OPTIMIZATION OF METHODS FOR PHAGE DISPLAY USING SINGLE-CHAIN VARIABLE FRAGMENT PHAGEMID LIBRARIES

By

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To my parents, siblings, and Matt. They, never doubted my abilities and always pushed me harder when I doubted myself.

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2xTY AG	2xTY medium containing 100 μg/mL and 1% (w/v) glucose
BSA	Bovine serum albumin
BSG	Phosphate-buffered saline containing 0.1% (w/v) gelatin
CFU	Colony forming unit
CT	Cholera toxin
ddH_2O	Deionized distilled water
dH_2O	Deionized water
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
HC	Hemorrhagic colitis
HRP	Horseradish peroxidase
HUS	Hemolytic uremic syndrome
HyΦ	Hyperphage
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MWCO	Molecular weight cut off
OD	Optical density
pAb	Polyclonal antibody
PBS	Phosphate-buffered saline
PBST-0.01	PBS containing 0.01% (v/v) Tween-20
PBST-0.05	PBS containing 0.05% (v/v) Tween-20
PC	Phosphate-citrate
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
PFU	Plaque forming unit
RF	Replication factor
RNA	Ribonucleic acid
S:N	Signal to noise
scFv	Single chain F variable
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Stx-2	Shiga-like toxin 2
TMB	3,3′,5,5′-tetramethylbenzidine
tu	Transducing unit
MOI	Multiplicity of infection
Vc	*Vibrio cholerae*
v/v	Volume per volume
w/v	Weight per volume

Abstract of Thesis Presented to the Graduate School
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OPTIMIZATION OF METHODS FOR PHAGE DISPLAY USING SINGLE-CHAIN VARIABLE FRAGMENT PHAGEMID LIBRARIES

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Detection assays for biological agents and their products are important to identify disease-causing organisms in water, air, food, and patient samples to enable prevention or treatment of the disease. A commonality in almost all detection assays are proteins that specifically bind to a target molecule. While monoclonal antibodies are the most common detection reagents used in detection assays, phage display reagents are becoming more prevalent. Phage display involves the display of recombinant peptides on bacteriophages. These recombinant phages can be panned against a target antigen to select recombinant peptides that bind specifically to the antigen. Optimization of protocols and reagents used in phage display was the goal of this thesis.

Optimization of whole cell panning with the Tomlinson J human synthetic $V_H + V_L$ (scFv) phagemid library revealed that a trypsin elution between 10 and 30 minutes eluted the highest number of phagemid particles; this may be because the phagemid particles were degraded by trypsin treatment after 45 minutes. A high throughput ELISA for the production and screening of scFv proteins was developed and demonstrated that scFv proteins produced in a microtiter well could be screened by ELISA and produce a detectable S:N even when diluted 1:10. A high throughput ELISA for the production and screening of phagemid particles was attempted, but

phagemid particles could not be produced in a microtiter well in titers high enough to make a high throughput ELISA acceptable for screening of phagemid particles.

Various phagemid particle amplification tools were analyzed. The hyperphage genome was incorporated into *E. coli* and maintained as a plasmid to enable phagemid particle amplification. However, these strains had low infection efficiencies with phagemid particles. Helper plasmids were incorporated into *E. coli* to enable phagemid particle amplification. However, these strains produced low yields of phagemid particles. The titer of laboratory-produced hyperphage was improved by use of *E. coli* MG1655 (pGTR203), expressing the M13 *gIII* gene. The improved high titer hyperphage was used to successfully amplify the Tomlinson J scFv phagemid library to ensure that the redundancy of phagemid clones was maintained. Using the newly amplified library to pan against an *E. coli* O157:H7 Stx2 toxin preparation resulted in 89% of phagemid particles from the third round of panning recognizing the Stx2 toxin preparation in an ELISA.

Previous panning procedures used in this laboratory resulted in no better than 1% of selected phagemids being specific to the target antigen, and often no usable specific clones were isolated. The optimization of panning procedures and reagents developed in this thesis greatly increased the efficiency of selection of phagemid particles specific to the target antigen. Using the improved reagents and procedures developed in this theis yielded over 80% positive phagemid clones selected against *E. coli* O157:H7 flagella by another lab member. The phagemids recognized the major flagellin protein by Western blot (data not shown). Therefore, the improvements on phage display techniques and tools described in this thesis are, in fact, useful and offer promise of success for continuing studies in the laboratory.

CHAPTER 1
INTRODUCTION

Methods for Bacteriological Detection and Analysis

There has always been a need for detection of biological agents. One basic reason for this need is for prevention of disease by identifying biological agents in food, water, or air. Of key interest are organisms that have potential as bioterrorism agents. With resent bioterrorist events, the need for rapid detection of bioterrorism agents has increased (1). Another reason for detection of biological agents is for the purpose of diagnosing disease by detecting agents in patient samples so that suitable treatment can be given. Detection is also of interest to epidemiology. By detecting agents that cause disease and analyzing them through epidemiology, measures can be implemented to control or prevent outbreaks. Various methods have been used to detect organisms or their products.

Since the discovery of microscopic organisms, methods for detecting the organisms have evolved. One of the first methods of detection was culturing an organism in enrichment medium, followed by selective medium. Afterwards, the organism would go through various biochemical and metabolic tests for identification. However, culture-based identification of organisms can take days or even weeks. As seen in the anthrax attacks in the fall of 2001 and the SARS virus outbreaks of 2002-2003, symptoms can occur within days of exposure and infection can spread rapidly. These reasons enforce the need for rapid detection of biological agents (2). The rapid detection of biological agents is necessary to treat individuals at risk, limit transmission of the disease, improve public health surveillance and epidemiology, and monitor environmental impact (3-5).

Methods have progressed to optimize detection of agents and their products in clinical and environmental settings. Over the last 25 years, assays for detection and identification of agents

have improved immensely. Particularly, the reagents and detection equipment have improved to allow for the detection and identification of agents in as little as a few minutes (2). An ideal detection system would be rapid, sensitive, selective, inexpensive, and would not require extensive training of personnel to operate the system. While there is no single optimal-detection method, there are numerous methods for detecting a variety of agents in a variety of environments.

Immunological tools are one of the most widely used and successful methods for detection of biological agents. Since the first radioimmunoassay was developed by Yalow and Berson in 1959 for the detection of human insulin (6), immunoassays have expanded and been used for the detection of a variety of agents. The main components of an immunoassay are summarized by Andreotti et al. as follows: "Immunoassays rely upon four basic components regardless of the application and underlying technology: (i) the antigen to be detected; (ii) the antibody or antiserum used for detection; (iii) the method to separate bound antigen and antibody complexes from unbound reactants; and (iv) the detection method. The efficacy of any given immunoassay is dependent on two major factors: the efficiency of antigen-antibody complex formation and the ability to detect these complexes."

The most important component of an immunoassay is the antibody. The discovery of different types of antibodies has altered the range and scope of immunoassays. Polyclonal antibodies have largely been supplanted by monoclonal antibodies. In the current age, recombinantly engineered antibodies are supplanting monoclonal antibodies (2). The demand for a variety of immunological assays reflects the growing number of assays developed to optimize their use by increasing their sensitivity, speed, handling, and cost. The specific binding of antibody to antigen has been coupled with a variety of detection applications including

fluorescence, enzymatic activity, chemiluminescence, electrochemiluminescence, metallic beads, and many more. The detection of these complexes can be assayed on a variety of platforms such as biosensors, flow cytometry, microarray, and lateral flow diffusion devices (1). Perhaps the most common immunological assays are Enzyme-Linked ImmunoSorbent Assays (ELISAs). Such assays either have antibody or antigen bound to a solid support that enables specific, sensitive, and quantitative detection of antigen or antibody by optical-density detection of a colorimetric signal.

Nucleic acid-based tools for detection have advanced in the past few decades. Of main interest is Polymerase Chain Reaction (PCR), which was invented in 1983 by Kary Mullis and coworkers (7). Polymerase chain reaction involves the amplification of DNA by use of oligonucleotide primers, heat stable DNA polymerase, and nucleotides in an exponential capacity. The original PCR has since been altered to give quantitative real time-PCR (q-PCR or kinetic PCR) and reverse transcription (RT)-PCR. Quantitative real time-PCR involves the amplification along with quantification of the DNA, while RT-PCR involves reverse transcribing a piece of RNA into DNA followed by PCR amplification of the DNA. Advances in PCR chemistry and thermocyclers have shortened the length of DNA amplification from a few hours to minutes. With small sample volumes in the amount of a few microliters and containing as little as one bacterial cell, PCR is one of the most sensitive assays available. However, this sensitivity increases the risk of contamination generating false positives. Field-based PCR amplification and identification is not common due to the complexity of the system and highly trained personnel required to operate and interpret the system. Perhaps the greatest constraint of nucleic acid-based detection assays is the availability of genomic sequence data of biological agents (2). Detection assays are continually being invented and improved to detect biological

agents. Because there are numerous detection environments, conditions, and agents, the development and improvement of detection assays will continue to progress.

Vibrio cholerae

Vibrio cholerae is a gram-negative, curved rod-shaped bacterium with a single polar flagellum found primarily in estuarine and marine environments. It is a facultative human pathogen causing the pandemic diarrheal disease cholera (8). Cholera is characterized by profuse watery diarrhea, vomiting, and leg cramps leading to dehydration and shock (9). Cholera infection occurs through the ingestion of food or water contaminated with the bacterium. Because cholera can be prevented by proper sanitation and hygiene, it is uncommon in industrialized countries and is most prevalent in the Indian subcontinent and sub-Saharan Africa (10).

Vibrio cholerae uses its toxin co-regulated pilus (TCP) to colonize the small intestine; once attached to the small intestine, the bacterium secretes cholera toxin (CT) (11). Cholera toxin binds to the epithelial cell receptor, $G_{M1,}$ and is transported into the cell. Cholera toxin is an AB toxin composed of a catalytically active A-subunit surrounded by a homopentameric B-subunit. Once the CT is internalized, it is transported in a retrograde pathway through the Golgi to the endoplasmic reticulum. In the endoplasmic reticulum it is retrotranslocated to the cytosol. In the cytosol CT catalyzes ADP-ribosylation of the GTP-binding protein G_s (8) causing adenylate cyclase to become constitutively activated. The increase in cAMP levels leads to secretion of Cl^-, HCO_3^-, and water from epithelial cells into the intestinal lumen causing diarrhea.

The loss of water can amount to 30 liters per day, and without proper rehydration treatment can lead to a 30% mortality rate (12). Under-reporting of cholera infections is a great problem. It is estimated that 3 to 5 million cases and 120,000-200,000 deaths occur worldwide annually (13). However, this number could easily be multiplied by a factor of ten due to unreported cases.

There are many different serogroups of *V. cholerae,* most of which do not cause acute diarrhea. *Vibrio cholerae* is classified into serovars or serogroups on the basis of its lipopolysaccharide (LPS) O-antigen (14). There are at least 200 known serogroups, of which serogroups O1 and O139 are the only ones that cause epidemic or endemic cholera. The O1 serogroup can be further distinguished into three serotypes. Ogawa and Inaba are the most common serotypes, with Hikojima being rarely reported. These serotypes can be further classified into two biotypes, El Tor and classical, that differ in their biochemical properties and phage susceptibilities (8).

With the enormous numbers of cases of cholera every year, there is a need for an effective diagnosis tool for patient and environmental samples. Detecting endemic serogroups of *V. cholerae* early in an outbreak is extremely important for control of an epidemic. A problem with detection of *V. cholerae* is that cholera is a disease of developing countries. Outbreaks normally occur around water-ravaged areas where laboratories are not prevalent. Therefore, field-based assays are the most effective tools for early detection of *V. cholerae.* Because there are many serogroups of *V. cholerae*, most of which present mild symptoms, it is important to distinguish the epidemic strains from the non-epidemic strains. *Vibrio cholerae* O1 and O139 are the epidemic strains. Determining features of these serogroups are the O-antigen of their LPS and their ability to produce CT. Therefore, field tests usually detect these specific antigens.

There are many rapid diagnostic tests for cholera. Some of these detect CT by passive-latex agglutination (15,16), while others detect the O-antigens of LPS from O1 (17-21) and O139 (22-24) strains of *V. cholerae*. A commonly used rapid *V. cholerae* diagnostic tool is the multistep colloidal gold-based colorimetric immunoassay known as SMART. This monoclonal antibody-based test was developed for the detection of *V. cholerae* O1 (25,26) and O139 (24)

strains in stool specimens. SMART is 95% sensitive and 100% specific to *V. cholerae* O1 strains (26) and 100% sensitive and 97% specific to O139 strains (24). A one-step immunochromatographic dipstick test for the detection of *V. cholerae* O1 and O139 LPS in stool samples was invented by the Institute Pasteur in Paris. This assay requires minimal technical skill and rapidly detects thresholds of purified LPS at 10 ng/mL for *V. cholerae* O1 and at 50 ng/mL for *V. cholerae* O139 strains in approximately 10 minutes (27). With continuing improvement and use of methods for detection of *V. cholerae,* outbreaks could be lessened or prevented with proper treatment and containment.

***Escherichia coli* O157:H7**

Escherichia coli O157:H7 is an enterohemorrhagic serotype of *E. coli* that causes hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The bacterium is primarily found on cattle farms and colonizes cattle, swine, and deer intestines with subclinical effects to the animals (28). Disease in humans comes from the ingestion of beef, milk, vegetables, and other products that are contaminated with *E. coli* O157:H7. Symptoms from an *E. coli* O157:H7 infection include mild diarrhea, abdominal pain, vomiting, bloody diarrhea, HC, strokes, and HUS (28).

Over the past 23 years, 146 Shiga-like toxin producing *E. coli* (STEC) outbreaks and sporadic cases of human illnesses have been traced to consumption of beef contaminated with various *E. coli* O157 strains (29). Most of these illnesses were caused by infection with *E. coli* O157:H7. Of the 146 outbreaks and sporadic cases 89% occurred in the United States (29). The large number of cases in the Unites States compared with the rest of the world can be explained by high levels of beef consumption and availability of *E. coli* O157 diagnostic methods in the United States. The Center for Disease Control and Prevention estimated that *E. coli* O157:H7 is

responsible for approximately 73,000 illnesses and more than 60 deaths per year in the United States (30).

