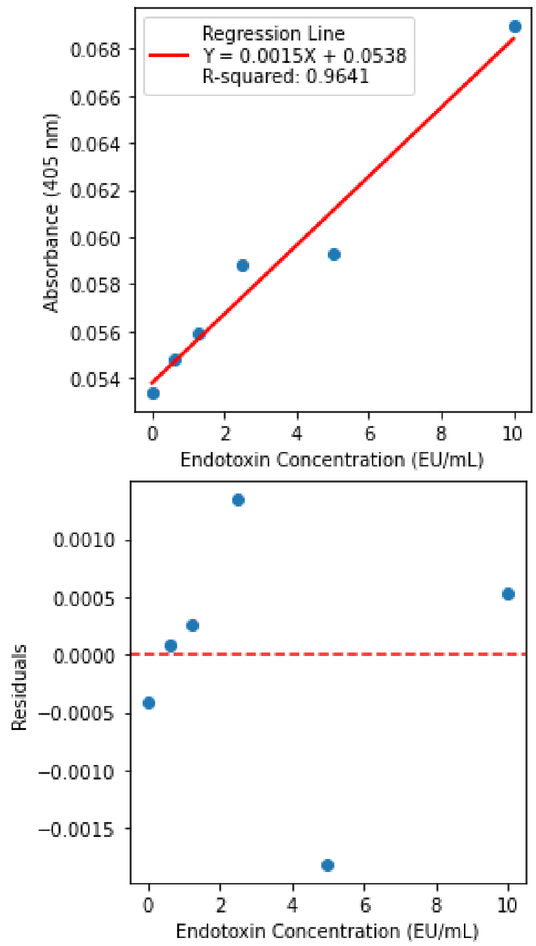

Figure 2.2.11: Calibration plots with averaged residuals for 10 – 0.625 EU/mL loading solution used in syringe filtration experiment using both SK2 and J1MH1 preparations.

3 Results and Discussion

3.1 Bacteriophage Temperature Stability

The effects of storage conditions on bacteriophage J1MH1 over a 2-month period are shown in **Figure 3.1.1**. Storage in phage buffer (4°C) resulted in the lowest loss of titer over the 2-month period, resulting in an average log reduction value (LRV) of 0.23. Storage in 15 % (v/v) glycerol – phage buffer solution (-20°C), 0.8 M sucrose -phage buffer (-20°C), and phage buffer (- 20°C), caused increasingly larger associated average LRVs of 0.30, 1.27, and 1.83 respectively. These results align with findings by Alvi et al which found that storage of bacteriophage in LB at 4°C resulted in the least loss of titer over the course of a year for 5 different phage species [70]. Additionally, storage of bacteriophage SK2 in phage buffer at 4°C was shown to maintain phage titer stability over the course of 1 year (as seen in **Figure A.1.1**), corroborating the results seen with J1MH1. As such, future stocks of bacteriophage would be stored in phage buffer at 4°C, as it not only resulted in the low loss of titer, but stocks in these conditions did not require a time-consuming thawing step, improving the ease of access to the stocks when needed which suited the needs of this work. Its important to note, that if titer stability across periods longer than 1-year is required, more long-term storage studies should be conducted. A study by Gonzalez – Menendez et al found that titer stability across 24 months in various storage conditions at storage temperatures of 4°C, -20°C, and -80°C using 4 bacteriophages was highly variable, with LRVs ranging from $<$ 1 – 3 [68]. As such, storage conditions should be individually optimized for each phage if long-term storage is necessary.

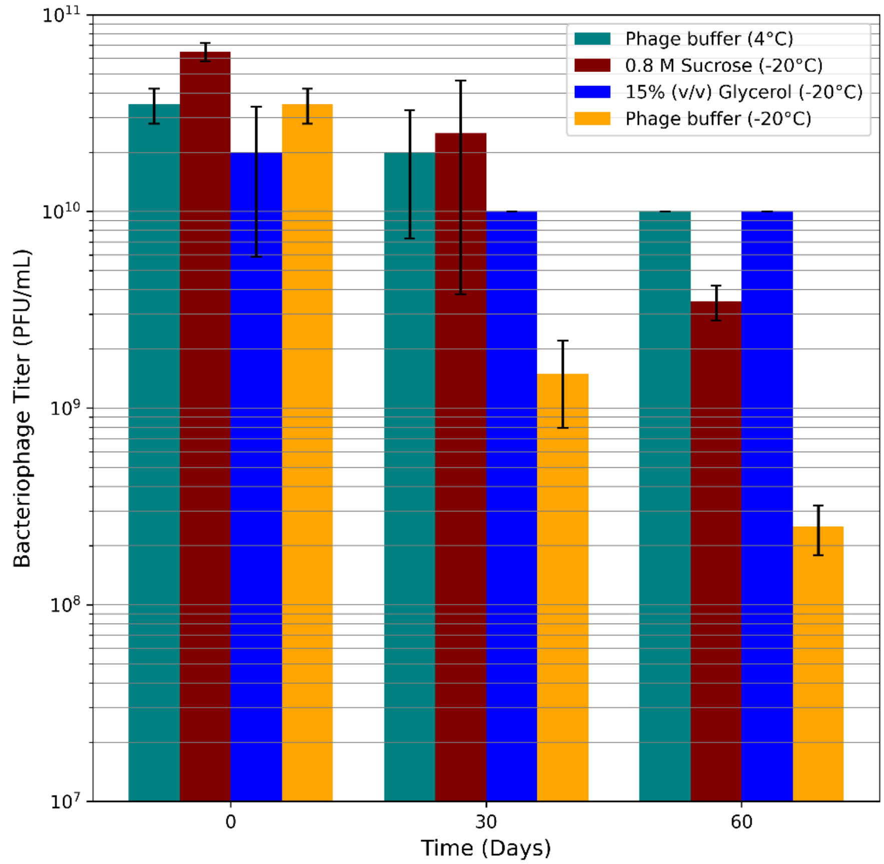

Figure 3.1.1: J1MH1 phage titer (PFU/mL) in various solution conditions across a study period of 60 days. Titer was quantified every 30 days. Error bars represent the standard deviation about the average of duplicate measurements.

3.2 High-throughput Screening Studies

3.2.1 Effect of dilution on bacteriophage binding

Preliminary high-throughput screening work carried out using J1MH1 sought to assess if binding of phage to an AEX membrane Natrix Q in phage buffer could be quantified and if any loss of titer to background binding (*i.e* to the device) or ambient conditions (room temperature) occurred**.** These early screening results indicated a lack of binding of phage to the membranes. These results also suggested that no titer is lost to background binding and ambient conditions as seen in **Figure A.2.1**

Following these preliminary results, a J1MH1 static binding experiment was conducted to examine how initial titer or dilution factor could affect bacteriophage binding to the Natrix Q membrane. **Figure 3.2.1** compares the average titer between no membrane controls vs membrane samples (top figure) and average LRV (bottom figure) achieved using four different preparations of J1MH1 feedstock at 6 h of contact time. These samples are the undiluted J1MH1 feedstock and 3 serial dilutions of that original feed stock (factor of 1:10, 1:100, 1:1000 respectively) representing preparations at initial titers of ~ 10^9 ($7.50 \times 10^9 \pm 5.00 \times 10^8$), 10^8 ($1.50 \times 10^8 \pm 7.00 \times 10^7$), 10^7 (4.00×10^7), and 10^6 ($2.50 \times 10^6 \pm 5.00 \times 10^5$) PFU/mL. The preparation representing a 1:1000 dilution (10^6 PFU/mL) of the feed stock generated an average LRV of 1.64, considerably higher than all other dilutions, which produced significantly smaller LRV's ranging from – 0.30 to 0.27. Additional experiments, at a contact time of 72 h partially corroborated these results, also showing that a 1:100 dilution factor produced a comparable LRV to a 1: 1000 dilutions as shown in **Figure A.2.2**. These results demonstrate that increasing the dilution of the bacteriophage preparation used in the high throughput screening studies improves the binding of phage to AEX membranes, with the best performance seen at a 1:1000 dilution of the bacteriophage stock.

Sterile filtered cell culture supernatant generally contains a significant amount of salt and other low molecular weight substances that can alter ionic strength unexpectedly and hinder bacteriophage binding to ligands. A study performed by Roshankhah et al, which discussed the use of an improved membrane chromatography device for the purification of phage T7, found that dilution of their phage feed was necessary to maximize phage binding to the AEX membranes, determining a 1:20 dilution using binding buffer to be optimal. [52]. This is further supported by an additional high-throughput screening experiments using Natrix Q at 24 h of contact time, which showed that controlled for titer (10^6 PFU/mL) SK2 preparation diluted with phage buffer (1: 1000 dilution) experienced significantly greater removal and therefore greater binding to the membrane, as compared to un-diluted lysate (as seen in **Figure A.2.3**). Although rapid saturation of membranes with bacteriophage may play a role in the poor removal performance seen with higher-titer, less diluted preparations, it is much more likely that the presence of a larger number of anionic elements in raw lysate significantly confound the binding of bacteriophage to membrane, with this effect being moderated through dilution. As such, all future high-throughput screening work would be conducted using bacteriophage preparations diluted by a factor of 1:1000 dilution factor, assuming the initial titer of a given feedstock was in the range of 10^9-10^{10} PFU/mL.

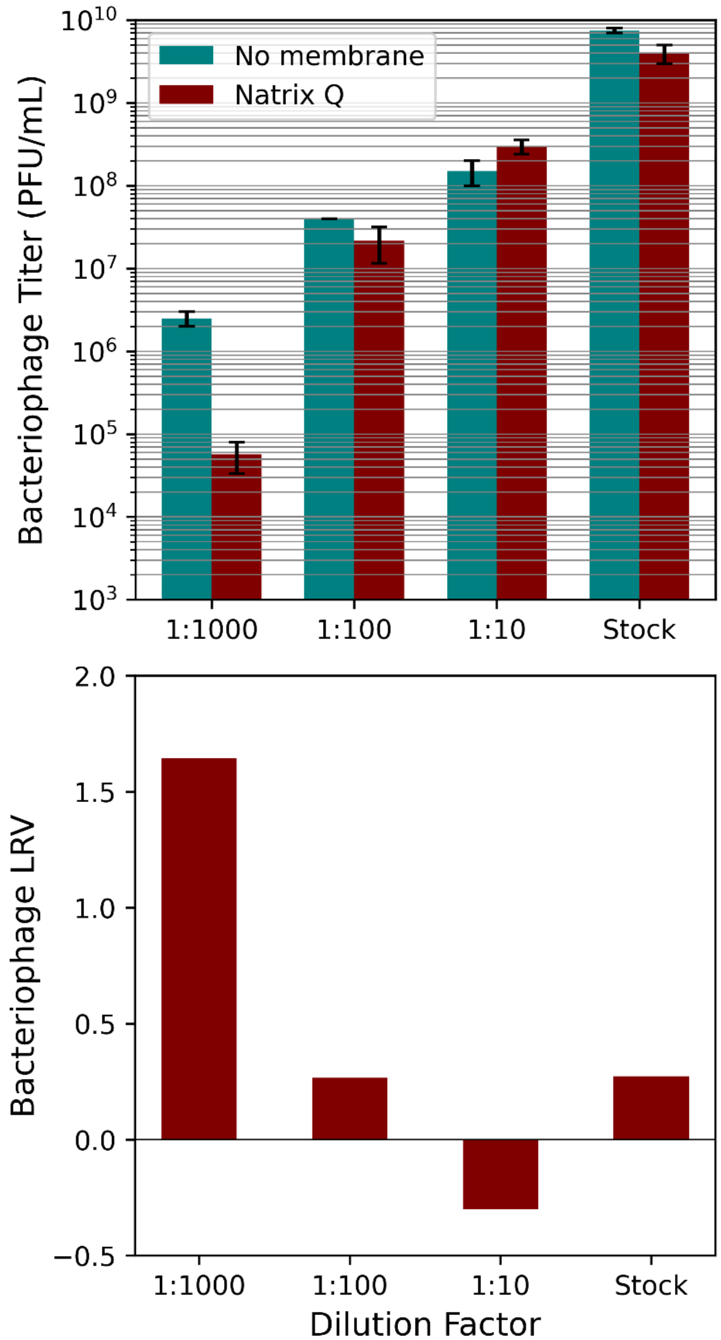

Figure 3.2.1: Effect of dilution on J1MH1 removal (LRV) using the Natrix Q AEX membrane at 6 h of contact time. A comparison of no membrane control and Natrix Q titer shown above. Error bars refer to standard error about the average (titer) of triplicate experiments. Each dilution represents an initial phage titer as follows; 1:1000 ($2.50 \times 10^6 \pm 5.00 \times 10^5$ PFU/mL), 1:100 (4.00×10^7 PFU/mL$^)$, 1:10 ($1.50 \times 10^8 \pm 7.00 \times 10^7$), Stock ($7.50 \times 10^9 \pm 5.00 \times 10^8$ PFU/mL).

3.2.2 Effect of time on bacteriophage binding

After optimizing sample preparation for high throughput screening experiments a study of SK2 binding to 3 AEX membranes (Natrix Q, Sartobind Q, Mustang Q) was carried out to better understand how adsorption of phage to this membrane were affected by contact times using our multi-well device. All experiments used SK2 at an initial titer of ~ 10^6 PFU/mL (1:1000 dilution of stock).

Figure 3.2.2 describes the binding of SK2 to the three membranes over a 24-hour period. SK2 removal using all 3 membranes increased over the 24-hour period, with average LRVs of 1.68, 1.50, 0.94 for Natrix, Sartobind, and Mustang Q respectively after 24 h of contact time. Removal of SK2 using Natrix Q increased the most rapidly, having the greatest LRVs at 1.5, 3, and 24 h of contact time as compared to the other 2 membranes. The sudden decrease in LRV at 6 h of contact from 0.91 at 3h to 0.53 LRV at 6 h is unexpected and seemingly contradicts the gradual increase in removal seen between prior time periods. This is primarily caused by unexpected variation seen between 2 sets of experiments. The average LRV at 6 h of contact time using Natrix Q was generated using values from 2 separate experiments (all samples and controls run in triplicate for both). When average LRVs are calculated for each independent experiment, experiment 1 produces an LRV of 0.33, while experiment 2 produces a 1.46 LRV, with the latter value in line with expectations as it is much closer to the LRV seen at 24 h and greater than the value seen at 3h. It remains unclear why the results in experiment 1 differed so greatly. Removal of SK2 using Sartobind Q exhibits a less gradual trend, remaining low initially, only increasing to 0.18 LRV over 3 h, before removal sharply increasing to an average LRV of 1.13 at 6 h. Mustang Q exhibits a similar trend with removal increasing to an average LRV of 0.22 at 6 h, before sharply increasing to 0.94 at 24 h. Interestingly, removal at 6 h for both Natrix Q (excluding values from experiment 1) and Sartobind Q seemed comparable to values at 24 h.

To understand the relative productivities of the membranes, a comparison was drawn between the average titer of SK2 at 24 h of contact time for each membrane (Natrix, Sartobind, Mustang) and the average titer of all controls across each membrane as seen in **Figure 3.2.3**. The average no membrane titer (in this case representing initial titer) of SK2 across all experiments was $2.19 \times 10^6 \pm 3.36 \times 10^5$ with average titer decreasing to $4.50 \times 10^4 \pm 1.12 \times 10^4$, $6.33 \times 10^4 \pm 3.50 \times 10^{4,}$ and $2.97 \times 10^5 \pm 2.52 \times 10^5$ PFU/mL using Natrix, Sartobind, and Mustang Q respectively. SK2 removal was greatest with Natrix Q supported by an associated average LRV of 1.68, although removal by Sartobind Q was comparable with an associated average LRV of 1.50, with the average titer of both samples being within the same order of magnitude. Removal by Mustang Q was noticeably lower when compared to the performance of the former membranes, achieving a smaller LRV of 0.98. These results demonstrate the higher productivity of Natrix and Sartobind Q as compared to Mustang Q, in the context of bacteriophage binding.

These differences in performances partially align with expectations. In the context of virus removal (Minute virus of mice (MVM) specifically) Mustang Q has been shown to have a lower ion-exchange binding capacity than that of Sartobind Q when assessing both static and dynamic binding capacity [71] . This supports the superior SK2 binding of Sartobind Q as compared to Mustang Q found in this study. Natrix Q is described as a porous, functionalized 3-dimensional polyacrylamide hydrogel supported by an inert polymeric web support. As such, when exposed to solution, Natrix Q swells to a significant degree [65]. Although, it remains unclear why SK2 binding to Natrix Q was more rapid than to Sartobind Q it can be surmised that the greater membrane volume from the drastic swelling of Natrix Q, may increase binding through the reduction of steric hinderance and spatial repulsion between individual bacteriophage [51], [72]. Additionally, the structures of membranes as seen in cross sectional SEM images in **Figure 2.1.1** may also corroborate this claim as both Natrix Q and Sartobind Q have less dense structures as compared to Mustang Q upon visual inspection. This may allow larger biomolecules such as phages to bind to a greater degree. Overall, this work demonstrates that phage binding to membranes progressively increases with as function of time over 24 h and supports that phage binding was greater to Natrix and Sartobind Q (as compared to the Mustang Q), making them ideal candidates for evaluation in later high-throughput screening experiments.

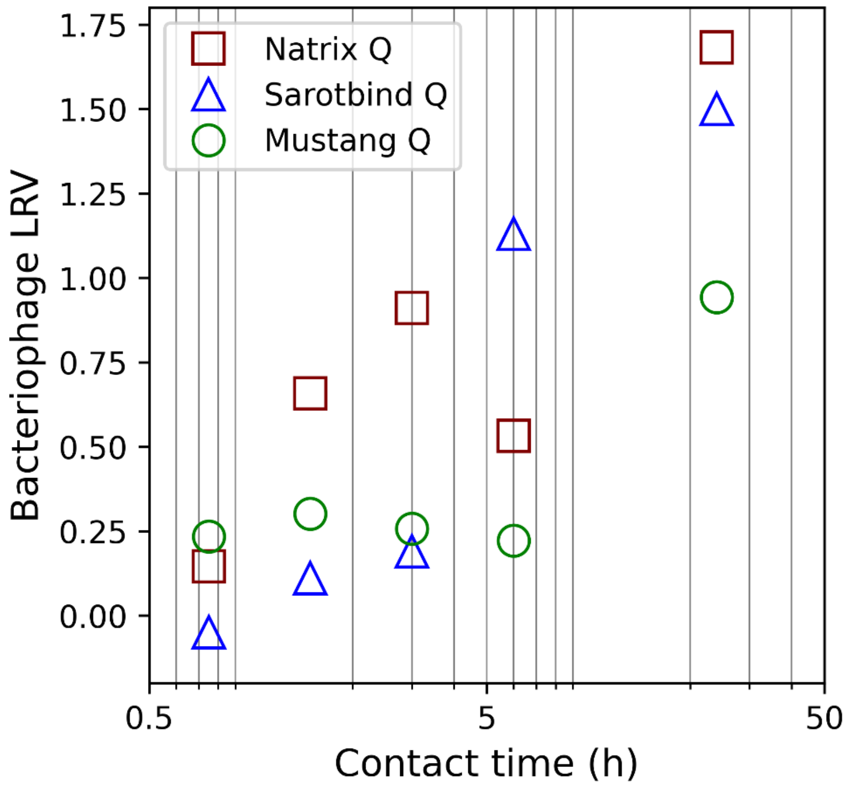

Figure 3.2.2: A comparison of SK2 Removal (LRV) at an initial titer of $\sim 10^6$ PFU/mL after 0.75, 1.5, 3, 6, 24 h of contact time using Natrix Q **(**□**)**, Sartobind Q **(**Δ**)**, and Mustang Q **(**○**)** in the multi-well device.

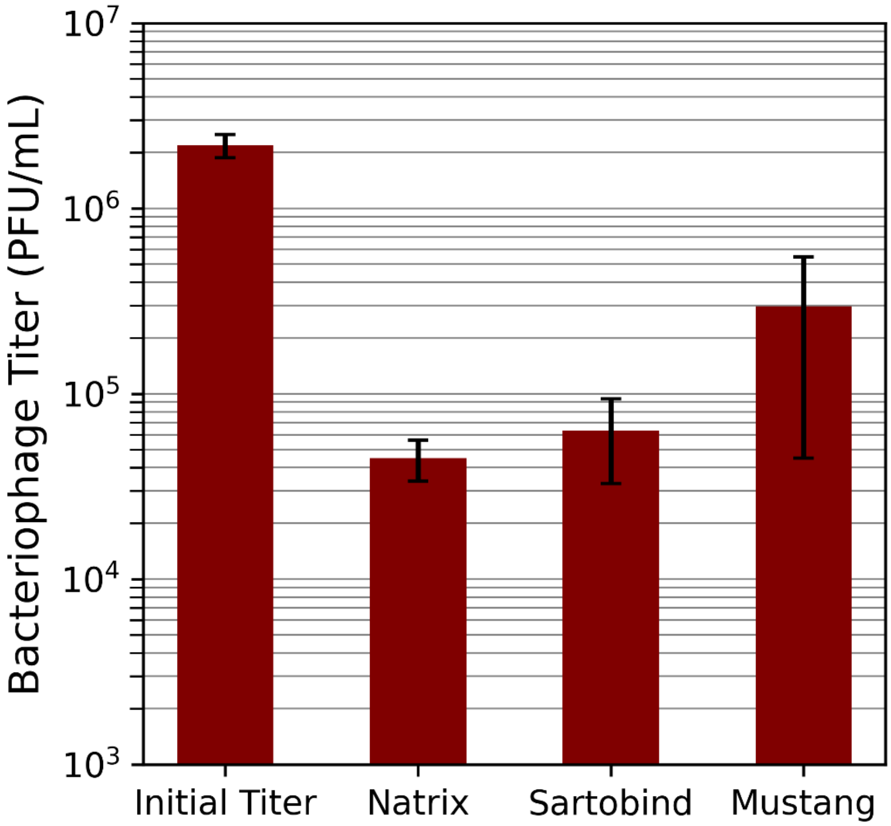

Figure 3.2.3: Comparison of the average bacteriophage (SK2) titer (PFU/mL) between membrane types (Natrix Q, Sartobind Q, Mustang Q) and control with standard error bars of replicate experiments after 24 h of contact time in phage buffer. Error bars for initial titer refer to standard error about the average (titer) of 15 experiments, while with Natrix and Sartobind it represents standard error about the average of 6 experiments. Error bars for Mustang represent standard error about the average of 3 experiments.

3.2.3 Solution Chemistry Effects

A key goal of this project was to evaluate the potential of using this multi-well device to screen and optimize solution conditions (conductivity, pH…) for larger scale purification work. This study sought to determine if the previously outlined methodology could screen varying solution conditions and generate removal of both endotoxin and phage that was in line with trends seen in the literature. To simplify the design space this study focused solely on the effects of varying NaCl molarities on endotoxins and bacteriophage (SK2 and J1MH1) removal using both Natrix Q and Sartobind Q.