Escherichia coli O157:H7 produces Shiga toxins that are responsible for human disease. These Shiga toxins produce severe cytopathic effects and have a high degree of homology with Shiga-toxin (Stx) of *Shigella dysenteriae* type 1 (28). The Stx toxin is a member of the AB toxin family. It is composed of a catalytically active A-subunit surrounded by a pentameric B-subunit. The B subunits specifically bind to glycosphingolipid globotriosylceramide (Gb3) receptors (31) of the renal glomerular endothelial, mesangial (32) and tubular epithelial cells (33). Upon entry into the cell, the catalytically active A1 subunit cleaves ribosomal RNA leading to the cessation of protein synthesis and cell death (34). Not only does Stx toxin damage host cells, but it may also increase the adherence of *E. coli* O157:H7 to epithelial cells leading to increased risk of colonization. Tissue culture experiments showed that Stx toxin evoked an increase in a eukaryotic receptor, nucleolin, that binds to the *E. coli* O157 attachment factor, intimin, leading to increased cell adherence (32).

There are two distinct toxin-converting bacteriophages (phages), 933J and 933W, in E. *coli* O157:H7 that generate two genetically related toxins that are antigenically distinct but create similar biologic effects (35). These two toxins are called Shiga toxins I and II (Stx1 and 2). Experimental studies suggest that *E. coli* that produces the Stx2 toxin is more virulent than *E. coli* that produces Stx1 or both Stx1 and Stx2 toxins (36). *Escherichia coli* strains producing Stx2 toxin are more frequently linked with the development of HUS than Stx1 toxin-producing strains (37,38). In mouse studies, the lethal dose of purified Stx2 toxin is 400 times lower than that of Stx1 toxin (39). In piglet studies, Stx2 toxin-producing *E. coli* strains caused more severe neurologic symptoms than strains producing both Stx1 and 2 toxins or only Stx1 toxin (40).

Detection of *E. coli* O157:H7 has become of increasing importance to the food industry in the United States as outbreaks continue to occur. Traditional methods of detection of *E. coli* O157:H7 involve plating and culturing, enumeration methods, biochemical testing, microscopy, and flow cytometry. Other methods have been developed, including immunoassays (41), immunomagnetic separations (42), nucleic acid probe-based methods based on hybridization and polymerase chain reaction (PCR) (43), and DNA microarrays (44). However, many of these assays are time-consuming and not suitable for rapid detection of *E. coli* O157:H7. Therefore, biosensors have been developed for the rapid detection of *E. coli* O157:H7 cells and Stx toxins. "An electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element" (45). Some advantages of biosensors include their continuous data acquisition ability, target specificity, fast response, mass produce feasibility, and the simplicity of sample preparation. A quartz-crystal microbalance (QCM) has been developed for detection of *E. coli* O157:H7 cells. Upon specific binding of *E. coli* O157:H7, the QCM uses its ultra sensitive mass-measuring sensor to detect decreases in the crystal-resonance frequency to enable detection of 2.0×10^2 CFU/mL of *E. coli* O157:H7 (42). An amperometric biosensor for *E. coli* O157:H7 cells made use of a dissolved-oxygen probe to enable detection of 50 CFU/mL in as little as 20 minutes of preparation and processing time. Upon binding of the bacterial cells, a decrease in enzyme activity results in a change in oxygen concentration that was detected with a Clark-type oxygen electrode probe (46). Polymerase chain reaction-based assays are also very common for detection of *E. coli* O157:H7 cells (47-51) and Stx toxins (50).

Phage Display

Phage display was initially described in 1985 by George P. Smith (52) as a means to display foreign proteins on filamentous bacteriophage. Filamentous phages are non-lytic phages with circular ssDNA genomes. Of the filamentous phages, the Ff family (f1, fd, and M13) phages infect F^+ *E. coli* through binding with their F pilus. These phages are useful tools to link genotype and phenotype of select recombinant proteins. This link was created by encoding a foreign polypeptide in-frame with a coat protein gene of the M13 phage. Phage display could theoretically be implemented with any phage, but filamentous phages have been the most widely used. Of the filamentous phages, M13 is the most commonly used.

The M13 phage (Fig. 1-1) replicates in *E. coli,* turning the bacterium into a phage-production factory (53). The bacteria harboring these phages do not lyse, but undergo reduced cell growth due to the stress of phage production. The M13 phage contains a 6.4-kb, circular, single-stranded DNA genome that encodes phage proteins I to XI. Five of these proteins are coat proteins. The major coat protein (pXIII) is present in approximately 2,700 copies and protects the genome in a cylindrical manner. The minor coat proteins pVII and pIX are necessary for efficient particle assembly, while the minor coat proteins pIII and pVI are necessary for particle stability and infectivity (54-56). The pIII protein mediates the binding of the phage to the F pilus and is necessary for viral uncoating and phage DNA transfer to the cytoplasm of the bacterium (55). Host enzymes then convert the ssDNA into supercoiled dsDNA, known as the replicative form (RF) (55). The RF is essential to the phage display system because it can be purified and manipulated just like a plasmid. Through the manipulation of the RF of M13 came some of the earliest cloning vectors (57). Through some of these vectors came the development of recombinant phage libraries.

During assembly of M13, the foreign protein is fused to a coat protein and displayed on the surface of the phage. The minor coat protein III (pIII) is the most common protein for fusions, but the major coat protein XIII (pXIII) and the other minor coat proteins of M13 have also been used for recombinant fusions (58,59). Phage displays using the pIII and pXIII proteins have different advantages. Using the pXIII protein as the fusion protein enables high copy display of the recombinant protein because there are over 2,700 copies of the pXIII protein on the surface of the phage. However, a drawback in using the pXIII protein as the fusion protein is its limitations in the size of the displayed protein (60). The pXIII protein can only display peptides less than six amino acids in length before the function of the coat protein becomes compromised and the number of infectious particles plummets. If the size of the display peptide increases to eight amino acids, only 40% of the phages are infective; if the display peptide increases to 16 amino acids, less than 1% of the phages are infective. Recombinant fusions using the pIII protein are not as restricted in the size of the display peptide (53). The pIII-fusion protein can display peptides of 100 amino acids or greater before the ability of the pIII protein to bind to the F pilus of *E. coli* becomes compromised (52). Also, since there are only five copies of the pIII protein on the surface of phage particles, the ability to select high affinity binding phage particles is greater than that of the ability of the pXIII-fusion protein.

Recombinant Phage Libraries

Recombinant phage libraries are composed of phages that display a fused protein to a coat protein of the phage. There are two main phage display libraries, phage and phagemid libraries. Phage libraries are M13 with the addition of a recombinant fusion to the *gIII* gene, while phagemid libraries are just plasmids that contain a recombinant fusion to the *gIII* gene. There are advantages and disadvantages to both systems. Phagemid libraries are advantageous due to their greater library diversity because of their higher ligation-transformation efficiency, and their

simplicity enables easier genetic manipulation than with phage vectors. Also, phagemid particles isolated from phagemid libraries are able to be produced to generate more phagemid particles or secrete the recombinant protein, which is usually the ultimate end product. A disadvantage of phagemid particles is their dependence on the aid of a helper phage to provide the rest of the M13 proteins in trans to enable phagemid particle production and assembly. Phage libraries are advantageous due to their lack of dependence on helper phages. Because phage libraries are M13 phage with some alterations, they are capable of propagating themselves by simple infection of *E. coli*. A disadvantage to phage libraries is that they are less stable than phagemid libraries (61). Also, phage particles are only able to produce phages and have to be further manipulated to be able to produce the recombinant protein alone.

Tomlinson I + J Human Synthetic $V_H + V_L$ Phagemid Libraries

The Tomlinson libraries are "naïve" libraries comprised of approximately 1 x 10^8 random phagemids derived from non-immunized human donors (62). Naïve libraries enable greater diversification of antibody genes, increasing the probability of isolating phagemids specific to a wide variety of targets. The Tomlinson libraries also encode greater diversity through random side-chain diversification. These phagemid libraries encode a single chain F variable (scFv) gene fusion to the *gIII* gene of a library vector plasmid contained in a M13 phage. The scFv (Fig. 1-2) is composed of a single polypeptide with V_H and V_L domains that are joined by a flexible glycine-serine linker. The Tomlinson libraries were constructed by use of reverse transcription and PCR to amplify the V_H and V_L antibody genes from B-lymphocytes of human donors. Universal degenerate primers were then used to anneal to the 5' end of the exons encoding the antibody V-gene, which is conserved in humans. The mRNA from the B lymphocytes was converted to cDNA, which represents the V_H and V_L antibody genes. The cDNA was then PCR assembled using an overlap extension technique and contained the

restriction enzyme sites for subcloning into the pIT2 library vector. The library vector, pIT2, encodes an M13 origin of replication, an ampicillin resistance gene (*bla*), and both His6 and myc tags (Fig. 1-3). The pIT2 phagemids were electroporated into *E. coli,* where they were superinfected with helper phage to generate phagemid particles displaying a scFv-pIII fusion protein. The phagemid particles display the scFv on the surface of the phage particles and encode the scFv gene in the phagemid genome, linking phenotype with genotype.

New England Biolabs 12mer Peptide Phage Library

The New England Biolabs 12mer Peptide Phage Library (Ph.D. System, New England Biolabs) is a combinatorial phage library that encodes a random sequence of twelve amino acids fused to the *gIII* gene of the M13 genome. The 12 random amino acids are fused to the N-terminus of the pIII protein, which is displayed on the surface of the M13 phage. The first residue of the mature protein is the first randomized position. The peptide is followed by a (Gly-Gly-Gly-Ser) spacer linked to the wild type pIII protein. The library is constructed in M13 phage with an insertion of the *lacZα* gene fragment into the genome. The insertion of the *lacZα* gene fragment enables distinction of *E. coli* that harbor phages from the library opposed to environmental phages that do not contain the *lacZα* gene. This is done by blue/white screening of phages on agar that contains X-gal and IPTG. When an *E. coli* strain has a functional *lacZ* gene it will produce β-galactosidase, which is a heterodimer composed of an α and an Ω peptide. Neither of these peptides have enzymatic activity on their own; therefore, if one component is missing then β-galactosidase enzymatic activity is lost and if they are complemented they spontaneously combine to generate an active structure. Beta-galactosidase cleaves X-gal into a product that becomes oxidized to generate an insoluble blue product. IPTG is an inducer of the *lac* promoter which drives transcription of the *lacZ* gene. Therefore, if phages are produced in *E. coli* cells with a nonfunctional *lacZα* gene and a functional *lacZΩ* gene and grown under

selection for the *E. coli* cells then bacteria containing a phage from the library, which encodes *lacZα*, will have a complemented *lacZ* gene to enable distinction from bacteria containing a phage from the environment. The NEB 12mer library consists of approximately 2.7 x 10^9 electroporated sequences that were amplified once to yield approximately 55 copies of each sequence. Sequencing of 104 clones from the library yielded six clones (5.8%) that did not contain a displayed peptide insert. Sequencing from the 98 other clones revealed a wide diversity of sequences with no obvious positional biases.

Tools for Production of Phages from Phagemids

To propagate phagemid particles, an amplification tool must be supplied that encodes the rest of the M13 genes necessary for phagemid particle production. These tools may be phages or plasmids and are referred to as "helpers" because they help the phagemid particles propagate by supplying the necessary phage genes in trans. Some often used phage amplification tools include helper phage, hyperphage, phaberge phage, ex-phage, and helper plasmids.

Helper Phage

Helper phage is the most common helper tool used to produce phagemid particles. There are many variations of helper phages, R408, VCSM13, and M13KO7 (63) that differ slightly. Of the various helper phages M13KO7 is the most commonly used. It is a derivative of M13 that has a couple differences including a kanamycin resistance gene and the P15A origin of replication, which allows the genome to be replicated as a plasmid in *E. coli.*

Because helper phages are basically M13 phages, they supply all of the genes necessary for production of phagemid particles. Just like M13, helper phages infect F^+ *E. coli* through the binding of the pIII protein to the F pilus. Helper phage amplifies phagemid particles to yield average titers of 2 x $10^{10\text{-}12}$ phagemids/mL (64). However, because helper phage encodes a wild type *gIII* gene, the phage particles produced will contain a mixture of wild-type pIII proteins and

pIII-fusion proteins. This heterogeneity in display of fusion proteins can result in progeny phagemids bearing all wild type pIII proteins or only a monovalent display of the recombinant pIII protein (65). The low level of display of recombinant pIII proteins results in low efficiency of selection of recombinant phagemid particles. Because the helper phages will also be produced and packaged, amplification of phagemids with helper phage generates a heterogeneous mix of phage particles encoding phagemids and helper phage genomes. The number of helper phages produced can sometimes be greater than or equal the number of phagemid particles generated (64). To get around the problems of helper phage, many alterations have been implemented to improve helper phage.

Helper Phage with *gIII* Mutations

There are many variations to helper phage. One of the key variations to helper phage is the deletion or mutation of the *gIII* gene in the helper phage genome, yielding hyperphage, Ex-phage, and Phaberge phage. All three *gIII*-mutated helper phages are derivatives of M13KO7 that lack full *gIII* gene functionality but still possess pIII proteins. These pIII proteins enable the helper phages to bind to the F pilus of *E. coli* and transfer their helper phage genomes into the *E. coli*. Hyperphage contains a partial deletion of the *gIII* gene, while Ex-phage and Phaberge phage have amber stop codons within the *gIII* gene. Ex-phage contains two amber stop codons, and Phaberge phage contains only one. The mutations in the *gIII* gene of the helper phages promote multivalent display of fusion proteins, which enhances the avidity of binding of phagemid particles to the target molecule. This increased avidity is desirable because it increases the chances of selecting positive clones.

In comparing the antigen-binding activity of phagemid particles produced using M13KO7, hyperphage-produced phagemid particles have over 400-fold increased activity (66), Ex-phage-produced phagemid particles have over 100-fold increased activity (65), and Phaberge-phage-

produced phagemid particles have over 5 to 20-fold increased activity (67). Of the three *gIII* gene mutated helper phages, hyperphage has the highest display of recombinant fusions. This is most likely due to partial read through of the amber stop codons in non-suppressor strains with Ex-phage or Phaberge phage. This partial readthrough generates display of wild-type and fusion-pIII proteins. Hyperphage does not have any readthrough of the wild-type *gIII* gene because of the partial deletion of the *gIII* gene, ensuring that all pIII proteins are recombinant fusions. Hyperphage not only has a higher display level of fusion-pIII proteins, but it also packages phagemid particles over 100 times more efficiently than Ex-phage or Phaberge phage (68).

Of the *gIII* gene-mutated helper phages, hyperphage is the most advantageous for use in phage display systems. Hyperphage enables multivalent display of pIII fusions, which makes it ideal in phagemid particle production. However, an issue with hyperphage is that the phagemid particle stocks generated using hyperphage have titers of $10^{9\text{-}10}$ phagemids/mL (64). This is a log or two lower than phagemid particles produced using helper phage. However, many of the phagemid particles generated by helper phage are useless because they bear no recombinant pIII proteins. Therefore, even though the quantity of phagemid particles produced with hyperphage is lower than those produced with helper phage, the quality of phagemid particles produced is much higher.

Helper Plasmids

Helper plasmids (64) are M13-based plasmids used for phagemid particle amplification that are engineered in three forms to overcome many disadvantages of helper phage and hyperphage. The plasmids are M13mp19 with a chloramphenicol resistance gene cloned in from pBSL121 to allow for selection of *E. coli* containing the helper plasmids. The M13 origin of replication was deleted and replaced with the p15a origin of replication from pMPM-K3. This

eliminates the ability of helper plasmids to be packaged in progeny phage particles, resulting in progeny phages that contain the phagemids but not the helper plasmids. The *gIII* gene of the helper plasmid was partially deleted or fully deleted to yield three helper plasmids with varying lengths of their *gIII* gene.

These three helper plasmids are maintained in *E. coli* and provide phagemid particles with all of the necessary structural proteins for phagemid particle amplification. One of the advantages of the helper plasmids is that they have a full (M13cp), deleted (M13cp-dg3), or truncated (M13cp-CT) *gIII* gene, which enables monovalent to multivalent display of recombinant proteins. Multivalent display phagemid particles possess high avidity binding ability, while monovalent display phagemid particles possess high affinity binding ability. Thus, if high affinity monovalent display phagemid particles are desired, M13cp would be used. If high avidity multivalent display phagemid particles are desired, M13cp-dg3 or M13cp-CT might be used. An additional advantage is that the helper plasmids are maintained in *E. coli*. This negates the need for superinfection, removing the limiting factor of the number of helper phages or hyperphages needed to amplify phagemid particles.

This thesis describes the efforts to optimize biopanning processes and associated reagents to improve the likelihood of isolating recombinant phages that specifically bind to biological agents or their products. The recombinant phages that specifically bind to biological agents or their products will eventually be used to enable detection of the agents.

The specific aims for this study are:

1. To optimize extraction methods and determine saturation limits of *V. cholerae* O1 LPS and use these methods to pan phage display libraries to *V. cholerae* LPS.

- A phenol-water method was a more effective extraction method than TRIzol Reagent method for the extraction of *V. cholerae* O1 LPS and 10-100 times more LPS could be bound to nitrocellulose paper as opposed to a microtiter well. Biopannings against

V. cholerae O1 LPS that was immobilized onto nitrocellulose paper or a microtiter well failed to yield phages that recognized *V. cholerae* O1 LPS.

2. To optimize panning and screening procedures to allow for a more efficient biopanning process that will be more likely to isolate and detect specific recombinant phagemid particles.

- In biopanning with the Tomlinson scFv phagemid library, a trypsin elution time of 10-30 minutes eluted the most phagemid particles. Screening scFv proteins by a high throughput ELISA was an acceptable screening process and enabled the possible screening of hundreds of clones per day. Screening phagemid particles by a high throughput ELISA was not an acceptable screening process because phagemid particles could not be produced in high enough titers in a microtiter well to make a high throughput ELISA screen effective.

3. To optimize the production of phagemid particles to ensure high quality and high quantity of phagemid particles.

- Phagemid particle amplification by *E. coli* harboring hyperphage or helper plasmids was not an effective method to produce phagemid particles. Homemade hyperphage that was produced in *E. coli* MG1655 (pGTR203) enabled high quality and high quantity production of hyperphage. With homemade hyperphage, the Tomlinson J library was effectively amplified to ensure that the redundancy of the clone population in the library was maintained.

4. To isolate specific recombinant phagemid particles to *E. coli* O157:H7 Stx2 toxin using the optimization techniques discovered in previous aims.

- Using the improved homemade hyperphage and the homemade hyperphage-amplified Tomlinson J scFv library to pan against a commercial Stx2 toxin preparation resulted in isolation of phagemid particles that recognized the Stx2 toxin preparation.

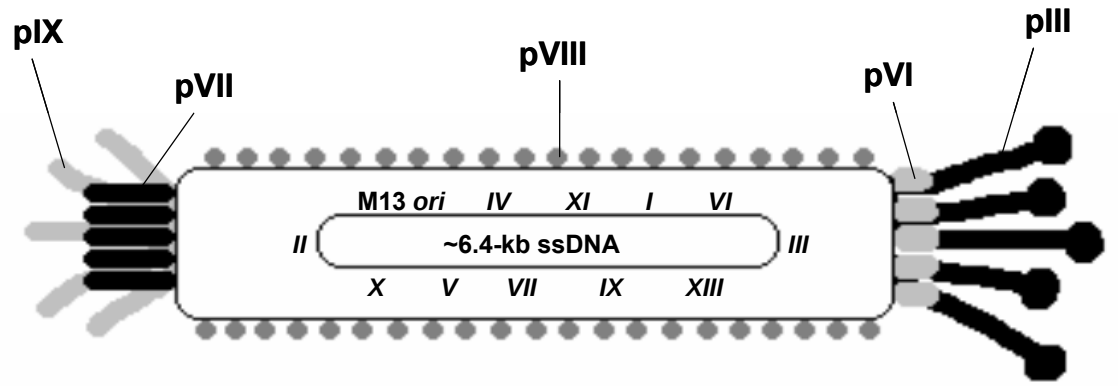


Figure 1-1. Structure of M13 phage. The M13 phage particle contains a ssDNA, circular, 6.4-kb genome that encodes genes I-XI and the M13 *ori*. The M13 phage has five coat proteins: the major coat protein (pXIII) that is present in 2,700 copies and the minor coat proteins (pIII, pVI, pVII, and pIX) that are present in 5 copies each.

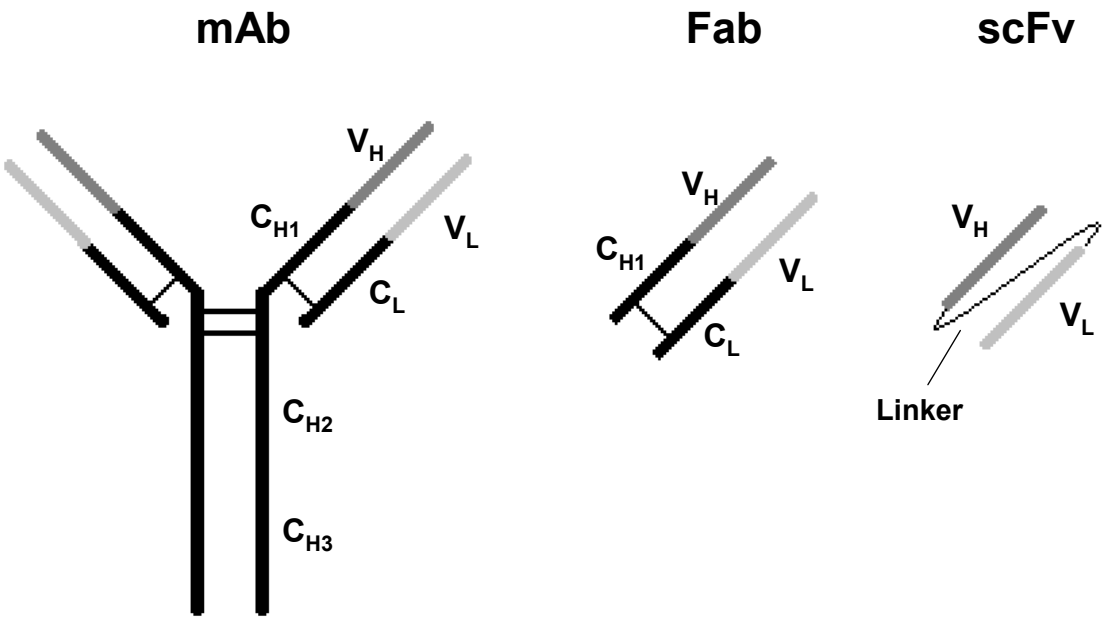


Figure 1-2. Structures of antibodies. mAb (monoclonal antibody), Fab (fragment antigen binding), scFv (single-chain variable fragment), V_H (variable heavy chain), V_L (variable light chain), C_{H1-3} (constant heavy chain domains 1-3), and C_L (constant light chain). The heavy and light chains of mAb and Fab antibodies are held together by disulfide bonds, and the heavy and light chains of the scFv antibody are fused together by a flexible glycine-serine linker.

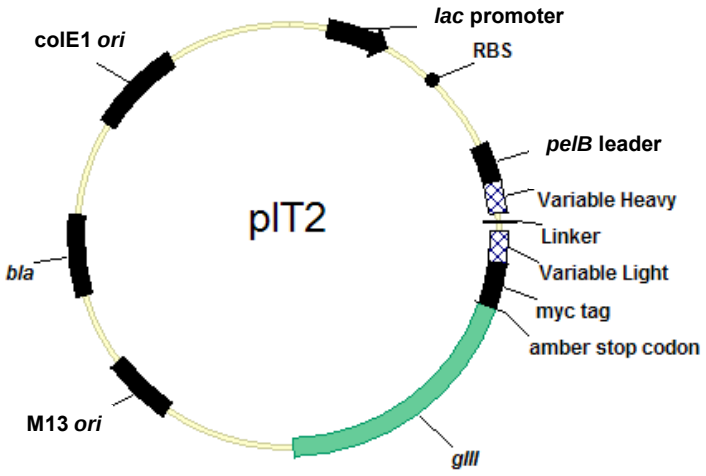


Figure 1-3. Genetic map of pIT2 phagemid vector from the Tomlinson scFv library. RBS-ribosome binding site. pelB leader peptide sequence promotes export of the scFv protein. Variable Heavy and Variable Light peptide sequences are fused together by a glycine-serine linker. An amber stop codon is at the junction of the c-myc tag and the gIII gene to enable conditional expression of the scFv-pIII fusion in an amber suppressor strain. The M13 origin of replication enables packaging into M13 phage particles, the bla gene encodes ampicillin resistance, and the colE1 origin of replication enables maintenance as a plasmid in E. coli.

CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Methods

The bacterial strains used and their genotypes are listed in Table 2-1. All *E. coli* strains were grown in LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 3 mL of 1 M NaOH in 1 L water) or on LB agar plates containing 1.5% (w/v) agar. Exceptions are *E. coli* TG1, *E. coli* DH5α (pNR100), and *E. coli* ER2738. *Escherichia coli* TG1 was grown in 2xTY broth (16 g tryptone, 10 g yeast extract, 5 g NaCl, and 3 mL of 1 M NaOH in 1 L water) or on 2xTY agar plates containing 1.5% (w/v) agar. *Escherichia coli* DH5α (pNR100) was grown in LB broth containing 100 μg/mL ampicillin or on LB agar plates containing 100 μg/mL ampicillin and 1.5% (w/v) agar. *Escherichia coli* ER2738 was grown in LB broth containing 20 μg/mL tetracycline or on LB agar plates containing 20 μg/mL tetracycline and 1.5% (w/v) agar. All strains of *V. cholerae* were grown in Luria Bertani broth containing with physiological saline (LB-N) (10 g tryptone, 5 g yeast extract, 8.5 g NaCl, and 3 mL of 1 M NaOH in 1 L water) or on LB-N agar plates containing 1.5% (w/v) agar. The most common medium used was 2xTY containing 100 μg/mL ampicillin and 1% (w/v) glucose; therefore, it was abbreviated as 2xTY AG. *Escherichia coli* strains harboring hyperphage were grown in broth or on plates as specified, with the addition of 40 μg/mL kanamycin. *Escherichia coli* strains harboring helper plasmids were grown in broth or on plates as specified above, with the addition of 30 μg/mL chloramphenicol.

All bacterial cultures were initially grown as a standing-overnight culture. A standing-overnight culture was made from 10 mL of specified medium that was inoculated with bacteria from an agar plate. The standing-overnight culture was grown overnight (~16 h) in a 37°C incubator. A log-phase culture was obtained by diluting a standing-overnight culture 1:40 in

medium and incubating the culture in a 37°C incubator with shaking until the optical density at 600 nm (OD600) was between 0.4 to 0.6.

The Tomlinson J Human Synthetic VH + VL phagemid library was constructed by Medical Research Council, Cambridge, U.K. (Human Single Fold scFv Libraries I + J (Tomlinson I + J). 2002. Cambridge, UK, MRC Laboratory of Molecular Biology, MRC Centre for Protein Engineering.) and was obtained from the Interdisciplinary Center for Biotechnology Research Hybridoma Core, University of Florida. The Ph. D. 12mer peptide library was obtained from New England Biolabs (NEB, Ipswich, MA). The hyperphage (66) used to amplify phagemid particles was obtained from Progen Biotechnik (Heidelberg, Germany).

Biopanning of Phage Display Libraries

Panning on Immunotubes

A polystyrene immunotube (Nunc, Rochester, NY) was coated with 2 mL of 50 µg/mL Shiga-like toxin 2 (Stx2 (Toxin Technology, Sarasota, FL)) in phosphate-buffered saline (PBS) (Cellgro, Manassas, VA) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3). The immunotube was rotated overnight on a labquake at 4°C. The next day the tube was washed three times with 4 mL of PBS to remove non-immobilized toxin. The tube was filled with casein blocker (Pierce, Rockford, IL) containing 0.05% (v/v) Tween-20 and incubated for two hours at room temperature (~25°C). Excess blocker was removed by rinsing the tube three times with 4 mL of PBS. The Tomlinson J library (for round one of panning) or amplified eluted phages (for subsequent rounds of panning) was added to the immunotube at a concentration of 1 x 10^{12} phages in 4 mL of casein blocker (Pierce) containing 0.05% (v/v) Tween-20. The library was incubated for one hour at room temperature on a labquake, followed by a one hour standing incubation. The unbound library was removed by aspiration, and the weakly bound phages were removed by washing the tube 10 times for round one and 20 times for rounds two and three with

4 mL of PBS containing 0.05% (v/v) Tween-20 (PBS-0.05T). The excess PBS-0.05T was aspirated, and bound phages were eluted with 0.5 mL of trypsin-PBS (10% (v/v) trypsin stock (10 mg/mL trypsin (Type XIII from Bovine Pancreas) (Sigma-Aldrich, St. Louis, MO), 50 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$ in water) in PBS) for 10 minutes at room temperature on a labquake. Ten microliters of the eluted phages were immediately titered, while the rest of the eluted phages were infected into 5 mL of *E. coli* TG1 at an OD_{600} of 0.4. The infected *E. coli* was incubated for 30 minutes at 37°C in a standing water bath. Following incubation, the infected cells were isolated by centrifugation at 13,776 x g for 10 minutes at room temperature. Cells were suspended in 0.6 mL of 2xTY medium and plated on three 2xTY AG plates to amplify the phagemid-containing *E. coli*. The plates were incubated overnight at 37°C. The next day 1.5 mL of 2xTY was added to the lawns of bacteria, and the bacterial lawns were scraped from the plate in the medium and pooled together. Of the pooled bacteria, 50 µL was inoculated into 100 mL of 2xTY AG medium and grown in a 37°C shaking incubator until the OD_{600} was 0.4. Once the OD was reached, 10 mL of the culture was superinfected with homemade hyperphage at a MOI of 20 and incubated for 30 minutes in a standing 37°C water bath. The superinfection was centrifuged at 13,776 x g for 10 minutes at room temperature. The resulting pellet was suspended in 100 mL of 2xTY containing 100 µg/mL ampicillin, 40 µg/mL kanamycin, and 0.1% (w/v) glucose and incubated overnight in a shaking 30°C water bath. The next day the culture was centrifuged at 13,776 x g for 10 minutes at 4°C. Phage-containing supernatant was precipitated with final concentrations of 13 mM Polyethylene Glycol (PEG) and 0.55 M NaCl. PEG and NaCl were dissolved in the supernatant at room temperature and then the PEG-containing supernatant was incubated overnight at 4°C. The next day the PEG-precipitated supernatant was centrifuged at 13,776 x g for 20 minutes at 4°C. The pellet was

suspended in 2 mL of PBS and centrifuged at 13,776 x g for 5 minutes at room temperature to remove bacterial debris. The supernatant was transferred to a new tube to be titered and stored at 4°C until used in further rounds of panning or screening.