Recall, that endotoxins (IEP = 2) generally will have a larger magnitude of charge than bacteriophage (IEP = 3.5 -7). As such manipulation of the ionic strength (in this case NaCl mM) in solution can introduce electrostatic shield effects, decreasing the magnitude of electrostatic interaction with a given ligand. The biomolecule with a greater magnitude of charge will often be more resistant to these shielding effects at higher ionic strengths, allowing for specific separation of two species. The available literature suggests that bacteriophage and endotoxin binding or removal is high (3.29 and 4.30 average LRV respectively) at NaCl molarities of < 50 mM NaCl (Tris-buffer at pH 7-8). Bacteriophage binding to AEX membranes is generally greatest at ~ 0 mM NaCl and as this concentration is increased to ~50 mM NaCl bacteriophage binding significantly decreases whereas at ≥ 50 mM NaCl little to no observable removal of phage is seen (~ 0 LRV). Endotoxin removal continues as ionic strength increases, but removal slightly decreases at 50 ≤ mM NaCl (4.3 to 3.5 LRV). As such, a range of NaCl molarities (0.068, 10, 30, 50, 100, 200, 300 mM NaCl) was selected to be screened at pH 7 (10 mM Tris-HCl buffer) using both SK2 and J1MH1 bacteriophage preparations as well as the two previously selected AEX membrane (Natrix Q and Sartobind Q) at 6 hours of contact time.

A preliminary experiment characterized background binding of endotoxin to the multi-well device in the previously described buffers. **Table 3.2.1** compares the initial concentration (stocks) of endotoxin to the concentration after 6 h of contact time in the multi-well device, without the use of membranes. The average endotoxin concentrations between the no-membrane samples and the stocks were comparable and within expected differences outside of the 50 mM (42. 5 ± 12 vs 70.3 EU/mL) and 200 mM (26.5 ± 2.0 vs 76.6 EU/mL) conditions. Excluding these results, the average recovery across all conditions is 98 %. Factors such as temperature, endotoxin concentration, and divalent cation concentration effect the aggregation of endotoxins, which in turn affects its activity [60], [73], [74]. All samples quantified in this study had endotoxin concentrations of 2 -10 EU/mL and were quantified under a consistent temperature of 37°C. Although 10 mM of $MgSO_4$ and $CaCl_2$ are in the initial bacteriophage preparation, samples used in this work have estimated $MgSO_4$ and $CaCl_2$ molarities of ~ 0.001 mM because of serial dilutions. The consistency of these factors worked to minimize the variability in measurements introduced by aggregation. Although it remains unclear why the endotoxin concentration of the 50 mM and 200 mM samples differed so greatly from the stocks, experimental error and error introduced by the necessary 1:10 dilutions applied to each sample (ensures endotoxin concentration is in the range of assay), and lack of repeats for stock endotoxin quantification are the likely reasons for the discrepancies.

Table 3.2.1: Comparison of endotoxin concentration between initial stocks and no-membrane samples after 6 h of contact time in multi-well device using SK2 preparation in 10 mM Tris HCl (pH 7.1, 0.068, 10, 20, 30, 50, 100, 200, 300 mM NaCl). All no-membrane sample values are averages of triplicate measurements while initial values for 0 and 10 mM NaCl represent averages of duplicates. Standard error of values is included for these values. Initial concentration values for 20 – 300 mM NaCl represent a single measurement.

NaCl (mM)	Initial Concentration (EU/mL)	No Membrane (EU/mL)	Recovery (% of initial)
0	79.6 ± 4.6	68.6 ± 3.5	86%
10	70.1 ± 5.6	58.0 ± 5.0	83%
20	41.3	39.5 ± 9.8	95%
30	44.6	58.7 ± 2.3	132%
50	70.3	42.5 ± 12.0	60%
100	56.0	56.4 ± 2.1	101%
200	76.6	26.5 ± 2.0	35%
300	58.0	61.3 ± 10.0	106%

Figure 3.2.4 illustrates the endotoxin removal achieved (LRV) at each condition for both membrane types using SK2 preparations. Using Natrix Q, endotoxin removal remained unchanged across < 50 mM NaCl (0.068 – 30 mM NaCl) with an average LRV of 0.26 over this range. At molarities 50 ≤ mM NaCl (50 – 300 mM NaCl), removal decreased to an average LRV of 0.13. The results using Sartobind Q exhibit a similar trend, where endotoxin removal remained unchanged at values < 50 mM NaCl, with an average LRV of 0.61 across this range, while across molarities of 50 ≤ mM NaCl average LRV was calculated to be 0.29. Interestingly, average endotoxin removal across all NaCl molarities using Sartobind Q (0.45 LRV) was greater than Natrix Q (0.20 LRV).

Figure 3.2.5 illustrates bacteriophage removal at all 8 of the conditions using SK2 preparations. Using Natrix Q, as NaCl molarity is increased from 0.068 to 30 mM there is a rapid decline in phage LRV from average values of 0.840 to 0.122, followed by negative average LRVs at 50 ≤ mM NaCl indicating little or no removal of phage at these salt concentrations. The average phage LRV for molarities < 50 NaCl was 0.395 LRV while for 50 ≤ NaCl mM it was significantly lower at -0.144 LRV. This trend is conserved in the Sartobind Q results, where phage removal gradually decreased from 1.49 to 0.08 LRV across concentrations of 0.068 to 30 mM NaCl before dropping to LRVs ranging from -0.09 to 0.02 across 50 ≤ mM NaCl, again indicating little or no removal of SK2. The average phage removal seen across molarities < 50 mM NaCl was 1.077 LRV while for 100 – 300 mM NaCl it was significantly lower at 0.0011 LRV. Like the endotoxin removal results, average bacteriophage LRV was higher across all conditions evaluated using Sartobind Q (0.544 LRV) as compared to Natrix Q (0.125 LRV).

Figure 3.2.6 illustrates the endotoxin removal achieved at each condition for both membrane types using J1MH1 preparations. Using Natrix Q, endotoxin removal across NaCl molarities < 50 mM NaCl was greater than removal across molarities 50 ≤ mM NaCl, with an average LRV of 0.51 compared to 0.21. The Sartobind Q results exhibit a similar trend, as average endotoxin LRV across concentrations < 50 mM NaCl was calculated to be 0.80, higher than the average LRV across 50 ≤ mM NaCl calculated to be 0.40. Again, average endotoxin removal across all conditions using Sartobind Q (0.45 LRV) was greater than Natrix Q (0.20 LRV).

Figure 3.2.7 illustrates bacteriophage removal using J1MH1 preparations. Using Natrix Q, as NaCl molarity was increased, bacteriophage removal remains consistent across all conditions outside of the results seen at 10 mM NaCl (LRV of -0.22). The average phage LRV for molarities < 50 mM NaCl was 0.11, while across values of 50 ≤ mM NaCl it was slightly higher at 0.21 LRV, this in contrast to the trends seen using SK2. Interestingly, the trend seen using Sartobind Q differed significantly, as the average phage LRV at NaCl molarities < 50 mM NaCl was 0.33, while across 50 ≤ mM NaCl it was significantly lower at an average of 0.01, better reflecting trends seen using SK2 preparation. Interestingly, average bacteriophage LRV was similar across all conditions evaluated when comparing Sartobind Q (0.17 LRV) as compared to Natrix Q (0.16 LRV).

As flow through membrane chromatography (removal of endotoxin but not phage) is of particular interest when working with larger biomolecules such as phages, a comparison of endotoxin membrane binding capacity was conducted. **Table 3.2.2** compares the endotoxin static binding capacity (SBC, EU bound/ mL membrane) of Natrix and Sartobind Q derived in this work to that of reported or calculated dynamic binding capacities (DBC) from literature (studies in **Table 1.2.2**). Endotoxin DBC values for both membranes were larger than the SBCs found in this work with values generated using Sartobind Q closer to expectations (factor of 10-100 X lower than DBC) as compared to Natrix Q SBC values, which were considerably smaller than the DBC value reported by the manufacturer (factor of 10^3 – 10^4 X lower than DBC) [58]. Although reported

DBC values for Natrix Q are larger than those reported or calculated for Sartobind Q (10^6 vs 10^4 EU/mL membrane), results from the screening experiments found that the SBCs were comparable when controlling for NaCl molarity.

As reported in literature, no or little removal of bacteriophage by AEX membranes occurs at NaCl molarities above 50 mM NaCl, with removal of phage inversely proportional to NaCl salt concentrations across values < 50 mM NaCl. Removal of endotoxin is significantly less affected by changes in NaCl molarities below 300 mM NaCl, with changes in average LRV below and above 50 mM NaCl much smaller than seen with phage. As such, 50 mM NaCl is viewed by this work as a key value around which analysis is conducted.

Endotoxin removal data generated using 0.068 – 300 mM NaCl buffer (pH 7) with the multi-well device seem to reflect the trends reported in literature. Comparing endotoxin LRVs from generated from solution conditions containing NaCl molarities < 50 mM NaCl (0.068 – 30 mM NaCl) to 50 ≤ mM NaCl (50 - 300 mM NaCl) across all other dependent variables (phage type and membrane), average LRVs at conditions within the former range are slightly higher than those found in the latter range with that difference ranging from 0.13 – 0.4 LRV. Although, this calculated change in LRV differs in magnitude from that calculated from literature (change of 0.6 reported in literature) it still follows reported trends, which is that of a moderate drop in average endotoxin LRV above molarities of 50 mM NaCl. As such, although the magnitude of average endotoxin LRVs seen in this work were lower than values reported in literature, the change of endotoxin removal in response to increases in ionic strength fell within expectations. Specifically, these results align with findings reported by Leuthold, which reported an endotoxin LRV of 3.5 using buffer conditions with NaCl molarities < 50 mM with a 0.5 decrease in average LRV to 3.0 when that concentration is increased above 50 mM NaCl (pH = 7-8) specifically using endotoxin spiked buffer and Sartobind Q [53]. The change in LRV here more closely reflects that found using Sartobind Q (change in average LRV of 0.32 for SK2 and 0.40 for J1MH1) in this work.

Removal of SK2 bacteriophage progressively declined as NaCl molarity increased, with effectively no or little removal (no binding of phage to membrane) in conditions above 50 mM NaCl irrespective of the membrane used. These reported trends seen using SK2 preparation are near identical to findings in literature such those reported by Leuthold who found that an average bacteriophage LRV of 2 across molarities < 50 mM NaCl, with that value dropping to 0 above 50 mM NaCl [53]. Using J1MH1, bacteriophage removal using Natrix Q remained consistent across all conditions, other than 20 mM NaCl, where no removal was observed. While using Sartobind Q, the broad trends were like that seen with the SK2 removal results, therefore more in line with expectations. It is unclear why average bacteriophage removal sharply increased as ionic strength increased from 0.068 – 20 mM NaCl, as removal is expected to be higher at ionic strengths closer to 0 mM NaCl. As discussed previously, strain homogeny concerns surrounding J1MH1 necessitated the switch to SK2 bacteriophage as the primary sample in an earlier study. As such, the presence of additional phage types with differing physiochemical properties may have confounded the phage removal results for J1MH1, explaining the unexpected trends seen for both the Natrix Q and Sartobind Q datasets and the lack of consistency between them. Interestingly, excluding the results associated with J1MH1 removal, Sartobind Q produced the greater average removal (LRV) of endotoxins and bacteriophage across all conditions evaluated. It is important to note that this does not necessarily demonstrate that Sartobind Q is the ideal membrane for later larger scale purification work as the goal is to maximize endotoxin removal while retaining (*i.e* low removal) as much of the initial phage titer as possible from a given feed stock.