Panning in Suspension

Vibrio cholerae N16961 was grown to log phase, and 10^7 to 10^9 cells were centrifuged at 10,621 x g for 5 minutes at room temperature. The bacterial pellet was suspended in 1 mL of PBS and transferred to a microcentrifuge tube. The Tomlinson J library (for round one of panning) or amplified eluted phages (for subsequent rounds of panning) was added to the bacterial suspension at a concentration of 1 x 10^{11} phages in 0.5 mL of PBS. The library and bacterial cells were incubated for 3 hours at 4°C while rotating on a labquake. The cell suspension was centrifuged at 10,621 x g for 5 minutes at room temperature. The supernatant, containing the unbound phage library, was stored at 4°C. The bacterial pellet containing the bound phages was suspended in 0.5 mL of PBS to wash off phages that were weakly bound to the bacteria. The suspension was centrifuged at 10,621 x g for 5 minutes at room temperature. The supernatant was discarded, and the pellet was suspended in 0.5 mL of PBS for a second wash. The suspended cells were transferred to a new microcentrifuge tube and centrifuged at 10,621 x g for 5 minutes at room temperature. The supernatant was aspirated, and phages bound to the bacteria were eluted with 0.5 mL of trypsin-PBS for 10 to 60 minutes at room temperature on a labquake. Ten microliters of the elution was immediately titered. The rest of the eluted phages were infected into 5 mL of *E. coli* TG1 at an OD_{600} of 0.4. The infected *E. coli* were incubated for 30 minutes in a 37°C standing water bath. Following incubation, the infected cells were isolated by centrifugation at 13,776 x g for 10 minutes at room temperature. The cells were suspended in 0.6 mL of 2xTY medium and plated on three 2xTY AG plates to amplify the phagemid-containing bacteria. The plates were incubated overnight at 37°C. The next day 1.5

mL of 2xTY was added to the lawns of bacteria, and the bacterial lawns were scraped from the plate in the medium and pooled together. Of the pooled bacteria, 50 µL were inoculated into 50 mL of 2xTY AG medium and grown in a 37°C shaking incubator till the OD_{600} was 0.4. Once the OD was reached, 10 mL of the culture was superinfected with homemade hyperphage at a MOI of 20 and incubated for 30 minutes in a 37°C standing water bath. The superinfection was centrifuged at 13,776 x g for 10 minutes at room temperature. The resulting pellet was suspended in 50 mL of 2xTY containing 100 µg/mL ampicillin, 40 µg/mL kanamycin, and 0.1% (w/v) glucose and incubated overnight in a 30°C shaking water bath. The next day the culture was centrifuged at 13,776 x g for 10 minutes at 4°C. The phage-containing supernatant was precipitated by slowly adding 12.5 mL of PEG solution (20% (w/v) PEG and 2.5 M NaCl in water) to the supernatant with continuous swirling. The PEG-containing supernatant was incubated overnight at 4°C. The next day the PEG-containing supernatant was centrifuged at 13,776 x g for 20 minutes at 4°C. The pellet was suspended in 1 mL of PBS and centrifuged at 10,261 x g for 5 minutes at room temperature to remove bacterial debris. The supernatant was transferred to a new tube to be titered and stored at 4°C until used in further rounds of panning or screening.

Panning on Microtiter Wells

A polysorp microtiter well (Nunc) was coated with 150 µL of 100 µg/mL *V. cholerae* 569 lipopolysaccharide (LPS) (Sigma-Aldrich) in carbonate coating buffer (0.1 M $NaHCO_3$, 0.02% (w/v) NaN_3 (pH 8.6)). Prior to coating, the LPS was sonicated by a Vibra Cell (Sonics & Materials Inc., Danbury, CT) for one minute. The microtiter well was coated overnight at 4°C in a humid chamber. The next day the microtiter well was equilibrated to room temperature for 15 minutes. The LPS was aspirated and blocked with 200 µL of carbonate blocking buffer for one hour at 4°C. The well was washed six times with 200 µL of 0.1 M Tris-buffered saline (TBS)

(81 mM Tris-HCl, 20 mM Tris-Base, 154 mM NaCl, pH 7.5) containing 0.1% (v/v) Tween-20 (TBS-0.1T) for the first round of panning and with 200 µL of 0.1 M TBS containing 0.5% (v/v) Tween-20 (TBS-0.05T) for subsequent rounds of panning. The washed wells were incubated with 2 x 10^{11} PFU of the Ph.D.-12mer peptide library (NEB) (for round one of panning) or amplified phages (for subsequent rounds of panning) diluted in 100 µL of TBS-0.1T for one hour at room temperature with gentle rocking. The unbound phages were aspirated and stored at 4°C. The wells were washed 10 times with TBS-0.1T for the first round of panning and with 200 µL of TBS-0.05T for subsequent rounds of panning. The phages were eluted with 100 µL of 0.1 M glycine (pH 2.2) for 10 minutes at room temperature with gentle agitation. The glycine containing the eluted phages was transferred to a microcentrifuge tube and neutralized with 15 µL of Tris-HCl (pH 9.0). Five microliters of the eluted phages were immediately titered. One hundred and ten microliters of the remaining elution was added to 20 mL of LB diluted 1:100 with a standing-overnight culture of *E. coli* ER2738. The culture was grown for 4.5 hours in a 37°C shaking incubator. The turbid culture was centrifuged at 13,776 x g for 10 minutes at 4°C. The phages were precipitated by slowly adding 3.3 mL of PEG solution (20% (w/v) PEG and 2.5 M NaCl in water) to the phage-containing supernatant with continuous swirling. The PEG-containing supernatant was incubated overnight at 4°C. The next day the PEG-containing supernatant was centrifuged at 13,776 x g for 20 minutes at 4°C. The pellet was suspended in 1 mL of 0.1 M TBS and centrifuged at 10,621 x g for 5 minutes at room temperature to remove bacterial debris. The phages were precipitated a second time by slowly adding 167 µL of PEG solution to the supernatant with continuous swirling. The PEG-containing supernatant was incubated one hour at 4°C and centrifuged at 13,776 x g for 15 minutes at 4°C. The pellet was suspended in 200 µL of 0.1 M TBS to be titered and stored at 4°C until used in further rounds of

panning or screening. Five rounds of panning were done with screening of clones after the fifth round of panning.

Panning on Nitrocellulose Paper

Six pieces of nitrocellulose paper (Bio-Rad) were cut to the surface-area dimensions of a microtiter well (5 mm x 20 mm). One and a half milliliters of approximately 340 μg/mL *V. cholerae* 569 LPS (phenol-water-extracted) in PBS was added to six microcentrifuge tubes, each containing a strip of nitrocellulose paper. Prior to coating, the LPS was sonicated by a Vibra Cell (Sonics & Materials Inc.) for one minute. The nitrocellulose papers were coated overnight at 4°C on a rotating labquake. The next day two pieces of LPS-coated nitrocellulose paper were each transferred to a new microcentrifuge tube and washed five times with 1 mL of PBS on a labquake with five minutes per wash. Washed nitrocellulose strips were transferred to new microcentrifuge tubes and blocked for one hour in PBS casein blocking blocker (Pierce) on a labquake at room temperature. Blocked nitrocellulose strips were transferred to new microcentrifuge tubes and washed five times with 1 mL of PBS on a labquake with five minutes per wash. Each of the washed pieces of nitrocellulose papers was transferred to a new microcentrifuge tube containing 1.5×10^{11} PFU of the Ph.D.-12mer peptide library (NEB) diluted in 1 mL of PBS. Nitrocellulose strips were incubated with the library for one hour at room temperature on a labquake. After one hour, the panned nitrocellulose strips were transferred to new microcentrifuge tubes and washed five times with 1 mL of PBS on a labquake with five minutes per wash to remove the unbound library. Washed nitrocellulose strips were transferred to new microcentrifuge tubes to be eluted. One piece of nitrocellulose was acid eluted by adding 250 μL of 0.1 M glycine (pH 2.2) to the strip and incubating it for 10 minutes at room temperature on a labquake. The acid eluted nitrocellulose paper was removed to a new microcentrifuge tube, and the glycine solution containing the eluted phages was neutralized with

37 µL of 1 M Tris-HCl (pH 9.0). The acid eluted phages were stored at 4°C until used for amplification. The other piece of panned nitrocellulose paper was antigen eluted by adding 250 µL of approximately 340 µg/mL of *V. cholerae* 569B LPS diluted in PBS to the piece of nitrocellulose paper and incubated one hour at room temperature on a labquake. The piece of nitrocellulose paper was removed, and the remaining LPS solution containing the eluted phages was acid eluted as described above. Five microliters of each eluted phage solution was titered, and the rest of the two elutions were each added to 20 mL of LB diluted 1:100 with a standing-overnight culture of *E. coli* ER2738. The cultures were grown for 4.5 hours in a 37°C shaking incubator. The turbid cultures were centrifuged at 13,776 x g for 10 minutes at 4°C. Five microliters of the amplified phages were titered. The rest of the phages were PEG precipitated by slowly adding 3.3 mL of PEG solution to the phage-containing supernatant with continuous swirling. The PEG-containing supernatant was incubated overnight at 4°C. The next day the PEG-containing supernatant was centrifuged at 13,776 x g for 20 minutes at 4°C. The pellet was suspended in 1 mL of PBS and centrifuged at 13,776 x g for 5 minutes at room temperature to remove bacterial debris. The supernatants were transferred to new microcentrifuge tubes, titered, and stored at 4°C.

After the first round of panning, the amplified eluted phages were negatively panned on. Two pieces of nitrocellulose paper (5 mm x 20 mm) were blocked for one hour in 1 mL of PBS casein blocking buffer (Pierce) on a labquake at room temperature. The blocked nitrocellulose strips were washed three times with 1 mL of PBS on a labquake at room temperature with five minutes per wash. One piece of blocked nitrocellulose paper was added to the acid-eluted-amplified phages from the round one panning and incubated one hour on a labquake at room temperature. The other strip of blocked nitrocellulose paper was added to the LPS and acid-

eluted-amplified phages from the round one panning and incubated one hour on a labquake at room temperature. The negatively panned pieces of nitrocellulose paper were removed, and the remaining amplified-eluted phages were used for two more rounds of panning. After the third round of panning the eluted phages were screen by ELISA.

Spot Titer of Phages

Escherichia coli TG1 was grown to log phase and centrifuged at 13,776 x g for 10 minutes at 4°C. The resulting pellet was suspended in PBS to yield an *E. coli* TG1 concentration of 1 x 10^{10} CFU/mL. One hundred microliters of the concentrated *E. coli* TG1 was spread on a 2xTY AG plate and set to dry for 30 seconds. Serially diluted phages in phosphate-buffered saline containing 0.1% (w/v) gelatin (BSG) was dropped onto the plate in 10 µL drops. Once the drops dried, the plate was incubated overnight at 37°C. The next day the colonies were counted, and the approximate titer was calculated.

Spread Titer of Phages

Phages were serially diluted in BSG. One hundred microliters of the diluted phages were added to 0.9 mL of log phase *E. coli* TG1. The infected *E. coli* TG1 was incubated 20 minutes in a 37°C standing water bath. One hundred microliters of the infections were plated on 2xTY AG plates. The plates were incubated overnight at 37°C. The next day the colonies were counted, and the approximate titer was calculated.

Amplification of Phages

A colony of *E. coli* TG1 containing a phagemid was picked from a plate with a sterile toothpick and swirled in 3 mL of 2xTY AG medium. The culture was grown overnight at 37°C. The overnight culture was diluted 1:20 in 3 mL of 2xTY AG medium and grown to log phase in a 37°C shaking incubator. Three hundred microliters of the log-phase culture was infected with hyperphage at a MOI of 10 and incubated in a 37°C standing water bath for 30 minutes. The

culture was added to 30 mL of 2xTY medium containing 100 μg/mL ampicillin, 40 μg/mL kanamycin, and 0.1% (w/v) glucose. The culture was grown overnight in a 30°C shaking incubator. The turbid culture was centrifuged at 13,776 x g for 10 minutes at 4°C. The phage-containing supernatant was titered and stored at 4°C.

High Throughput Production of Soluble Antibody Fragments (scFv antibodies)

A colony of *E. coli* HB2151 containing a phagemid was picked from a plate with a sterile toothpick and swirled in 200 μL of 2xTY containing 100 μg/mL ampicillin, 0.1% (w/v) glucose, and 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) in a 96-well polystyrene microtiter plate (Corning, Corning, NY). The plate was incubated overnight at 37°C. The next day the plate was centrifuged at 4,667 x g for 10 minutes at 20°C. Supernatants were analyzed by ELISA.

Deoxyribonucleic Acid Manipulations

Plasmid Extractions

Plasmid extractions for cultures of 3 mL were performed with the QIAprep Spin Miniprep kit (Qiagen, Germantown, MD), while cultures of 100 mL or greater were performed with the Plasmid Midi kit (Qiagen). Extraction procedures were performed as directed in the instruction manual.

Agarose Gel Electrophoresis

Deoxyribonucleic acid samples from 1-5 μL were added to 2 μL of 10 x Gel Loading Buffer (Invitrogen, Carlsbad, CA). The samples were loaded onto a 0.7% (w/v) agarose gel using Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 10 μg/mL ethidium bromide. The gel was electrophoresed at 100 V until the loading dye was two-thirds down the gel. The DNA bands were analyzed on a Gel Doc XR (Bio-Rad, Hercules, CA).