The discrepancy in magnitude of endotoxin and phage LRV seen using in this work as compared to those reported in literature can be partially attributed to feed stock or sample preparation. Currently, endotoxin spiked buffers are the most popular form of feedstock used when assessing endotoxin removal performance for a given AEX membrane or process rather than cell or phage lysate (raw or diluted). This reduces the amount (or eliminates) of additional host cell derived anionic species such as host cell proteins, RNA, and DNA which could compete with endotoxins (and phage) for ligands present in the membranes [56]. Additionally, groups studying bacteriophage removal are sometimes unclear about how the phage feed stocks are prepared. Studies which explicitly utilize cell lysate preparations show better agreement with the results of this work. For simple comparison, groups utilizing Sartobind Q have found that bacteriophage LRV at ~ 0 mM NaCl to be in the range of 1-2 LRV, while endotoxin LRV to be ~ 1.4 LRV. These values are much closer to the 0.64 (SK2), 0.75 (J1MH1) endotoxin LRV's and 1.49 SK2 phage LRV achieved in this work using Sartobind Q [52], [53]. Additionally, as these groups used different bacteriophage strains to the ones used in this study, differences in removal performance are expected. Overall, these differing removal performances highlight a need for broader applications of bacteriophage lysates or more representative samples in this space if a more comprehensive understanding of the fundamental interactions between membranes, phages, and endotoxins are to be developed.

The discrepancy between DBCs and SBCs can be caused by a wide range of factors such as sample type (lysate vs spiked buffer), differences in binding capacity calculation and determination, and the influence of flow paths. Interestingly, calculated SBC values using Sartobind Q were more like reported DBC values (10^2- 10^3 vs 10^4 EU/mL membrane) as compared to Natrix Q (10^2-10^3 vs 10^6 EU/mL membrane), although SBC values were similar across both membrane types controlling for phage type and NaCl molarity. Oddly, the SBC of both membranes was noticeably lower at solution conditions ≥ 50 mM NaCl as compared to values < 50 mM NaCl, when using SK2 (decrease from ~ 10^3 to 10^2 EU/ mL membrane) but not J1MH1 (binding capacity remains at ~10^3 EU/mL membrane). The larger difference in SBC seen using SK2 above 50 mM NaCl unexpected considering little SK2 is bound at NaCl molarities ≥ 50 mM, in theory producing a greater capacity to bind endotoxins.

The final aim of this study (as reported in Section 3.3) was to assess the translatability of the data collected using the high-throughput screening technique to a larger scale purification system (ex. Syringe filtration). As such, a limited evaluation was conducted, first identifying an "optimal" NaCl molarity where significant amounts of endotoxin would be expected bind to the membrane, while bacteriophage would not. Retaining as much phage as possible was important, as such, Natrix Q was chosen as the initial membrane to evaluate as average phage LRV was lower when compared to Sartobind Q across all conditions evaluated in the high-throughput screening work. Average bacteriophage LRV at 30 mM NaCl for SK2 and J1MH1 were lower (0.12 and 0.24 LRV respectively), than the corresponding endotoxin LRVs (0.21 and 0.72 respectively) using Natrix Q, meaning a greater proportion of endotoxin was expected to bind to the membrane. Additionally, the potential of 30 mM NaCl seemed to agree with the trends reported in literature as endotoxin removal is expected remain quite high at this ionic strength (~ 4.3 LRV) while bacteriophage removal should be low in comparison. As such, 30 mM NaCl (10 mM Tris-HCl, pH 7) using Natrix Q were selected as the operational parameters for the larger scale purification experiment. Inspired by literature, a flow through mode of purification, specifically syringe filtration, was chosen. The results and discussion pertaining to this work can be found in Section 3.3.

Overall, this study demonstrated that the multi-well device can be used to study the effects of varying NaCl molarity on endotoxin and phage binding to AEX membranes. Endotoxin removal data generated using both SK2 and J1MH1 preparation generally showed good agreement with the trends found in literature, with partial agreement being found with the bacteriophage removal data. Despite these promising results, the average endotoxin and bacteriophage LRVs found in this work were significantly lower than expected.

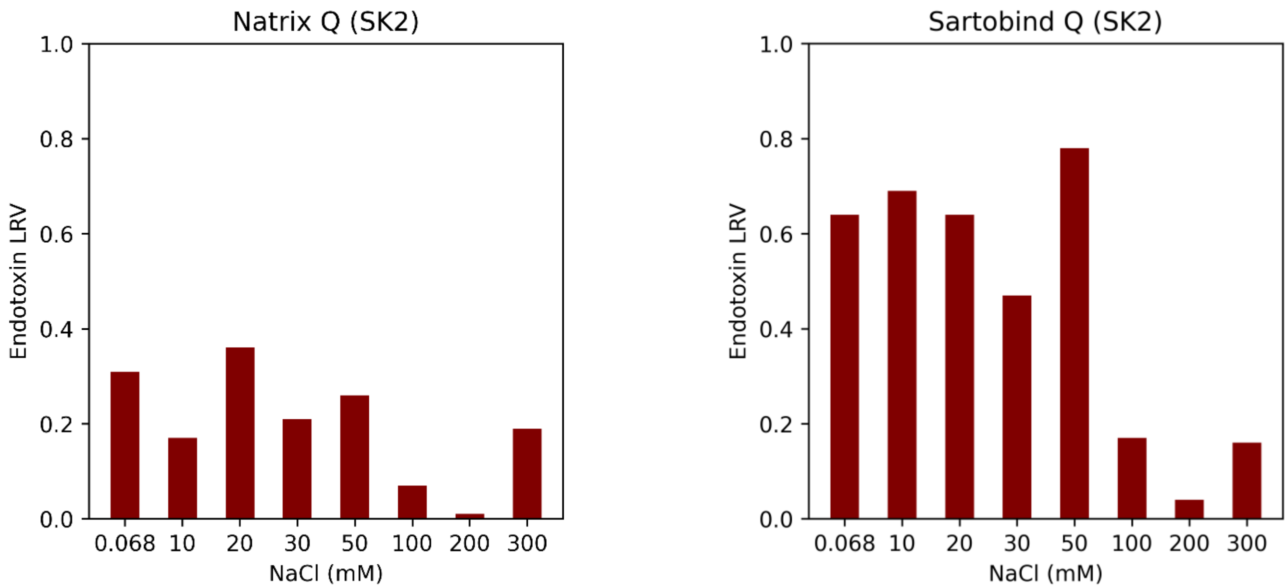

Figure 3.2.4: Comparison of average endotoxin LRVs generated using Natrix Q and Sartobind Q with SK2 bacteriophage preparation in the multi-well device after 6 h of contact time in 10 mM Tris HCl (pH 7.1, 0.068, 10, 20, 30, 50, 100, 200, 300 mM NaCl). All samples were run in triplicate, while controls were run in duplicate.

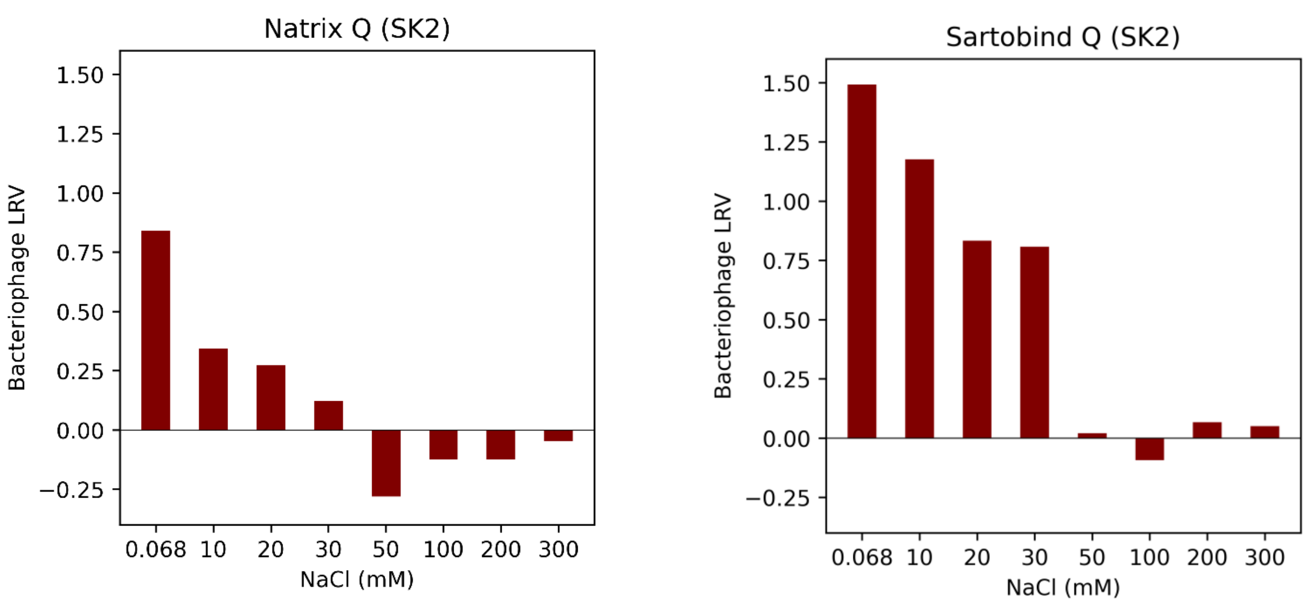

Figure 3.2.5: Comparison of average phage LRV generated using Natrix Q and Sartobind Q with SK2 bacteriophage preparation in the multi-well device after 6 h of contact time in 10 mM Tris HCl (pH 7.1, 0.068, 10, 20, 30, 50, 100, 200, 300 mM NaCl). All samples were run in triplicate, while controls were run in duplicate.

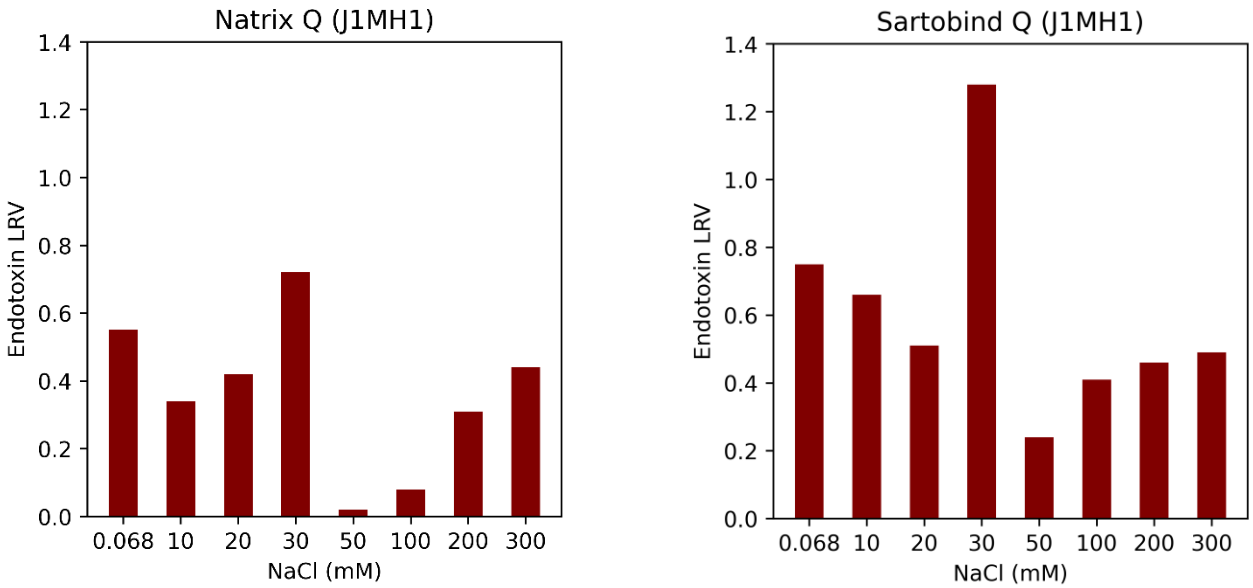

Figure 3.2.6: Comparison of average endotoxin LRV generated using Natrix Q and Sartobind Q with J1MH1 bacteriophage preparation in the multi-well device after 6 h of contact time in 10 mM Tris HCl (pH 7.1, 0.068, 10, 20, 30, 50, 100, 200, 300 mM NaCl). All samples were run in triplicate, while controls were run in duplicate.

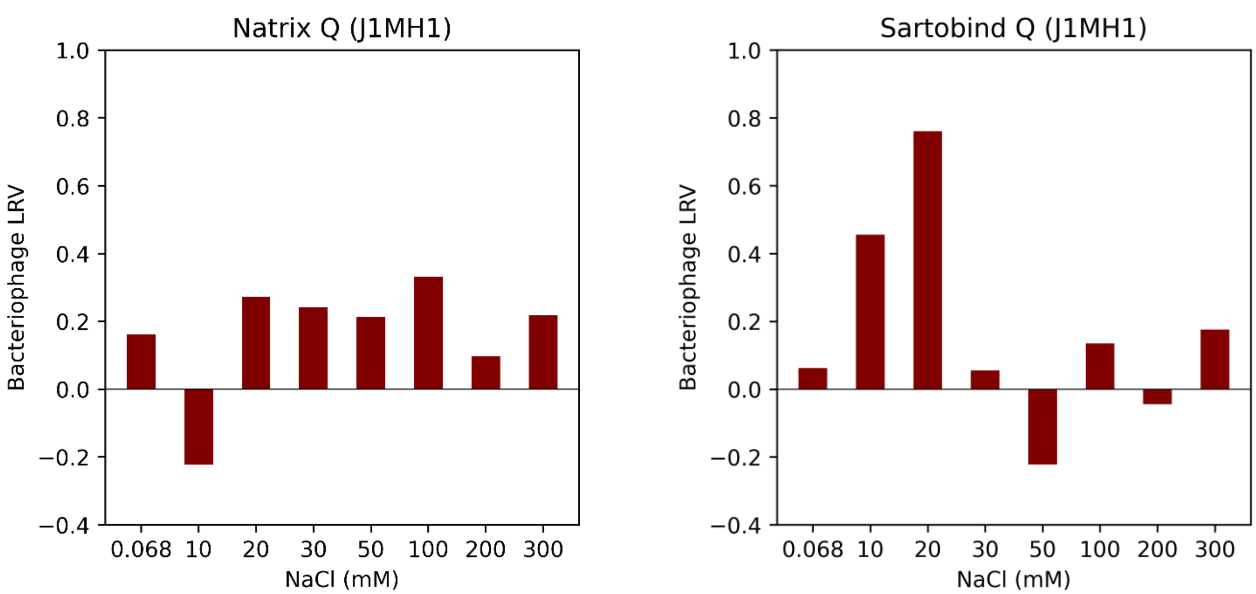

Figure 3.2.7: Comparison of average phage LRV generated using Natrix Q and Sartobind Q with J1MH1 bacteriophage preparation in the multi-well device after 6 h of contact time in 10 mM Tris HCl (pH 7.1, 0.068, 10, 20, 30, 50, 100, 200, 300 mM NaCl). All samples were run in triplicate, while controls were run in duplicate.

Table 3.2.2: Comparison of calculated endotoxin SBC with standard error to reported or calculated DBCs for the Natrix and Sartobind Q membranes at given ranges of NaCl molarity using Tris-HCl buffer at pH 7-8. DBC for Sartobind Q are averages of reported or calculated values from literature derived from literature in **Table 1.2.2**. DBC values for Natrix Q were reported by the manufacturer. SBC values derived using SK2 are on top of the values derived using J1MH1.

Membrane	NaCl (mM)	SBC (EU/mL membrane)	DBC (EU/mL membrane)
Natrix Q	< 50	$1.01 \times 10^3 \pm 2.24 \times 10^2$ **(SK2)** $2.90 \times 10^3 \pm 4.07 \times 10^2$ **(J1MH1)**	9.00×10^6 [58]
	≥ 50	$1.10 \times 10^2 \pm 1.58 \times 10^2$ $2.32 \times 10^3 \pm 4.46 \times 10^2$	9.00×10^6 [58]
Sartobind Q	< 50	$2.45 \times 10^3 \pm 1.57 \times 10^2$ $4.20 \times 10^3 \pm 5.57 \times 10^2$	2.36×10^4
	≥ 50	$5.47 \times 10^2 \pm 4.03 \times 10^2$ $3.37 \times 10^3 \pm 6.00 \times 10^2$	1.72×10^4

3.3 Scaling up from high throughput format to syringe filtration

As mentioned in Section 2.2.5 membranes were pre-wet using 20 mL of 30 mM NaCl 10 mM Tris-HCl (pH 7) buffer as the absence of prewetting results in complete removal of quantifiable phage titer as seen in **Figure A.3.1**. The final experiment of this work sought to examine the translatability of a select solution condition from the previous screening study to a larger scale purification modality. As identified previously, 30 mM NaCl (10 mM Tris-HCl, pH 7) – Natrix Q was evaluated. Both SK2 and J1MH1 (1:1000 dilution of stocks) preparations were utilized in this work.

Table 3.3.1 and Table 3.3.2 illustrate the endotoxin removal using both SK2 and J1MH1 preparations. Across both sets of the ten, 1 mL fractions collected, Natrix Q achieved an average LRV of 2.19 using SK2 preparation, higher than the 1.65 average LRV achieved using J1MH1 preparation. Although, the SK2 preparation had a significantly higher initial endotoxin concentration at 782.06 ± 288.79 EU/mL compared to the J1MH1 preparation (184.09 ± 80.40 EU/mL), the average endotoxin concentration across the 10 fractions for both were similar at 5.09 ± 1.80 and 4.25 ± 1.08 EU/mL respectively. Interestingly, complete loss of quantifiable bacteriophage titer was observed for both SK2 and J1MH1 (initial titers of 1.0 x 10^6 and 3.33 x 10^6 ± 8.82 x 10^5 PFU/mL respectively) as described in **Table 3.3.3 and 3.3.4**. Note that the lowest titer of bacteriophage that can be quantified using this spot dilution assay is within an order of magnitude of 10^3 PFU/mL. As such it can be assumed that the Natrix Q membrane in these conditions achieved an average removal of > 3 LRV using both bacteriophage preparations.

The result seen in the filtration study are consistent with data from the literature as buffers with ionic strengths of 0 – 50 mM NaCl (Tris-buffer, pH 7-8) are typically found to achieve an average bacteriophage LRV of 2-7 using AEX membranes, aligning with the > 3 LRV achieved in this work. Alternatively, the endotoxin removal results from this study seem to contradict data specific to Natrix Q. In a communication by Millipore Sigma they report that the Natrix Q could achieve a 4 LRV in 25 mM Tris buffer at both ~ 40 mM NaCl and ~120 mM NaCl in a > 1000 EU/mL endotoxin spiked buffer [58]. As compared to the results in this study which employed diluted lysate, achieving only 1.6 – 2.2 endotoxin LRV using samples with lower initial endotoxin loads of 180 - 780 EU/mL. As mentioned previously, the use of an endotoxin spiked buffer as opposed to lysate may improve the removal of endotoxins as it will lack a significant degree of low molecular weight anionic species (*e.g.* DNA, RNA, and HCP). This is supported by work done by Roshankhah et al which achieved a similar performance of 94% removal of endotoxins (1.22 LRV) using diluted bacteriophage lysate (T7) [52].

As seen in **Table 3.3.5** the calculated endotoxin DBCs of Natrix Q in this study where significantly lower than those reported by the manufacturer (~10^2-10^3 vs 10^6 EU/mL membrane). Interestingly, Natrix Q endotoxin SBC results (30 mM NaCl) from the screening studies were comparable to the syringe filtration DBC values across both phage types (both ~ 10^2 - 10^3 EU/mL membrane). Although it is difficult to directly compare the calculated endotoxin DBCs to reported values due to different operating parameters (flow rate, membrane format, initial endotoxin load, solution conditions), the presence of phage seems to influence endotoxin binding capacity, which is inline with expectations.

Interestingly, the results derived from the syringe filtration study differ from the expectations set by the previous screening studies, suggesting bacteriophage LRVs of 0.122 – 0.24. Additionally, syringe filtration produced greater removal of endotoxins at 1.65 (J1MH1) and 2.19 (SK2) LRV. These values are significantly higher than expectations set by the results of the screening studies, which suggested LRVs of 0.72 and 0.21 respectively. Although, the precise mechanisms of this discrepancy remain unclear, an important limitation of this work to consider is the lack of bacteriophage and endotoxin penetration analysis. As the multi-well device operates using a gravity driven tangential flow pattern, it remains uncertain if larger (> 100 nm) biomolecules such as bacteriophage and endotoxin complexations can penetrate AEX membranes to the same degree in a normal flow, pressure driven process such as syringe filtration. Although previous work has demonstrated green fluorescent protein (GFP) (~4.2 nm) has comparable penetration into membranes regardless of the modality, this has yet to be confirmed with bacteriophage (30 – 200 nm) and endotoxin aggregates (50 -500 < nm) [10], [60], [64], [75].

As such, the endotoxin and phage binding seen with HTS using this select solution condition did not translate well to syringe filtration, with far greater removal of both endotoxin and bacteriophage than expected. Furthermore, it remains unclear whether this is discrepancy is due to limitations of the device itself or the need for further optimization of the methodology. Future work should focus on validating the penetration of phages and endotoxins into AEX membranes (*e.g.* GFP labelled bacteriophage) to better understand this discrepancy. Despite these discrepancies calculated DBCs in the syringe filtration experiments were comparable to the SBC values calculated by HTS using 30 mM NaCl buffer.

Table 3.3.1: Comparison of endotoxin concentration in control and permeate fractions (F1- F10) with associated LRV using the Natrix Q and SK2 preparation. All endotoxin concentrations are averages of triplicate measurements with standard deviation.

Sample	Endotoxin Concentration (EU/mL)	LRV
Control	782.06 ± 288.79	-
F1	4.45 ± 1.30	2.25
F2	5.42 ± 1.57	2.16
F3	5.62 ± 1.84	2.14
F4	4.31 ± 1.07	2.26
F5	6.84 ± 6.21	2.06
F6	6.02 ± 0.75	2.11
F7	5.88 ± 2.73	2.12
F8	4.61 ± 0.75	2.23
F9	3.79 ± 1.28	2.32
F10	3.97 ± 0.53	2.29
Average (Fractions)	**5.09 ± 1.80**	**2.19**

Table 3.3.2: Comparison of endotoxin concentration in control and permeate fractions (F1- F10) with associated LRV using the Natrix Q and J1MH1 preparation. All endotoxin concentrations are averages of triplicate measurements with standard deviation.

Sample	Endotoxin Concentration (EU/mL)	LRV
Control	184.09 ± 80.40	-
F1	3.54 ± 0.22	1.72
F2	4.00 ± 1.24	1.66
F3	4.43 ± 1.16	1.62
F4	5.38 ± 1.11	1.53
F5	4.32 ± 1.28	1.63
F6	3.26 ± 0.63	1.75
F7	5.63 ± 1.85	1.51
F8	3.15 ± 0.75	1.77
F9	2.63 ± 1.81	1.84
F10	6.22 ± 0.75	1.47
Average (Fractions)	**4.25 ± 1.08**	**1.65**

Table 3.3.3*:* Comparison of SK2 titer in control and permeate fractions (F1- F10) with associated LRV using the Natrix Q. All endotoxin concentrations are averages of triplicate measurements with standard deviation.