Electroporation of Plasmids

Electroporation was performed to transform plasmids into *E. coli.* DNA (30-1000 ng/μL) was added to 50 μL of electrocompetent cells (see below) in a microcentrifuge tube. The tube was flicked to mix the DNA with the cells. Once the DNA and cells were mixed, they were transferred to a chilled 0.1 cm electroporation cuvette and electroporated at 1.25 kV/cm with a MicroPulser (Bio-Rad). Nine hundred and fifty microliters of 2xTY medium was immediately added to the transformation and transferred to a small culture tube where it was incubated in a 37°C static water bath for 1 hour. The electroporation was serially diluted in BSG and plated on appropriate plates. The plates were incubated overnight at 37°C. The next day the colonies were counted, and the number of transformations was calculated.

Electrocompetent Cells

Electrocompetent cells were made by diluting a standing overnight culture 1:100 in 1 L of medium containing 1% (w/v) glucose and appropriate antibiotics. The culture was incubated in a 37°C shaking incubator until the OD_{600} was between 0.4 and 0.6. All centrifuge rotors, wash buffers, and centrifuge tubes that were used were pre-chilled to 4°C. Once the OD_{600} was between 0.4 and 0.6, the culture was chilled on ice in a 4°C cold room for 45 minutes or more until the culture was ~4°C. The culture was centrifuged at 13,776 x g for 10 minutes at 4°C, and the supernatant was decanted. The pellets were suspended in 1.2 L chilled ddH_2O by vortexing or pipeting until the cell suspension was homogeneous. The suspended pellets were centrifuged to pellet the bacterial cells. The supernatant was decanted, and the pellets were suspended in 400 mL of chilled ddH_2O. The bacteria were pelleted by centrifugation, and the supernatant was decanted. The pellets were suspended in 200 mL of chilled ddH_2O into one centrifuge tube. The bacteria were pelleted by centrifugation, and the supernatant was decanted. The pellet was suspended in 25 mL of chilled ddH_2O and transferred to a 35 mL centrifuge tube. The bacteria

were pelleted by centrifugation, and the supernatant was decanted. The pellet was suspended in 25 mL of chilled 10% (w/v) glucose in water and centrifuged to pellet the bacteria. The supernatant was decanted, and the bacteria were suspended in 0.5-1 mL of chilled 10% (w/v) glucose in water. The suspended bacteria were either used directly for transformation or aliquoted and frozen on dry ice in 95% (v/v) EtOH and stored at -80°C.

Enzyme-Linked ImmunoSorbent Assays (ELISAs)

Enzyme-linked immunosorbent assays were performed for the screening and characterization of phage particles, scFvs, LPS, bacterial protein preparations, monoclonal antibodies, and polyclonal antibodies. The coating antigen or antibody was diluted in PBS (Cellgro) or carbonate/bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$ in water), and 100 µL was loaded onto a 96-well polystyrene flat-bottomed microtiter plate (Becton-Dickinson, Franklin Lakes, NJ). Concentrations used of whole cells (10^8 tu/mL), LPS (1 µg/mL), toxin (10 µg/mL), and proteins (10 µg/mL) were kept relatively consistent. The coated microtiter plate was incubated overnight in a humid chamber at 4°C. The next day the microtiter plate was equilibrated to room temperature for 30 minutes. Wells were aspirated with an EL*x* 800 Strip Washer (Bio-Tek, Winooski, VT) and washed once with 300 µL of PBS. If the coating antigen was whole cells, LPS, or toxin, then the coating antigen was removed by vacuum or pipet and sterilized. Two hundred microliters of PBS casein blocking buffer (Pierce) containing 0.05% (v/v) Tween-20 was added to each well and incubated two hours at room temperature. Blocker was aspirated by the EL*x* 800 Strip Washer (Bio-Tek). One hundred microliters of primary antibody diluted in PBS casein blocking buffer (Pierce) containing 0.05% (v/v) Tween-20 was added to each well and incubated one hour at room temperature. Concentrations used of phagemid particles were 10^7 to 10^9 tu/mL and antibodies were 1 to 10 µg/mL. The primary antibody was aspirated and washed three times with 300 µL of PBS-0.05T by the EL*x* 800 Strip

Washer (Bio-Tek). One hundred microliters horseradish peroxidase (HRP) conjugated secondary antibodies were diluted in PBS casein blocking buffer (Pierce) containing 0.05% (v/v) Tween-20 and added to each well and incubated 30 minutes at room temperature. The secondary antibody was aspirated and washed three times with 300 µL of PBS-0.05T by the ELx 800 Strip Washer (Bio-Tek). Substrate was prepared by dissolving one capsule of phosphate-citrate (PC) buffer (0.05 M phosphate-citrate buffer (pH 5.0), 0.03% (w/v) sodium perborate) (Sigma-Aldrich) in 100 mL of water. A ten milligram tablet of 3, 3', 5, 5'-tetramethylbenzidine substrate (Sigma-Aldrich) was added to 10 mL of the PC buffer to give a final concentration of 1 mg/mL. Two hundred microliters of substrate was added to each well and incubated for 30 minutes at room temperature. The plate was read in an ELx 800 UV plate reader (Bio-Tek) at 630 nm. The data were analyzed with KcJunior (Bio-Tek) software.

Infection Efficiency

Infection efficiency experiments were done to compare the infection efficiency of phagemid particles to bacterial strains harboring helper plasmids to the same strains not harboring helper plasmids. *Escherichia coli* standing-overnight cultures were diluted 1:40 in 3 mL of medium containing 1% (w/v) glucose for strains containing no helper plasmids and in medium containing 1% (w/v) glucose and 30 µg/mL chloramphenicol for strains containing helper plasmids. The diluted cultures were grown in a 37°C shaking incubator till the OD_{600} was between 0.4 and 0.6. Once the desired OD_{600} was obtained, 3×10^7 bacteria were infected with phage at a MOI of 0.1 and incubated for 20 minutes in a 37°C standing water bath. The transductions were titered using the spread-titer method. Transduced *E. coli* strains containing helper plasmids were titered on 2xTY AG plates containing 30 µg/mL chloramphenicol. Transduced *E. coli* strains containing no helper plasmids were titered on 2xTY AG plates.

Protein and Lipopolysaccharide (LPS) Manipulations

Phenol-Water Extraction of LPS

A phenol-water extraction (76) was performed to extract *V. cholerae* 569B LPS from whole cells. One and a half liters of *V. cholerae* 569B was grown to log phase in a 37°C shaking incubator. Bacteria were pelleted at 13,776 x g for 10 minutes at 4°C and suspended in 10 mL of 1% (w/v) NaCl in water. The bacteria were centrifuged and suspended in another 10 mL of 1% (w/v) NaCl in water. Next, the bacteria were centrifuged and suspended in 10 mL of ddH_2O. This suspension was warmed to 70°C, along with 24 mL of 90% (w/v) phenol (made directly before extraction). Ten milliliters of warmed 90% (w/v) phenol was added to the 10 mL cell suspension and vortexed. The twenty milliliter suspension was warmed in a 70°C water bath for 20 minutes while vortexing frequently. The phenol suspension was transferred to an ice bath where it was swirled in ice water for 5 minutes. The phenol suspension was centrifuged at 2,284 x g for 25 minutes at 4°C. The aqueous phase of the phenol extraction was transferred to a new centrifuge tube and stored on ice. The same volume of water as that extracted was added back into the phenol suspension tube and heated to 70°C for 20 minutes with frequent vortexing. The extraction step was repeated, and the two aqueous phase extractions were combined into the same tube. Aqueous extractions were placed in a 70°C water bath for 15 minutes. Twelve milliliters of warmed 90% (w/v) phenol was added to the warmed aqueous extraction and incubated at 70°C for 10-15 minutes with frequent vortexing. Two back extractions were performed on the aqueous extractions. The final extraction was dialyzed (MWCO: 3,500) for 24-48 hours at 4°C. The dialyzed extraction was digested with DNase (Qiagen) (20 µg DNase/mL dialysis volume) and RNase (Qiagen) (40 µg RNase/mL dialysis volume) in MgCl (1 µL 20% (w/v) MgCl/mL dialysis volume) overnight at 37°C. The digested extraction was

dialyzed (MWCO: 3,500) for 24-48 hours at 4°C. The final extraction was used in a Tsai-Frasch silver stain to analyze the LPS.

TRIzol Reagent Extraction of LPS

TRIzol Reagent (Invitrogen), a mono-phasic solution of phenol and guanidine isothiocyanate (77), was used to extract *V. cholerae* N16961 LPS from whole cells. Ten milliliters of log phase (OD_{600} ~ 0.4-0.6) *V. cholerae* N16961 were centrifuged at 13,776 x g for 10 minutes at 4°C. The supernatant was decanted, and the cells were suspended in 200 µL of TRIzol Reagent and incubated at room temperature for 10 to15 minutes. Twenty microliters of chloroform per mg of cells was added to the mixture, vortexed, and incubated at room temperature for 10 minutes. The mixture was centrifuged at 12,000 x g for 10 minutes at room temperature. The resulting aqueous phase was transferred to a new microcentrifuge tube and stored on ice. One hundred microliters of ddH_2O was added to the TRIzol-Reagent tube, and a back extraction was performed. Two more back extractions were performed following the first back extraction to give a total of four extractions. The extractions were dried with a Savant SpeedVac Concentrator (Global Medical Instrumentation, Ramsey, MN) for 2.5 hours. The pellet was suspended in 0.5 mL of 0.375 M MgCl in 95% (v/v) EtOH that was chilled to 4°C. The suspension was centrifuged at 12,000 g for 15 minutes at room temperature. The resulting pellet was suspended in 200 µL of ddH_2O. The final LPS extraction was analyzed in a Tsai-Frasch silver stain (see below).

Extraction of Periplasmic Proteins

Periplasmic proteins from *E. coli* were extracted using a Tris-EDTA-Sucrose (TES) extraction. A standing-overnight culture of an *E. coli* culture was diluted 1:400 in 100 mL of LB medium and incubated overnight in a 37°C shaking incubator. The next day the cells were centrifuged at 13,776 x g for 10 minutes at 4°C. The supernatant was stored at 4°C. The pellet

was suspended in 25 mL of PBS by vortexing. The suspension was centrifuged at 13,776 x g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was suspended in 10 mL of TES (20% (w/v) D-sucrose, 30 mM Tris-HCl, 1 mM EDTA) in water. The suspended pellet was incubated for 15 minutes at room temperature with occasional swirling. The suspension was centrifuged at 13,776 x g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was gently washed with 10 mL of 0.5 mM $MgCl_2$ without dislodging the pellet. The 10 mL of the 0.5 M $MgCl_2$ wash was discarded, and the pellet was suspended by vortexing in 10 mL of 0.5 mM $MgCl_2$. The suspension was incubated in an ice bath for 15 minutes with occasional swirling. The suspension was centrifuged at 21,525 x g for 30 minutes at 4°C. The supernatant was collected and concentrated with 10,000 MWCO Amicon (Millipore, Billerica, MA) filters and stored at 4°C. The pellet was suspended in 10 mL of PBS and stored at 4°C.

Determination of Protein Concentration

Protein concentration was determined by DC-Protein Assay (Bio-Rad). Protein samples were diluted in PBS. Five microliters of each sample was added to a microtiter well followed by 25 µL of Reagent A and 200 µL of Reagent B. All wells were performed in triplicate. Protein standards used were Bovine Serum Albumin (BSA) in concentrations from 0-1.4 mg/mL. The reaction took place for 15 minutes at room temperature and was read at 750 nm with an ELx 800 UV plate reader (Bio-Tek). Protein concentrations were analyzed using KC Junior (Bio-Tek) software.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to resolve *V. cholerae* LPS and protein preparations. ReadyGel Tris-glycine 12% (w/v), 15% (w/v), and 4-20% (w/v) PAGE gels (Bio-Rad) were used with the MiniProtean-Electrophoresis system (Bio-Rad) for SDS-PAGE analysis of samples. The LPS samples were sonicated by a Vibra Cell

(Sonics & Materials Inc.) for 2 minutes and diluted in Laemmli sample buffer (Bio-Rad). Protein samples were vortexed and diluted in Laemmli sample buffer. After dilution in Laemmli sample buffer, the samples were boiled for 10 minutes and loaded in the gels. The samples were electrophoresed for 1 hour at 100 Volts in electrode buffer (25 mM Tris, 0.17 M glycine, and 0.1% (w/v) SDS).

Coomassie Blue Staining

Proteins resolved by SDS-PAGE were stained with 0.05% (w/v) Coomassie Blue R, 50% (v/v) methanol, and 10% (v/v) glacial acetic acid in water and agitated gently for 10 minutes. The gel was rinsed with ddH_2O to remove excess stain. The gel was destained with 5% (v/v) methanol and 7% (v/v) glacial acetic acid in water overnight while gently shaking. The next day a picture was taken of the gel, and the gel was dried.

Tsai-Frasch Silver Staining

Tsai-Frasch silver staining (78) was performed on SDS-PAGE resolved LPS that was extracted from *V. cholerae.* Carbohydrates resolved by SDS-PAGE were fixed in 40% (v/v) ethanol and 5% (v/v) glacial acetic acid in water overnight. Carbohydrates were oxidized by incubating the fixed gel in 0.7% (w/v) periodic acid, 40% (v/v) ethanol, and 5% (v/v) glacial acetic acid in water for five minutes. The gel was washed three times in 500 mL of ddH_2O with 15 minutes per wash. Reagent A (1% (v/v) 10 M NaOH and 6.7% (v/v) NH_4OH in water) and Reagent B (20% (w/v) $AgNO_3$ in water) were made immediately before staining. The stain was made by drop wise titration of Reagent B into Reagent A until a hazy brown color to the solution appeared and remained. The titrated reagent was diluted in 115 mL of ddH_2O to make the final stain solution. The gel was placed in the staining solution and stained for 10 minutes with agitation. The gel was washed three times in 500 mL of ddH_2O with 15 minutes per wash. The gel was developed with 1 x Developer (5 x Tsai-Frasch developer: 1.3 mM citric acid and 0.25%

(v/v) of 37% (v/v) formaldehyde in ddH_2O) for 3 to 10 minutes with constant gentle shaking. The developed gel was soaked in 1% (v/v) acetic acid in water for 10 minutes and transferred into 50 mL of ddH_2O. A picture was taken of the gel, and then the gel was dried.