Sample	Bacteriophage Titer (PFU/mL)	LRV
Control	1.0×10^6	-
F1	0	> 3
F2	0	> 3
F3	0	> 3
F4	0	> 3
F5	0	> 3
F6	0	> 3
F7	0	> 3
F8	0	> 3
F9	0	> 3
F10	0	> 3
Average (Fractions)	**0**	**> 3**

Table 3.3.4: Comparison of J1MH1 titer in control and permeate fractions (F1- F10) with associated LRV using the Natrix Q. All endotoxin concentrations are averages of triplicate measurements with standard deviation.

Sample	Bacteriophage Titer (PFU/mL)	LRV
Control	$3.33 \times 10^6 \pm 8.82 \times 10^5$	-
F1	0	> 3
F2	0	> 3
F3	0	> 3
F4	0	> 3
F5	0	> 3
F6	0	> 3
F7	0	> 3
F8	0	> 3
F9	0	> 3
F10	0	> 3
Average (Fractions)	**0**	**> 3**

Table 3.3.5: Comparison of the calculated Natrix Q endotoxin SBC and DBC using SK2 and J1MH1 preparation to a reported DBC value (reported by the manufacturer). Endotoxin SBC values for SK2 and J1MH1 were generated using data from the 30 mM NaCl screening experiments. Values reported for calculated DBCs represent an average with associated standard error calculated using final endotoxin concentrations across all 10 fractions collected in the syringe filtration study.

Sample	SBC (EU/mL membrane)	Calculated DBC (EU/mL membrane)	Expected DBC (EU/mL membrane)
SK2	$9.21 \times 10^{2} \pm 2.30 \times 10^{2}$	$3.88 \times 10^{3} \pm 4.57 \times 10^{2}$	9.00×10^{6} [58]
J1MH1	$6.73 \times 10^{3} \pm 9.70 \times 10^{2}$	$8.99 \times 10^{2} \pm 1.27 \times 10^{2}$	

4 Conclusions and Future Directions

4.1 Conclusions

Bacteriophage therapy is a promising alternative treatment for AMR bacterial infections. However, the production of phages produces harmful contaminants, most notably endotoxins, which face strict regulations regarding the level present in therapeutic preparations. Current methods of bacteriophage purification are labor-intensive and lack the productivity required to address the demand for commercial-scale production of these phage products. Membrane adsorbers in the context of anion-exchange chromatography could be a suitable alternative, but the variability in physical properties of bacteriophages has been found to necessitate strain-specific process development. As such, this thesis introduces a high-throughput screening methodology to address the challenges of membrane chromatography recipe development for bacteriophage purification. After confirming bacteriophage titer stability in phage buffer at 4ºC, high throughput screening revealed that the binding of bacteriophage to AEX membranes increased as a function of dilution factor and time, identifying an optimal dilution factor of 1:1000. Through comparison of bacteriophage binding at 24 h, both Natrix Q and Sartobind Q were identified as promising membranes. Later screening work assessing both bacteriophage and endotoxin removal using AEX membranes in buffers of varying NaCl molarity revealed that solution effects observed using the multi-well device are generally consistent with those reported in the literature, although LRVs significantly different from expectations. Unfortunately, the application of a promising screened solution condition to membrane filtration resulted in unexpected results, where no phage was recovered. This highlights the need for further work to understand this discrepancy, as it remains unclear why performance did not more closely reflect data generated from the screening study.

4.2 **Future Work**

It remains unclear why the syringe filtration results differed so significantly from those of the high-throughput screening work. The binding of bacteriophage to LPS on the outer membrane of gram-negative bacteria is often a critical step in host infection [76]. The binding of free endotoxins to phages may result in the formation of large aggregates causing the size exclusion of phage and endotoxin complexes, causing phage and endotoxin removal to be greater than expected. Considering this interaction, a multimodal purification approach may be beneficial where a pretreatment of samples would work to disrupt the interactions between phage and endotoxin, allowing for more effective removal of endotoxins in parallel with improved phage recoveries. For example, the addition of detergents (*e.g.* octyl – β- D – glucopyranoside) has been found to facilitate the dissociation of proteins from endotoxins, allowing a greater quantity of endotoxin to affinity ligands [77]. A pre-treatment of phage preparations may yield the same benefit allowing a greater degree of endotoxins to bind to the membrane, whiling increasing the net recovery of phages. This work's HTS method could be used to screen pretreatment parameters alongside chromatography recipes, optimizing this multimodal purification process for each given phage type. Although previous work has demonstrated GFP (~4.2 nm) has comparable penetration into membrane regardless of modality, this has yet to be confirmed with bacteriophage and endotoxin [64], [75]. As such future work could seek to image membranes (Natrix Q, Sartobind Q, Mustang Q, etc.) tested using GFP expressing recombinant phages to understand how factors such as flow path, phage size, and membrane structure may affect penetration. Alternatively, Helium Ion Microscopy (HIM) may be useful as it allows for the 3D dimensional imaging of membrane structures like that of SEM without the need for a harmful metallic sputter coating which allows for the accessible imaging of biological structures such as bacteriophages [78]. Phages have yet to be imaged bound to AEX membranes and a methodology will need to be developed. As such, there is room to make considerable contributions to this space. These suggestions will serve to validate phage binding to membranes and understanding the discrepancy seen in translatability between the high-throughput system and larger scale syringe filtration.

As the focus of this current body of work was to validate the usefulness of the tool and identify the basic operating parameters for use with bacteriophage preparations, future work should seek to further expand on potential of this device for screening and optimizing chromatography recipes. Additional parameters such as pH, temperature, and divalent cations should be screened alongside NaCl molarity (or ionic strength) in future studies to further optimize bacteriophage purification. [52]. Additionally, future work should seek to utilize bacteriophage preparations more representative of feedstocks to be used in full scale purification processes (*i.e* less diluted). To this end, work should be done to understand and potentially quantify the impact of other ionic species (DNA, RNA HCP) on the interaction of phage and endotoxin with membranes. Incorporation of model phages (*e.g.* T4) in this work maybe useful to this end as key physical properties (IEP, size, zeta potential in select conditions) of these phage would be well characterized [52]. Designing a methodology or framework for the combined optimization of the removal of endotoxin alongside other key contaminants would be a significant contribution to the field [2].

Additionally, although a "sterilization" procedure was applied to the device, sterility was never comprehensively assessed. As such, future work should move to appropriately assess the sterility of the outlined protocols. This can be accomplished using biological indicators which are systems consisting of standardized bacterial spore preparations (*e.g Bacillus stearothermophilus*) with a known resistance to a given sterilization process [79]. These tests useful as binary tests (*i.e* sterile or non sterile) to assess the efficacy of wet heat, dry heat, ethylene oxide, and radiation based sterilizations methods [79], [80].

Finally, it should be noted that the assays used to quantify bacteriophage titer suffers from high variability and error stemming from human operators, limiting the overall accuracy of metrics (*e.g.* LRV) used to compare phage removal or recovery performance. As such, the development of improved bacteriophage quantification techniques is critical to the further development high throughput screening work and the translatability of screened data to larger scale modalities. Although there exists an array of alternative method of viral particle quantification such as quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and flow cytometry, these assays present potential limits such as an inability to quantify infectious titer and limited sensitivity [81]. As such, multiple techniques could be used to validate titer measurements. Alternatively, imaging technologies such as the Incucyte, have attempted to utilize real-time live cell imaging and associated software to calculate infectious titers, introducing a method of objective cell counting, rather than relying on human observation [82]. Regardless, these techniques should be faster and less variable analytical methods compared to the traditional plaque assays. Additionally, due to the high cost of commercial LAL endotoxin quantification assays and future work should focus on the parallel development of cheaper and higher throughput techniques for the quantification of endotoxins. Currently, commercial LAL kits are very expensive with reagents that have poor storage life once resolubilized. As an example, the kit used in this work was $ 889 CAD (April 2024), with a capacity to quantify only 300 samples (not including standards). On average a single high throughput screening experiment cost ~$ 293 CAD in endotoxin quantification reagents alone, significantly impacting the financial viability of this work. Additionally, these kits have narrow quantification ranges with 10 EU/mL as their upper limit of detection for many kits. As samples used in this work and in literature have endotoxin concentrations > 10 EU/mL, serial dilutions are often necessary to quantify samples thus introducing additional error. As such, there needs to be a focus on the development of a simple, rapid, and cost-effective alternative, which can ideally quantify a higher upper threshold of endotoxin concentration. To this end a broad range of alternative assays or biosensors have been developed, ranging from biochips to cell-based assays, although LAL assays maintain their place as the gold standard [83]. As such, any novel endotoxin detection assay developed should be compared to a traditional LAL kit to confirm its accuracy and precision.

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Appendix A: Supplementary information for Section 3

A.1 Supplemental Information for Bacteriophage Stability study

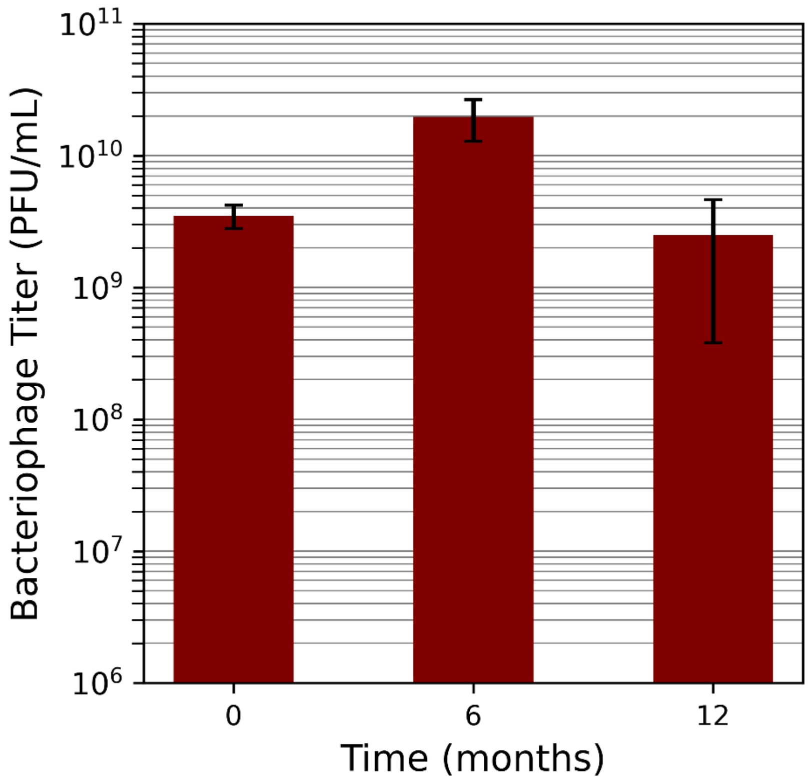

Figure A.1.1: Titer (PFU/mL) of SK2 in phage buffer stored at 4ºC across a sampling period of ~1 year. Individual bars represent the mean of duplicate measurements with associated standard deviation bars.