Western Blot

Proteins resolved by SDS-PAGE were transferred onto a nitrocellulose membrane, followed by reaction with antibodies and development with enhanced chemiluminescent (ECL) (Pierce). A Mini-TransBlotting cell (Bio-Rad) was used for the transfer of proteins to the nitrocellulose membrane. The transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol) was pre-chilled to 4°C. The transfer occurred at 100 V for one hour at 4°C. Transferred proteins were analyzed by Ponceau S staining. Fifteen milliliters of 0.3% (w/v) Ponceau S dissolved in 3% (v/v) trichloroacetic acid (TCA) was added to the transferred membrane and incubated 10 minutes at room temperature with gentle agitation. The stained membrane was gently washed in 25 mL of dH_2O to remove excess stain. The membrane was photographed to view the stained proteins. The membrane was blocked in 25 mL of PBS casein blocking buffer (Pierce) containing 1% (v/v) Tween-20 for two hours at room temperature or overnight at 4°C with gentle shaking. Primary antibodies were diluted in PBS casein blocking buffer (Pierce) containing 0.05% (v/v) Tween-20 to give a final volume of 10 mL and were added to the blocked membrane for one hour at room temperature with gentle shaking. The membrane was washed three times with 20 mL of PBS-0.05T while shaking for 15 minutes for each wash. Secondary antibodies conjugated with HRP were diluted in PBS casein blocking buffer (Pierce) containing 1% (v/v) Tween-20 to give a final volume of 10 mL and were added to the membrane for 30 minutes at room temperature with gentle shaking. The membrane was washed three times with 20 mL of PBS-0.05T while shaking for 10 minutes for each wash. The

blot was incubated in 8 mL of ECL substrate (Pierce) for three minutes at room temperature and developed on CL-XPosure Film (Pierce) by an X-omat 2000 processor (Kodak, Rochester, NY).

Lipopolysaccharide Saturation to Nitrocellulose Paper

The LPS saturation experiment to nitrocellulose paper was performed to determine the concentration of *V. cholerae* 569B LPS needed to saturate a piece of nitrocellulose paper. Phenol-water-extracted *V. cholerae* 569B LPS was serially diluted from 100 to 1 μg/mL in PBS. Twelve pieces of nitrocellulose paper were cut to dimensions of 5 mm x 20 mm. Nitrocellulose strips were put in a microcentrifuge tube containing 256 μL of the LPS dilutions or PBS for a control. LPS and PBS coated strips were done in duplicates in separate tubes. The strips were coated overnight at 4°C on a labquake. Coated nitrocellulose strips were transferred to new microcentrifuge tubes. Strips were washed 3 times with 1 mL of PBS on a labquake with 5 minutes per wash. Strips were blocked with 1 mL of casein blocking buffer (Pierce) for one hour at room temperature on a labquake. Strips were incubated with 0.5 mL of rabbit anti-*V. cholerae* O1 LPS polyclonal antibody (Accurate Chemical, Westbury, NY) at a dilution of 1:400 in casein blocking buffer (Pierce) for one hour at room temperature on a labquake. Strips were washed 3 times with 1 mL of PBS on a labquake with 5 minutes per wash. Strips were incubated with 0.5 mL of goat anti-rabbit peroxidase conjugated monoclonal antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a concentration of 1:1000 in casein blocking buffer (Pierce) for 30 minutes at room temperature on a labquake. Strips were washed 3 times with 1 mL of PBS on a labquake with 5 minutes per wash. Strips were incubated in 5 mL of ECL substrate (Pierce) for three minutes at room temperature and developed on CL-XPosure Film (Pierce) by an X-omat 2000 processor (Kodak).

Colony Blot with scFv

To test whether bacteria produce scFv that are specific to a target molecule, a colony blot was developed to screen scFv in a high throughput manor. A Petri dish size piece of nitrocellulose paper (Bio-Rad) was coated overnight at 4°C with 10 mL of target antigen at a concentration that the target antigen was panned. The next day the coated nitrocellulose paper was washed three times with 15 mL of 0.1 M TBS with five minutes per wash on a labquake. The nitrocellulose paper was then blocked for one hour at room temperature with 10 mL of casein blocker (Sigma-Aldrich) on a labquake. After being blocked, the nitrocellulose paper was overlaid onto a plate containing bacterial colonies and stamped with a metal-plate stamp. The bacterial-colony-containing nitrocellulose was washed three times with 15 mL of 0.1 M TBS with five minutes per wash on a labquake. The nitrocellulose paper was incubated with Protein L-peroxidase (Sigma-Aldrich) that was diluted 1:2,000 in 10 mL of casein blocker (Sigma-Aldrich) for one hour at room temperature on a labquake. The nitrocellulose paper was washed three times with 15 mL of 0.1 M TBS with five minutes per wash on a labquake. The nitrocellulose paper was incubated in 5 mL of ECL substrate (Pierce) for three minutes at room temperature and developed on CL-XPosure Film (Pierce) by an X-omat 2000 processor (Kodak).

Table 2-1. Bacterial strains and plasmids used.

Strain	Genotype / Description	Source / Reference
E. coli DH5α	F^-φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR, recA1, endA1*, *hsdR*17(r_k^- m_k^+), *phoA, supE44*, λ^-, *thi-1, gyrA96, relA1*	Bethesda Research Laboratories, Rockville, MD
E. coli DH5α (pNR100)	F^-φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR, recA1, endA1, hsdR17*(r_k^- m_k^+), *phoA*, supE44, λ^- thi-1, *gyrA96, relA1* (pNR100)	(69)
E. coli EC100D	F^- *mcr*A *Δ(mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G *pir*$^+$(DHFR)	Epicentre, Madison, WI
E. coli EC100D (pGTR203) (HyΦ)	F^- *mcr*A *Δ(mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G *pir*$^+$(DHFR) (pGTR203) (HyΦ)	This paper
E. coli ER2738	F′ *lacI*^{q}, Δ(*lacZ*)M15 *proA*$^+$*B*$^+$, zzf::Tn10(*TetR*) / *fhuA2, supE, thi,* Δ(*lac-proAB*), Δ(*hsdMS*$^-$*mcrB*)5, (r^- m^- McrBC$^-$)	New England Biolabs, Ipswich, MA
E. coli HB101 (pMJ100)	F-, *hsdS*20(r_B- m_B), *recAB*3, *ara*-14, *proA*2 *lacYl*, *galK*2, *rpsL*20(Sm^R), *xy*l-5 *mtl*-l, *supE44,* λ^-, (pMJ100)	(70)
E. coli HB2151	*ara*, Δ(*lac-pro*), *thi*/ F'*proA*$^+$*B*$^+$, *lacI*^{q}ZΔM15	Amersham Pharmacia, Piscataway, NJ
E. coli JM109	*endA1, recA1, gyrA96, thi, hsdR17* (r_k^-, m_k^+), *relA*1, *supE44*, Δ(*lac-proAB*) /F′ *traD36*, *proAB*, *laq*I^{q}ZΔM15	Invitrogen, Carlsbad, CA
E. coli MG1655	F^-, *λ*$^-$, *ilvG*$^-$, *rfb*-50, *rph*-1	(71)
E. coli MG1655 (pGTR203) (HyΦ)	F^-, *λ*$^-$, *ilvG*$^-$, *rfb*-50, *rph*-1 (pGTR203) (HyΦ)	This paper
E. coli O157:H7 EDL933	*Stx1*$^+$, *Stx2*$^+$, *gyrA*	(72)
E. coli O157:H7 87-23	*Stx1*$^-$, *Stx2*$^-$, *gyrA*	(73)

Table 2-1. Continued

Strain	Genotype / Description	Source / Reference
E. coli TG1	Δ*(lac-proAB)*, *supE, thi, hsdD*5/F' *traD36*, *proA*$^{+}$*B, lacI*^{q}*, lacZ*ΔM15	MRC, Cambridge, UK
E. coli TG1 (pGTR203)	Δ*(lac-proAB)*, *supE, thi, hsdD*5/F' *traD36*, *proA*$^{+}$*B, lacI*^{q}*, lacZ*ΔM15 (pGTR203)	Gopal Sapparapu
HyΦ	Hyperphage genome (contains all M13 genes except *gIII*)	(66)
M13cp	M13mp19; Cam^{R}; full length *gIII*	(64)
M13cp-CT	M13mp19; Cam^{R}; truncated *gIII*	(64)
M13cp-dg3	M13mp19; Cam^{R}; deleted *gIII*	(64)
pGTR203	pACYC184 with M13 *gIII;* Cam^{R}	Gopal Sapparapu
pMJ100	pBluescript with slt-II; Amp^{R}	(70)
pNR100	Stx2 toxoid; Amp^{R}	(69)
V. cholerae 569B	Classical, Inaba	(74)
V. cholerae N16961	El Tor, Inaba	(75)

CHAPTER 3
RESULTS

Rationale for Study

There exists a strong need for rapid, sensitive, and selective methods of detection for biological agents. Detecting biological organisms and their products has many uses. One reason is for diagnosis of patient samples. Detection and identification of a disease-causing agent in patient samples allows for the proper treatment to be given. Detecting biological agents in food, water, and air allows for preventative measures to be enforced to prevent or inhibit the spread of disease. A common component in almost all detection assays is a protein that specifically binds to a target molecule. This thesis describes the optimization of protocols used to isolate specific proteins that bind to target molecules by using phage display.

The specific aims of this study are:

1. To optimize extraction methods and determine saturation limits of *V. cholerae* LPS and use these methods to pan phage display libraries to *V. cholerae* LPS.
2. To optimize panning and screening procedures to allow for a more efficient biopanning process that will be more likely to isolate and detect specific recombinant phagemid particles.
3. To optimize the production of phagemid particles to ensure high quality and high quantity of phagemid particles.
4. To isolate specific recombinant phagemid particles to *E. coli* O157:H7 Stx2 toxin using the optimization techniques discovered in previous aims.

Specific Aim 1: Panning to V. cholerae LPS

The first goal of this work was to isolate phage display reagents that specifically recognized V. cholerae O1 LPS. There are many serogroups of *V. cholerae* but only O1 and O139 cause epidemic cholera. Therefore, O1 LPS serves as a useful target because it is a distinguishing feature of *V. cholerae* O1 strains. Previous attempts in the laboratory to obtain phage display reagents that specifically recognized *V. cholerae* O1 LPS may have failed because the *V. cholerae* O1 LPS was panned with the Tomlinson scFv phagemid library. Phagemid

particles, which only encode the *gIII* gene, require phage amplification tools to provide phage genes I-XI to enable phagemid particle production. We chose the Ph.D. 12mer phage library (NEB) because the Tomlinson scFv phagemid library was disadvantageous due to the poor quality of current methods to amplify phagemid particles. Because the Ph.D. 12mer phage library is M13 phage, it does not require the aid of tools to amplify phages like a phagemid library requires. Before the Ph.D. 12mer phage display library was to be panned against *V. cholerae* O1 LPS, an extraction method for *V. cholerae* O1 LPS and the best solid support for LPS binding had to be determined. Two LPS extraction methods were compared to determine which method generated the best quality of LPS to pan against. Also, two binding supports (microtiter well and nitrocellulose) paper were analyzed to determine which binding support could bind the most LPS.

The Phenol-Water Method Extracted *V. cholerae* LPS Most Closely Resembled the Commercially Acquired *V. cholerae* LPS

The phenol-water method for the extraction of LPS (76) was the previous lab procedure used to extract LPS. However, a new method for LPS extraction involving TRIzol Reagent (Invitrogen) was described by Yi and Hackett (77) that was stated to be a more efficient method for LPS extraction than the phenol-water method. The TRIzol Reagent LPS extraction can be executed in one day, while the phenol-water LPS extraction requires nearly a week. The TRIzol Reagent method for LPS extraction is also stated to be a cleaner method than the conventional phenol-water method for LPS extraction by extracting LPS with less degradation and contamination. TRIzol Reagent is composed of phenol and guanidinium thiocyanate in aqueous phase, and upon addition of chloroform the TRIzol Reagent can be used to extract LPS. *Vibrio cholerae* N16961 LPS was extracted by the TRIzol Reagent extraction of LPS (see Materials and Methods). The TRIzol Reagent-extracted *V. cholerae* LPS was resolved by SDS-

PAGE and stained with Coomassie blue for visualization of proteins (Fig. 3-1A) and with Tsai-Frasch silver stain for visualization of carbohydrates (see Materials and Methods) (Fig. 3-1B). The Coomassie blue stain of the TRIzol Reagent-extracted LPS, which was diluted 1:2 in Laemmli sample buffer, showed no detectable proteins. The limit of detection for proteins with Coomassie blue is 0.3-1 μg of protein/band (79); therefore, there was less than 0.3 μg of any single protein loaded onto the gel that was stained by Coomassie blue. The Tsai-Frasch silver stain for the TRIzol Reagent LPS extraction showed non-staining bands between 20 and 30 kDa. The Tsai-Frasch silver stain primarily stains carbohydrates and poorly stains proteins and lipids (78); therefore, the non-staining bands may be lipids or proteins. However, because the Coomassie blue stain for the TRIzol-extracted LPS showed no detectable proteins and due to the thickness of the non-staining bands, which correlates to concentration, then the non-staining bands were most likely due to lipid contamination. The LPS banding pattern of the TRIzol reagent LPS extraction in the silver stain was very similar to the commercial *V. cholerae* 569B LPS (Sigma-Aldrich). On the silver stain, both LPS preparations had bands at approximately 14 and 20 kDa and no bands larger than 50 kDa. Previously published silver stains of *V. cholerae* O1 LPS had bands at ~10 and 14 kDa (which was stated to be the lipid A-core of LPS), and ~20-50 kDa (which was stated to be the lipid A-core plus repeating O-antigens) (80,81).

To determine the concentration of the TRIzol-Reagent-extracted LPS, the intensities of the bands were compared to those of the commercial LPS in the silver stained gel (Fig. 3-1B). The intensity of the band of the commercial LPS (1 mg/mL) that was diluted 1:5 was approximately 1.5 times stronger than the intensity of the band of the TRIzol-Reagent-extracted LPS that was diluted 1:2. Therefore, we estimated the concentration of the TRIzol-Reagent-

extracted LPS was approximately 260 μg/mL. The purity of this LPS extraction was less than 0.2 μg of protein/μg of LPS.

The phenol-water LPS extraction (76) was performed to compare results with the TRIzol Reagent LPS extraction. The phenol-water LPS extraction method differs little from the original phenol-water LPS extraction described by Westphal, Luderitz, and Bister in 1952 (82). The phenol-water LPS extraction method utilizes the property that most proteins but not LPS are soluble in phenol, and that LPS is soluble in water. At temperatures above 68°C, phenol and water are miscible. Upon cooling to 5-10°C and centrifugation, three phases result: an aqueous phase containing LPS, a phenol phase containing proteins, and a solid phase containing water- and phenol-insoluble compounds. Removal and purification of the LPS-containing aqueous phase follows the separation of the phases.

One and a half liters of log phase (OD_{600} ~0.4-0.6) *V. cholerae* 569B was used for extraction of LPS by the phenol-water extraction protocol (see Materials and Methods). The four aqueous extractions were dialyzed and digested by DNase (Qiagen) and RNase (Qiagen) because nucleic acids are also extracted into the aqueous phase. Prior to digestion the dialyzed extraction was diluted 1:100 in ddH_2O, and the absorbance was measured at 260 nm and 280 nm (A_{260} and A_{280}). The $A_{260,}$ which correlates to DNA concentration, predigestion was 0.277 and post-digestion was 0.148. The A_{280}, which correlates to protein concentration, predigestion was 0.127 and post-digestion was 0.059. The final phenol-water extraction was examined for proteins by Coomassie blue stain (Fig. 3-2A) and for carbohydrates by Tsai-Frasch silver stain (see Materials and Methods) (Fig. 3-2B). The Coomassie blue stain for phenol-water-extracted *V. cholerae* LPS showed a protein band at approximately 15 kDa. This could possibly have been due to the 0.04 mg/mL RNase, 13.7 kDa, present in the extraction. The band intensity correlated

with the concentration of RNase present in the sample. Therefore, the protein contamination was most likely not from bacterial proteins that were co-extracted. The Tsai-Frasch silver stain for phenol-water-extracted *V. cholerae* LPS showed similar banding patterns to that of the *V. cholerae* 569B LPS (Sigma-Aldrich). On the silver stain, both LPS preparations had bands at approximately 13, 20, and 23 kDa. The phenol-water extraction showed distinct banding around 20-50 kDa that correlates with published data of where the banding of the O-antigen of LPS occurs. The commercially obtained LPS had smearing around 20-50 kDa. *Vibrio cholerae* O1 LPS has 12-18 O-antigen groups (83); therefore, the additional banding in the phenol-water-extracted LPS may be due to the ability of our phenol-water method to extract LPS with higher numbers of O-antigen side chains than that of the method used for the extraction of the commercial LPS that was phenol extracted and purified by gel-filtration chromatography.

To determine the concentration of the phenol-water-extracted LPS, the intensities of the bands were compared to those of the commercial LPS in the silver stained gel (Fig. 3-2B). The intensities of the bands of the commercial LPS (1 mg/mL) that was diluted 1:8 were between the intensities of the bands of the phenol-water-extracted LPS that was diluted 1:2 and 1:4. Therefore, we estimated the concentration of the commercial LPS at a dilution of 1:8 was comparable to the concentration of the phenol-water-extracted LPS at a dilution of 1:3. Because the commercial LPS was at a concentration of 1 mg/mL, the concentration of the phenol-water-extracted LPS was approximately 375 μg/mL. The purity of this LPS extraction was approximately 1 μg of protein/μg of LPS. However, if the protein band from the Coomassie blue stain was from RNase, the phenol-water-extracted LPS would have less than 0.3 μg of bacterial protein contamination/ μg of LPS, which is the same protein concentration in the TRIzol-extracted LPS.

Based on the results described above, we decided to use the phenol-water method for the extraction of *V. cholerae* O1 LPS over the TRIzol Reagent method. The phenol-water-extracted LPS most closely resembled *V. cholerae* 569B LPS (Sigma-Aldrich) when examined by silver stain. The phenol-water extraction method even appears to be a more sensitive and delicate extraction method than that used to extract the commercial LPS, because more precise banding from O-antigens was detected in the phenol-water-extracted LPS than in the commercial LPS by silver stain. The phenol-water LPS extraction extracted more protein than the TRIzol Reagent LPS extraction; however, for using the LPS extraction for panning it is more important to have intact O-antigens than minor protein contamination.

More V. cholerae LPS Can Be Bound to Nitrocellulose Paper than to a Microtiter Well

In most panning procedures the target molecule to be panned against is immobilized onto a solid support. We wanted to compare the saturation limits for LPS binding to a microtiter well and to a piece of nitrocellulose paper of equal dimensions. Enzyme-linked immunosorbent assays were performed to determine the saturation limits of the amount of phenol-water-extracted LPS that could be bound to a microtiter well. A 96-well Maxisorp plate (Nunc) was coated with 0.1-200 μg/mL of *V. cholerae* 569B LPS that was extracted by the phenol-water method. Two different primary antibodies were used to detect the LPS: a mouse anti-*V. cholerae* O1 LPS monoclonal antibody, α-Vc O1 LPS mAb, (Austral Biologicals, San Ramon, CA) and a rabbit anti-*V. cholerae* O1 polyclonal antibody, α-Vc O1 LPS pAb (Accurate Chemical, Westbury, NY). The primary antibodies were used at their manufacturer recommended concentration or dilution: a concentration of 1 μg/mL for α-Vc O1 LPS mAb and a dilution of 1:100 for the α-Vc O1 LPS pAb. Standard ELISA procedures were used for the assay (see Materials and Methods) (Fig. 3-3). When using the α-Vc O1 LPS mAb a significant difference ($p \leq 0.05$) between the signal to noise (S:N) values (signal on antigen-coated

well/signal on PBS-treated well) of LPS bound to α-Vc O1 LPS mAb reacting to HRP-conjugated anti-mouse antibody was not reached until the concentration of the LPS used to coat the microtiter plate decreased from 1 μg/mL to 0.1 μg/mL (0.1 to 0.01 μg LPS) ($p = 0.01$). When using the α-Vc O1 LPS pAb, a significant difference in the S:N was not reached until the concentration of the LPS decreased from 10 μg/mL to 1 μg/mL ($p = 0.01$). Both the α-Vc O1 LPS mAb and the α-Vc O1 LPS pAb when used at the manufacturer recommended concentrations saturated LPS-coated microtiter wells when the microtiter wells were coated with LPS at a concentration of 10 μg/mL. For the sake of conserving the LPS, the concentration of LPS used to coat microtiter wells was decided to be 1 μg/mL. While there was a significant difference in the S:N when using 10 μg/mL of LPS as opposed to 1 μg/mL of LPS with the α-Vc O1 LPS pAb, the S:N difference was only 0.5. This decrease in the S:N by 0.5 did not merit the need to use 10-fold more LPS; therefore, for further experiments *V. cholerae* LPS was used at a concentration of 1 μg/mL to coat microtiter wells.

The saturation limits for the primary antibodies were analyzed using 1 μg/mL *V. cholerae* LPS to coat a microtiter plate in an ELISA experiment. The α-Vc O1 LPS mAb was serially diluted to concentrations from 1.3 to 0.04 μg/mL by 2-fold dilutions. The α-Vc O1 LPS pAb was serially diluted from 1:100 to 1:3,200 by 2-fold dilutions. When using 1 μg/mL LPS to coat a microtiter well, the saturation limit of the α-Vc O1 LPS mAb was not reached (Fig. 3-4A). There were significant differences ($p=0.01$-0.03) between the S:N of each dilution step when using the α-Vc O1 LPS mAb at concentrations from 1.3-0.04 μg/mL. Therefore, when using 1 μg/mL LPS the saturation limit for the α-Vc O1 LPS mAb was greater than 1.3 μg/mL. When using 1 μg/mL LPS to coat a microtiter well, a significant difference in the measured S:N between dilution steps when using the serially diluted α-Vc O1 LPS pAb was not reached until a

dilution of 1:400 (p=0.01) (Fig. 3-4B). Therefore, the saturation limit of the α-Vc O1 LPS pAb was reached at a dilution of 1:400.

Because a microtiter well coated with 1 μg/mL LPS was never saturated by the α-Vc O1 LPS mAb, the α-Vc O1 LPS pAb was used to characterize the saturation limits of nitrocellulose paper. Pieces of nitrocellulose paper were cut to dimensions (5 mm x 20 mm) that were approximately equivalent to the surface area of a microtiter well. The same phenol-water-extracted *V. cholerae* LPS used for the microtiter well saturation experiment was also used to characterize the saturation of nitrocellulose with LPS. Serially diluted LPS from 100-1 μg/mL was used to saturate strips of nitrocellulose paper. These strips of LPS-coated nitrocellulose paper were examined in an altered Western blot protocol (see Materials and Methods for LPS Saturation to Nitrocellulose Paper) using the α-Vc O1 LPS pAb at a dilution of 1:400 (Fig. 3-5). The nitrocellulose paper was not saturated with LPS until 100 μg/mL LPS (250 μg) was used to coat the nitrocellulose paper. Therefore, nitrocellulose paper can bind approximately 250 to 2,500 times more LPS than a microtiter well.

Panning to V. cholerae LPS Failed to Yield Phages that Were Specific to V. cholerae LPS

Vibrio cholerae 569B LPS (Sigma-Aldrich) was immobilized onto a polystyrene Maxisorp microtiter well and panned against the NEB Ph.D. 12mer phage display library (see Materials and Methods). Five rounds of panning were performed with amplification of phages after the first four rounds of panning. Each round of panning promotes selection of phages that specifically bind to the target molecule. Amplifying the selected phages should increase the percentage of specific phages present in the library to pan with. Panning and amplifying for five rounds should greatly improve the chances of isolating phages that specifically bind to the target molecule.

One hundred phage clones from the fifth round of panning were amplified and screened by ELISA against *V. cholerae* 569B LPS, carbonate coating buffer, and PBS-treated microtiter wells. The α-Vc O1 LPS mAb was also tested against the three coating antigens and generated a S:N of 15.5, proving that the LPS successfully bound to the microtiter plate. Anti-BSA phagemid particles were used in the ELISA against BSA (10μg/mL), carbonate coating buffer, and PBS-treated microtiter wells and generated a S:N of 22, proving that the ELISA was a successful assay for the detection of phages. All 100 clones gave a S:N <1.4. A positive signal was classified as a S:N ≥2. Therefore, all of the clones screened by ELISA were negative, meaning that they failed to specifically bind to *V. cholerae* 569B LPS.

Panning the Ph.D. 12mer phage library against *V. cholerae* O1 LPS immobilized onto a microtiter well failed to yield phage clones specific to *V. cholerae* O1 LPS. It was therefore attempted to improve the panning procedure. Instead of using a microtiter well as a binding support for LPS, nitrocellulose paper was used as a binding support. Nitrocellulose paper can bind approximately 250 to 2,500 times more LPS than a microtiter well; therefore, if more antigen is present to pan against then the chances of isolating clones that specifically bind to LPS may improve. Also, the elution method was tested. The previous elution method was with glycine (pH 2.2). To determine if a glycine elution or an elution with a high concentration of the target antigen is more successful in eluting phages, both elution methods were tested in parallel. The theory behind an antigen elution is that a high concentration of antigen in solution will rapidly bind phages bound to the antigen on the nitrocellulose paper when the phages are transiently released. The end result is that these phages will "elute" off of the antigen-coated nitrocellulose paper and bind to antigen in solution. Antigen elution is supposed to be a less harsh treatment than acidic or basic elutions, which may denature the peptides.

Vibrio cholerae 569B LPS that was phenol-water-extracted was immobilized onto a strip of nitrocellulose paper and panned with the Ph.D. 12mer phage display library (NEB) (see Materials and Methods). Two parallel panning experiments were performed; one panning used a glycine (pH 2.2) elution, while the other panning used a *V. cholerae* 569B LPS elution. Three rounds of panning were performed with a negative panning performed between rounds one and two. The negative panning involved panning the library against a strip of nitrocellulose paper that was blocked in casein blocking buffer (Sigma-Aldrich) to remove phages that were specific to nitrocellulose paper or casein blocking buffer. Amplification of phages was performed after rounds one and two to increase the number of selected phages to improve the chances of isolating phages specific to the target molecule. Two hundred clones for each panning were selected from the round 3 elution and amplified for screening of phages by ELISA. The 400 phage clones were screened in an ELISA against *V. cholerae* 569B LPS and PBS. The α-Vc O1 LPS mAb was also tested against LPS and PBS-treated wells and generated a S:N of 10, proving that the LPS successfully bound to the microtiter plate. Anti-BSA phagemid particles were used in the ELISA against BSA (10μg/mL) and PBS-treated microtiter wells and generated a S:N of 15, proving that the ELISA was a successful assay for the detection of phages. All 400 clones gave a S:N less than 1.7. Therefore, all of the clones screened were negative and failed to specifically bind to *V. cholerae* O1 LPS.

Conclusion of Specific Aim 1

In comparing the phenol-water and the TRIzol Reagent methods for extraction of *V. cholerae* O1 LPS, the phenol-water method was better. The phenol-water extraction for LPS yielded LPS that most closely resembled commercially obtained *V. cholerae* O1 LPS by silver stain of SDS-PAGE-resolved LPS. The phenol-water method even extracted LPS that appeared to have more O-antigens than the commercially obtained *V. cholerae* O1 LPS. In comparing the

saturation limits of *V. cholerae* O1 LPS bound to microtiter wells and to nitrocellulose paper, the nitrocellulose paper bound approximately 250 to 2,500 times more LPS than did microtiter wells. Panning the Ph.D. 12mer phage display library against *V. cholerae* O1 LPS immobilized onto nitrocellulose and onto microtiter wells failed to yield phages that were specific to *V. cholerae* O1 LPS in an ELISA.

Specific Aim 2: Improve Panning and Screening Process of Biopanning

There are many steps in the biopanning and screening process. By analyzing certain key steps and optimizing them, the chances of isolating phagemid particles that are specific to the target molecule panned against should increase. Optimization of the panning process was performed with *V. cholerae* O1 whole cells as a target. Whole cells were chosen as the target because they are the target antigen for the biosensors under development by our collaborators. Optimization of the concentration of whole cells were analyzed to determine if higher or lower concentrations of the antigen were more likely to select for specific phagemid particles. Increasing the concentration of antigen should increase the amount of phagemid particles selected. This could mean an increase in phagemid particles that are specific and/or non-specific to the target molecule. Decreasing the concentration of antigen should decrease the amount of phagemid particles selected. This could mean a decrease in phagemid particles that are specific and/or non-specific to the target molecule. An ideal concentration would select a high number of phagemid particles that are specific to the target molecule and a low number of phagemid particles that are not specific to the target molecule.

The Tomlinson scFv libraries have a trypsin cleavable site between the scFv peptide and the pIII peptide of the phagemid particle. Cleaving this site separates the phagemid particle from the antigen, leaving the scFv bound to the antigen. Optimizing the time of trypsin elution may improve the chances of isolating specific phagemid particles. Eluting with trypsin for too short

of a time may fail to elute specific phagemid particles, but eluting for too long of a time may increase the amount of nonspecific phagemid particles eluted or may even cause degradation of the phagemid particles.