A.2 Supplemental Data for High-throughput Screening Studies

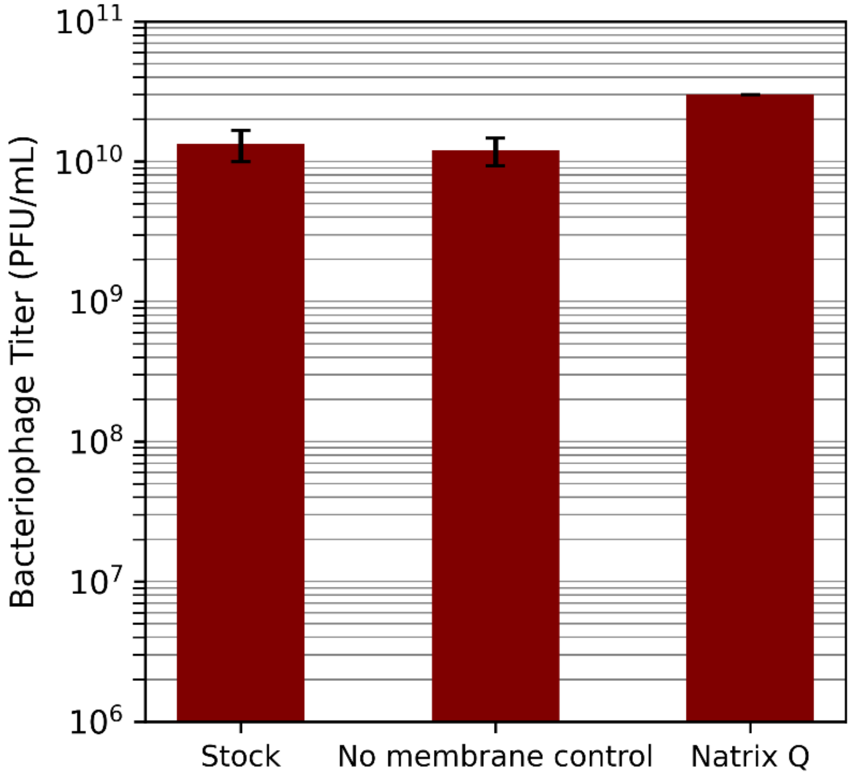

Figure A.2.1: Comparison of stock, no membrane control, and membrane the average bacteriophage titer (PFU/mL) using J1MH1 preparation with standard error bars of triplicate experiments in the multi-well device over 24 hours of contact time in phage buffer

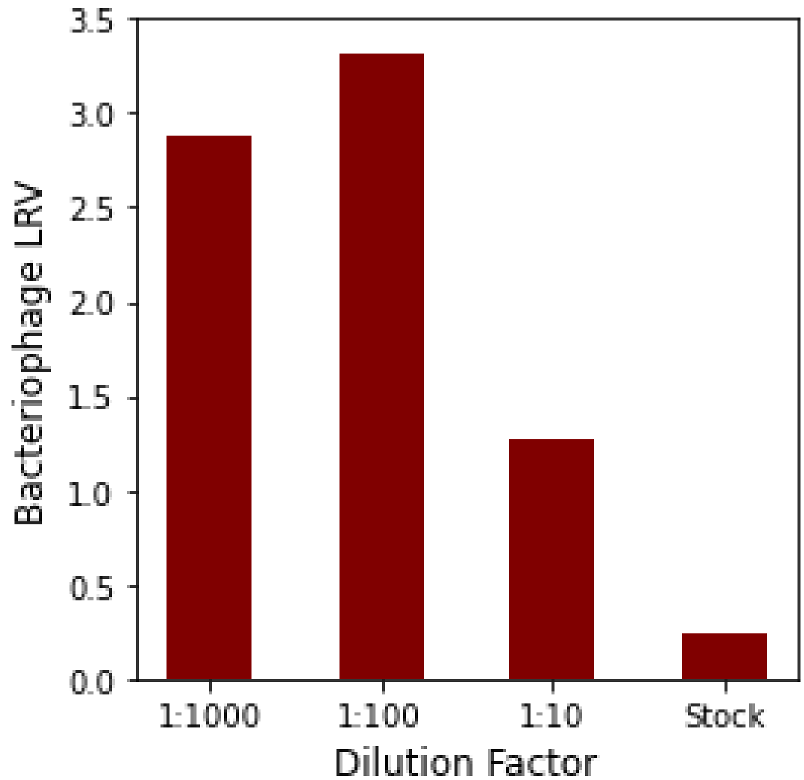

Figure A.2.2: Effect of dilution on J1MH1 removal (LRV) using the Natrix Q AEX membrane after 72 h of contact time. Each dilution represents an initial phage titer as follows; 1:1000 ($2.50 \times 10^6 \pm 5.00 \times 10^5$ PFU/mL), 1:100 (4.00 x 10 PFU/mL^7), 1:10 ($1.50 \times 10^8 \pm 7.00 \times 10^7$), Stock ($7.50 \times 10^9 \pm 5.00 \times 10^8$ PFU/mL).

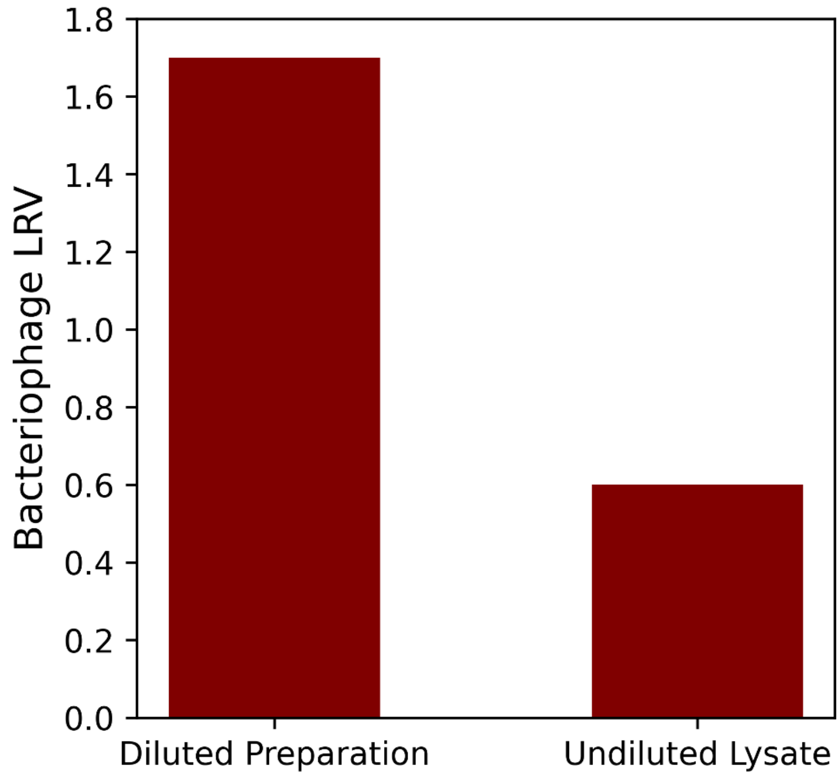

Figure A.2.3: Comparison of SK2 LRV achieved after 24 h of contact time with Natrix Q using diluted SK2 preparation (1:1000 dilution) and undiluted SK2 lysate both at a comparable initial titer ($2.17 \times 10^6 \pm 4.77 \times 10^5$ and $4.00 \times 10^6 \pm 3.61 \times 10^6$ PFU/mL respectively). Both preparations are suspended in phage buffer.

A.3 Supplemental data for Syringe Filtration Study

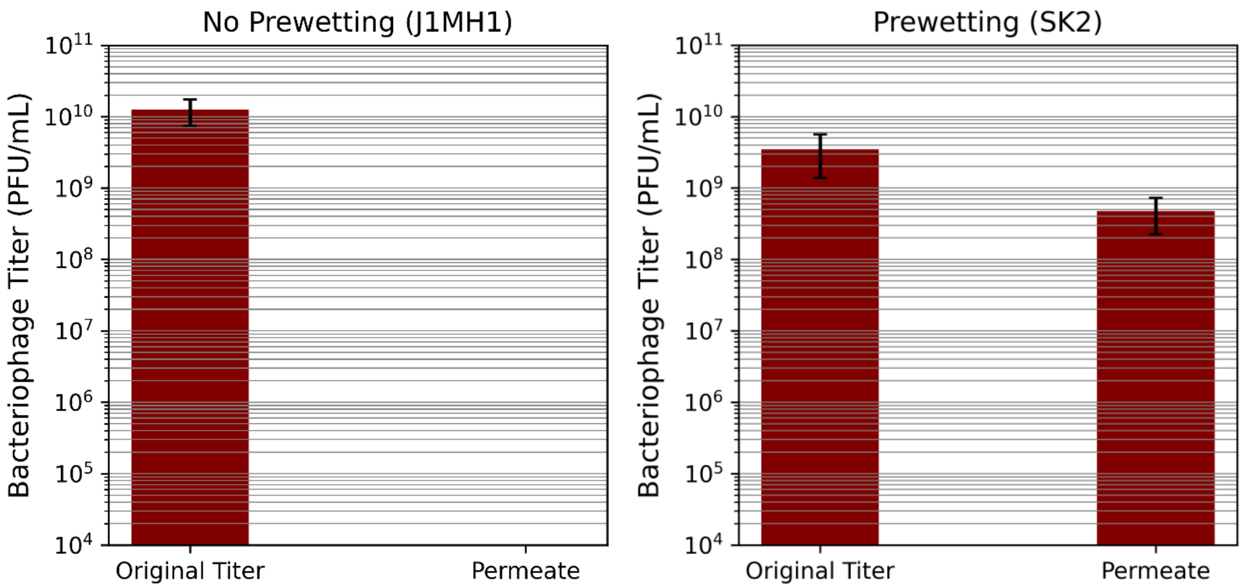

Figure A.3.1: Effect of prewetting Natrix Q membrane using 4 mL of phage buffer before conducting filtration experiment using undiluted bacteriophage lysate at a flow rate of 1 mL/min. All given titers given for Natrix Q in the figures represents the average titer across four, 1 mL fractions collected during each experiment. Titers represent averages with associated standard error values. Note that J1MH1 was used in filtration experiment without a prewetting step, while SK2 was used in the latter.

A.4 Helium Ion Microscopy (HIM) Images of Membranes

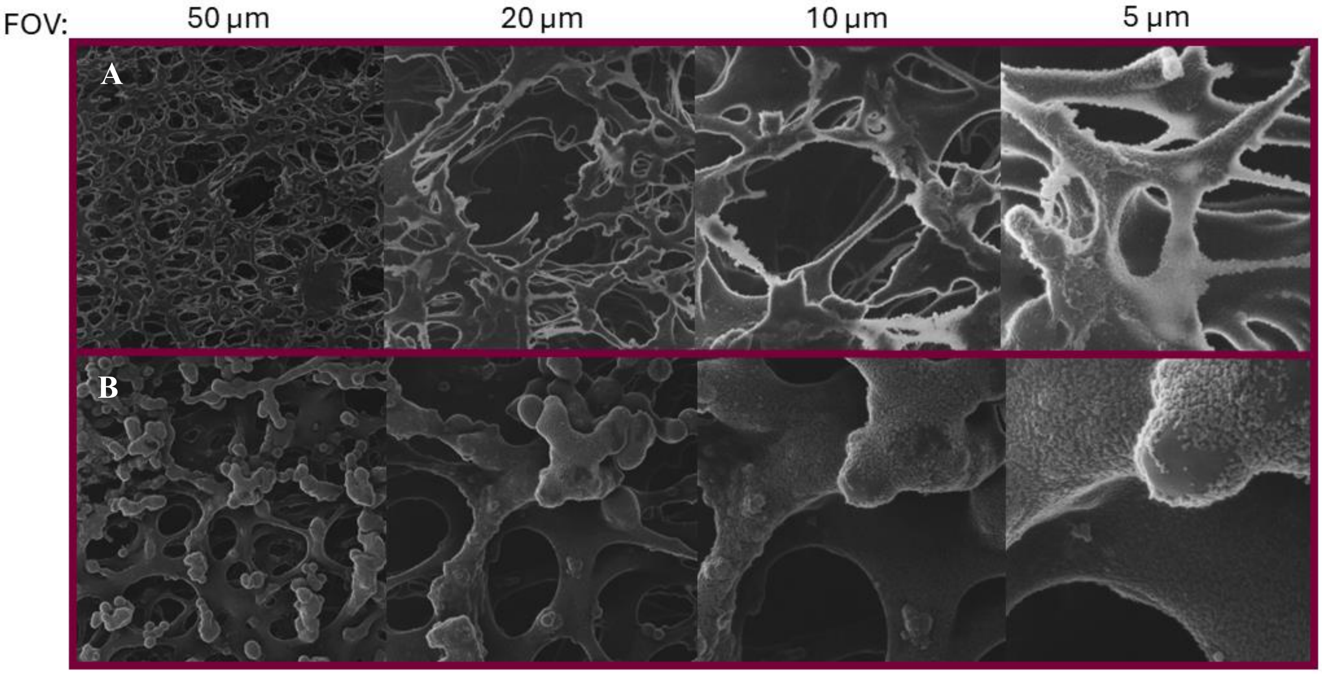

Figure A.4.1: HIM Images of 8 mm disc of Sartobind Q membrane samples after 6 h of contact in **A)** 10 mM NaCl (10 mM Tris-HCl, pH 7) buffer or **B)** 10^6 PFU/mL SK2 preparation in 10 mM NaCl (10 mM Tris-HCl, pH 7) buffer at varying fields of view (FOV).

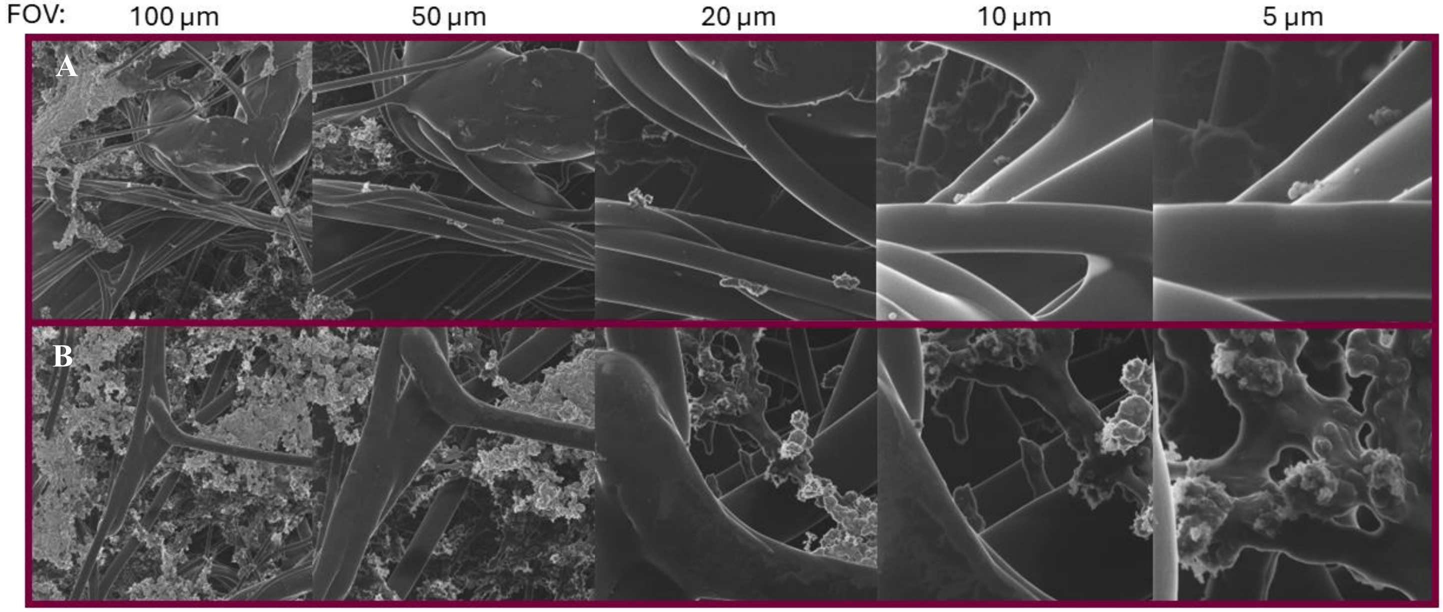

Figure A.4.2: HIM Images of 8 mm disc of Natrix Q membrane samples after 6 h of contact in **A)** 10 mM NaCl (10 mM Tris-HCl, pH 7) buffer or **B)** 10^6 PFU/mL SK2 preparation in 10 mM NaCl (10 mM Tris-HCl, pH 7) buffer at varying fields of view (FOV).

A.5 Evaluation of Sterilization Methodology for Multi-well device

To evaluate the efficacy of the ethanol bath and U.V exposure used to achieve sterility in the multi well device, A_{600} measurements were used to quantify microbial load in LB incubated in previously contaminated multi-well units using SK2 preparation.

5 conditions were evaluated, they were as follows: Condition A: no sterilization procedure, Condition B: 20 min ethanol soak and following 30 min U.V exposure, Condition C: 20 min ethanol soak, Condition D: 30 min U.V exposure, and Condition E: Gamma sterilization (no SK2 incubation). All samples were run in triplicate. An image of the experimental format can be seen in **Figure A.5.1**.

The procedure used was as follows. 1 mL of sterile filtered undiluted SK2 preparation was added to all multi well units aside from Condition E and allowed to incubate at room temperature for 1 hour. Following incubation SK2 samples were removed and each multi well unit was washed using Milli-Q water (3 washes). Then each multi well unit had its respective sterilization protocol applied. Once completed for all units 1 mL of LB was added to all wells of multi well unit and subsequently covered with parafilm. Samples were left to incubate at room temperature for 24 hours after which 800 µL of LB was extracted from each well. A_{600} nm of all samples was quantified to assess bacterial growth.

As seen in **Figure A.5.2** all conditions had comparable A_{600} nm values at ~ 0.46. It was found that the SK2 preparation used across all high throughput studies did not introduce a considerable bacterial load when incubated with the multi-well device. As such, the efficacy of the "sterilization" protocols used in Section 2 are inconclusive.

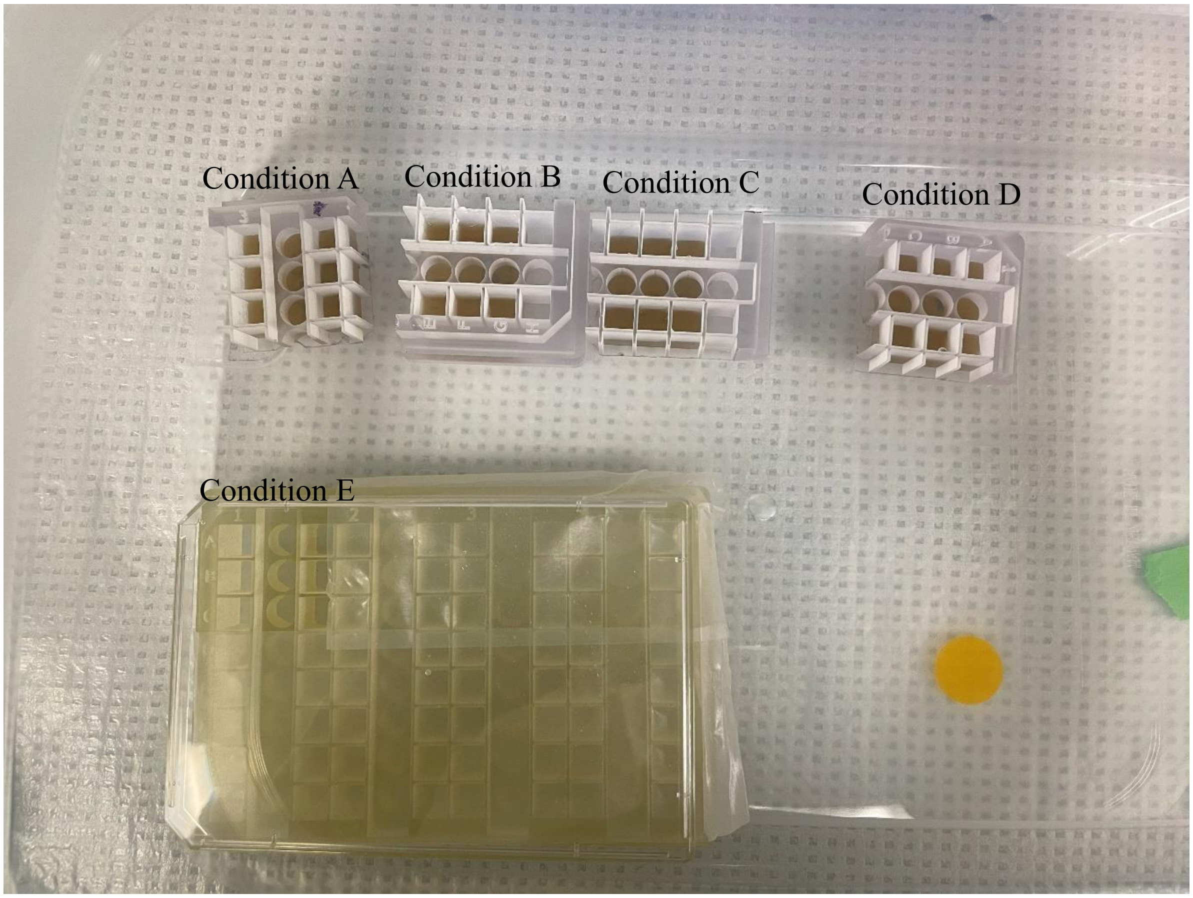

Figure A.5.1: Experimental setup of sterility experiment, showing incubation of all samples with SK2 preparation (1 h). Individual multi- well units contained 3 separate wells allowing triplicates for each experimental condition. Note that condition E served as a positive control so SK2 preparation was not added to the wells of the experimental unit.

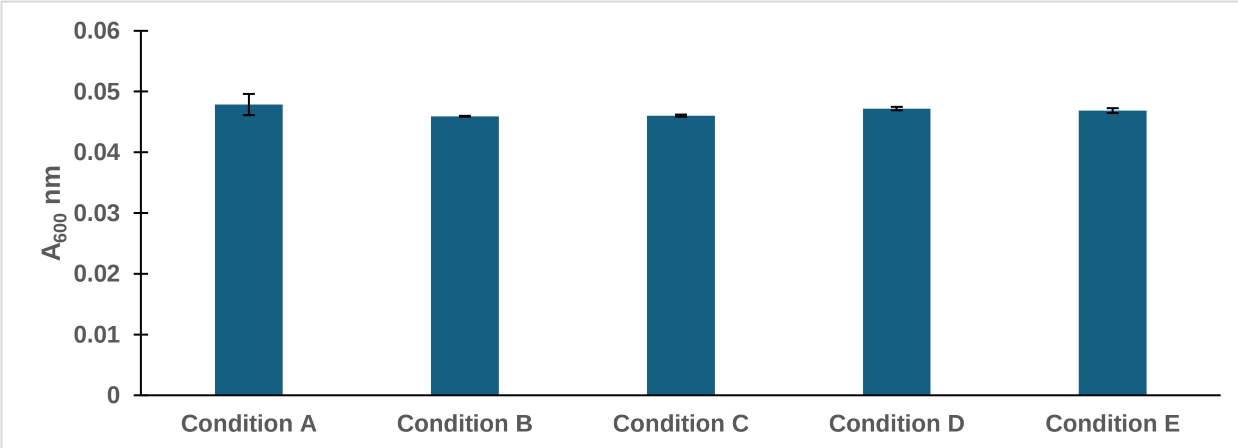

Figure A.5.2: Comparison of A_{600} measurements of LB samples after 24 h of incubation in multi well units which have undergone various sterilization procedures, other than Condition A and Condition E which serve as negative and positive controls respectively The conditions as follows: Condition A: no sterilization procedure, Condition B: 20 min ethanol soak and following 30 min U.V exposure, Condition C: 20 min ethanol soak, Condition D: 30 min U.V exposure, and Condition E: Gamma sterilization (no SK2 incubation). Values represent averages of triplicate samples with associated standard error bars.