Elution of Bound Phages by Trypsin during Panning Was Optimal between 10 and 30 Minutes

A one-round panning procedure was done to determine the optimal concentration of whole cells in suspension and the optimal trypsin elution time for biopanning. The purpose of the experiment was to compare the differences between using 1 x 10^8 or 1 x 10^9 whole cells of *V. cholerae* N16961 and trypsin elution times of 15, 30, 45, and 60 minutes in the biopanning process. Four parallel pannings were performed. Two were panned with the Tomlinson J library (2.5 x 10^{10} tu), one against 1 x 10^8 and the other against 1 x 10^9 whole cells of *V. cholerae* in suspension. The two other pannings used the Tomlinson J library (2.5 x 10^{10} tu) containing 1% (~2.5 x 10^8 tu) of Vc86 phagemid particles (an scFv-producing phagemid particle previously isolated in this lab by Dr. Rebecca Moose-Clemente from the Tomlinson scFv library that recognizes *V. cholerae* 1019 and *E. coli* O157:H7 whole cells). Panning against a library that is already enriched with phagemid particles that recognize the target molecule should help determine the optimal panning parameters. If more phagemid particles are eluted with the enriched library than the non-enriched library at a certain panning parameter then it would lead to the conclusion that the phagemid particles that recognized the target molecule were eluted at that parameter. One panning used 1 x 10^8, and the other used 1 x 10^9 whole cells of *V. cholerae* in suspension. Standard suspension panning procedures (see Materials and Methods) were performed. When the trypsin was added to the bound-phagemid particles, an aliquot of the eluted phagemid particles was removed at 15, 30, 45, and 60 minutes after the start of the elution and was titered immediately (Fig. 3-6). Elution of phagemid particles with trypsin was optimal

between 15 and 30 minutes. The phagemid particle titer decreased by approximately 80-93% from 30 to 45 minutes for the four pannings. More phagemid particles were eluted when panned against 1 x 10^9 whole cells. However, there were not enough data to do statistical analysis to determine if using 1 x 10^9 whole cells eluted significantly more phagemid particles than using 1 x 10^8 whole cells in the biopanning process.

Because the best whole cell concentration and trypsin elution time was not definitively obtained in the first experiment, a second one-round panning was done to analyze these factors. The purpose of the experiment was to compare the differences between using 1 x 10^7, 1 x 10^8, and 1 x 10^9 whole cells of *V. cholerae* N16961 and trypsin elution times of 10, 20, and 30 minutes in the biopanning process. The same protocol was used as the first one-round panning protocol above, except an additional concentration of whole cells was added, so there were six parallel pannings instead of four. When the trypsin was added to the bound-phagemid particles, an aliquot of the eluted phagemid particles was removed at 10, 20, and 30 minutes after the start of the elution and was titered immediately (Fig. 3-7). There were no significant differences in the titers of eluted phagemid particles when comparing the concentrations of whole cells and trypsin elution times used in the second one-round panning experiment. The panning was expected to yield distinct differences between eluted titers of phagemid particles from the libraries with and without 1% Vc86 phagemid particles. A certain parameter was supposed to be optimal and yield higher elution titers from the library with 1% Vc86 phagemid particles. Because there were not any significant differences in the titers of the eluted phagemid particles at the different parameters an optimal panning condition could not be determined. To determine which panning parameter elutes the most phagemid particles that recognize *V. cholerae* whole

cells, another one-round panning was performed with analysis of phagemid particle specificity via ELISA.

A third one-round panning was performed to analyze whole cell concentration and trypsin elution time further. The purpose of the experiment was to compare the differences between using 1 x 10^8 and 1 x 10^9 whole cells of *V. cholerae* N16961 and trypsin elution times of 10 and 30 minutes in the biopanning process. The same protocol was used as for the first one-round panning protocol above, except that only two elution time points were taken. When the trypsin was added to the bound phagemid particles, an aliquot of the eluted phagemid particles was removed at 10 and 30 minutes after the start of the elution and titered immediately (Fig. 3-8). When panning with the Tomlinson J scFv phagemid library that contains 1% (~2.5 x 10^8 tu) Vc86 phagemid particles, the amount of eluted phagemid particles did not significantly change ($p=0.50$) from 10 to 30 minutes when panning with 1 x 10^9 whole cells. When panning with the library that contains 1% (~2.5 x 10^8 tu) Vc86 phagemid particles, the amount of eluted phagemid particles significantly decreased ($p=0.04$) from 10 to 30 minutes when panning with 1 x 10^8 whole cells. Because the amount of eluted phagemid particles either stayed the same or decreased from 10 to 30 minutes in the pannings with 1% Vc86 phagemid particles, it suggested that eluting for 30 minutes had no advantage over eluting for 10 minutes.

To test if the eluted phagemid particles at the various time points were specific to *V. cholerae*, an ELISA was performed. The eluted phagemid particles from the third one-round panning experiment were amplified in *E. coli* TG1 (HyΦ-lg), which is *E. coli* TG1 that was already infected with hyperphage, and used at a concentration of 1 x 10^8 tu/mL in an ELISA. The phagemid particles were analyzed by ELISA against *V. cholerae* whole cells (3 x 10^8 CFU/mL) and PBS-treated microtiter wells. A standard ELISA protocol (see Materials and

Methods) was performed (Fig. 3-9). Of the different panning variations, the amplified phagemid particles from the panning with the library that contained 1% Vc86 phagemid particles, 1 x 10^8 whole cells *V. cholerae,* and an elution time of 30 minutes generated a significantly higher S:N (p=0.01-0.04) in an ELISA compared to the amplified phagemid particles from the other panning variations. This suggested that panning with 10^8 whole cells with a trypsin elution time of 30 minutes was optimal for selecting phagemid particles that were specific to the panned whole cells compared to using 10^9 whole cells or a trypsin elution of 10 minutes.

Screening of scFv Proteins by a High Throughput ELISA Was Acceptable

Once the panning procedure is complete, screening of phages or scFvs is performed. The previous method of screening scFvs involved inducing phagemid-containing bacteria to produce scFvs in culture tubes or flasks and screening the scFvs by ELISA. However, this method was very time consuming. Optimizing the screening process to enable high throughput screening of clones should increase the chances of finding clones that specifically bind to the target molecule. A colony blot was attempted to try to enable high throughput screening of scFv proteins. The colony blot (see Materials and Methods) involved the transfer of bacterial colonies that were grown to secrete scFvs onto a piece of nitrocellulose paper that was coated with a target antigen and blocked with casein blocking buffer. Following the transfer, the piece of nitrocellulose paper was washed with TBS to remove unbound scFv proteins, incubated with Protein-L peroxidase (Sigma-Aldrich) to bind to the kappa light chains of the scFv, washed with TBS to remove excess Protein-L peroxidase, and developed with ECL (Pierce). Upon development with ECL, scFv-producing clones specific to BSA failed to produce a positive signal against a piece of nitrocellulose paper coated in 10 μg/mL BSA but did produce a positive signal against pieces of nitrocellulose paper coated in PBS or *V. cholerae* whole cells. Also, clones that were not specific to *V. cholerae* whole cells in an ELISA produced positive signals against pieces of

nitrocellulose paper coated with *V. cholerae* whole cells, PBS, or 10 μg/mL BSA. The colony blot was determined to be neither selective nor specific. Because the colony blot was not functioning, a way to improve the culture tube method for the production of scFvs was performed.

A high throughput ELISA was contrived to enable screening of hundreds of clones per day. This high throughput procedure involved picking bacterial colonies containing a phagemid and growing them overnight in a 96-well plate to produce scFvs (see Materials and Methods). The next day the 96-well plate containing the turbid cultures was centrifuged to remove the bacterial cells. These scFvs in the supernatant were examined in an ELISA for screening of their specificity. To determine how well scFvs could be produced in a microtiter well, as opposed to a culture tube or flask, a bacterial colony containing a phagemid that encodes an scFv specific to BSA was grown in a microtiter well. When analyzed by ELISA, the anti-BSA (α-BSA) clone was diluted 1:2 in casein blocking buffer and produced a S:N of 11.5 in an ELISA against BSA. Anti-BSA scFv produced in a flask produced an average S:N value between 10 and15; therefore, producing scFv proteins in a microtiter well may not be optimal, but it was acceptable for the purpose of a high throughput screen.

We were successful in producing scFvs in a microtiter well. The next test was to determine how far the scFv can be diluted and still produce a positive signal. The α-BSA clone was grown in a microtiter well to produce scFvs. The produced scFvs were diluted 1:2, 1:4, 1:6, and 1:10 in casein blocking buffer and analyzed by ELISA (Fig. 3-10). Diluting the scFv supernatant 1:10 in casein blocking buffer yielded a S:N of 5.6. This S:N was high enough for detection of a positive clone and was not significantly different ($p=0.13$) than the S:N produced when using α-BSA phagemid particles that were diluted 1:6. Therefore, diluting scFv

supernatants as far as 1:10 with other clones can enable detection of positive clones. However, the α-BSA clone was the strongest clone that we had; therefore, weaker clones might fail to be detected if analyzed at a dilution of 1:10. For the purpose of a high throughput ELISA screen for scFvs I would recommend diluting the scFv proteins only 1:4 to 1:6 to lessen the chances of not detecting weaker binding scFvs.

Screening of Phagemid Particles by a High Throughput ELISA Was Not Optimal

Once the panning procedure is complete, screening of phagemid particles or scFvs is performed. Previous methods of screening phagemid particles involved the amplification of eluted phagemid particles in culture tubes or flasks in 2xTY containing 100 μg/mL ampicillin, 40 μg/mL kanamycin, and 0.1% (w/v) glucose at 30°C overnight, followed by screening by ELISA. This process was time-consuming; therefore, it was tested if phagemid particle production could be performed in a high throughput manor as with scFvs. Vc86 and α-BSA phagemids in *E. coli* TG1 (HyΦ-sm) (*E. coli* TG1 that was previously infected with hyperphage) were produced in a 96-well plate in 2xTYcontaining 100 μg/mL ampicillin, 40 μg/mL kanamycin, and with or without 0.1% (w/v) glucose at 30°C and 37°C overnight. Protocols recommend producing phagemid particles in medium that has 0.1% (w/v) glucose, but due to catabolite repression of the *lac* promoter, which drives transcription of the *gIII* gene, phagemid particle production might increase without the presence of glucose in the growth medium. The next day, the bacterial cells were removed by centrifugation, and the phage-containing supernatants were examined in an ELISA at a dilution of 1:2 in casein blocking buffer (Fig. 3-11). There was not a significant difference in the S:N ($p=0.07-0.48$) of phagemid particles produced in microtiter wells with and without 0.1% (w/v) glucose. Vc86 phagemid particles produced in microtiter wells with or without glucose at 30°C and 37°C did not produce a S:N higher than one. It was also noted that the cultures in the microtiter plates were only slightly turbid. The Vc86 phagemid particles

produced in a flask (S:N ~4) yielded a significantly higher S:N ($p=0.05$) compared to Vc86 phagemid particles produced in microtiter wells. When α-BSA phagemid particles were produced at 30°C, the S:N was significantly higher ($p<0.04$) than when α-BSA phagemid particles were produced at 37°C at either glucose concentration. The α-BSA phagemid particles produced in a flask (S:N ~12) yielded a significantly higher ($p=0.02$) S:N compared to α-BSA phagemid particles produced in microtiter wells (S:N~7).

The experiment was repeated a second time with only the α-BSA phagemid particles (Fig. 3-12). Anti-BSA phagemid particles produced in microtiter wells yielded significantly higher signals ($p<0.03$) when produced at 30°C than at 37°C at either glucose concentration. There was not a significant difference ($p=0.34$) in the signals produced by phagemid particles that were produced with or without glucose at 30°C. When phagemid particles were produced in microtiter wells at 37°C, there was a significantly higher ($p=0.02$) signal when phagemids were produced with no glucose compared to 0.1% (w/v) glucose. There was not a significant difference in the signal ($p=0.09$) produced by phagemid particles produced in microtiter wells compared to phagemid particles produced in a flask.

When phagemid particles were diluted 1:2, phagemid particles yielded significantly higher signals in an ELISA when produced at 30°C compared to 37°C. Whether the growth medium included 0.1% (w/v) glucose or not when phagemid particles were incubated at 30°C did not significantly affect the signal of the phagemid particles; therefore, 0.1% (w/v) glucose was not enough to cause catabolite repression of the *lac* promoter. Also, phages produced in flasks yielded higher signals than phages produced in microtiter wells. To determine if low titers of phagemid particles produced in a microtiter well were a factor in the lowered ELISA signals for phagemid particles, the phagemid particles were titered. When α-BSA and Vc86 phagemid

particles were produced in microtiter wells at 30°C, the titers were approximately 10^7 tu/mL. When α-BSA and Vc86 phagemid particles were produced in microtiter wells at 37°C, the titers were approximately 10^6 tu/mL. Growing phagemid particles at 30°C in microtiter wells yielded approximately 10-fold higher tu/mL titers than phagemid particles that were produced in microtiter wells at 37°C. Whether the growth medium contained 0.1% (w/v) glucose or not did not affect the titers of phagemid particles. All phages produced in microtiter wells were amplified by *E. coli* TG1 (HyΦ-sm). *Escherichia coli* TG1 (HyΦ-sm) is *E. coli* TG1 that was already infected with hyperphage. Having hyperphage already present in *E. coli* removes an additional step in phagemid particle production to enable a more high throughput screen When α-BSA and Vc86 phagemid particles were produced in flasks at 30°C with 0.1% (w/v) glucose in the medium, the titers were approximately 10^8 to 10^9 tu/mL. The phagemid particles produced in the flasks were amplified by superinfection with hyperphage, instead of by *E. coli* TG1 harboring hyperphage. Growing phagemid particles by superinfection with hyperphage may have resulted in higher titers of phagemid particles compared to phagemid particles produced from *E. coli* that already contained hyperphage. The high throughput phagemid particle ELISA was not optimal for screening phagemid particles because phagemid particles could not be produced in high enough titers in microtiter wells to make screening of phagemid particles effective in a high throughput manor.

Conclusion of Specific Aim 2

When panning with the Tomlinson J human synthetic $V_H + V_L$ phagemid library, trypsin elution was best between 10 and 30 minutes. Phagemid particles could possibly be degraded by trypsin treatment of 45 minutes or greater. When screening scFvs, a high throughput ELISA was acceptable. The scFv proteins can be diluted 1:10 in an ELISA with other scFv proteins and still produce high enough signals to be detected. When screening phages, screening in a high

throughput ELISA was not optimal. Phagemid particles cannot be produced in high enough titers when produced in microtiter wells to be useful in a high throughput manor.

Specific Aim 3: Improve Phagemid Particle Production

Phagemid particle production is one of the most important steps in biopanning. Because the only M13 gene in a phagemid is the *gIII* gene, use of phagemids requires an amplification tool to be provided in trans to supply the additional M13 genes to enable phagemid particle production. There needs to be high quality and high quantity phagemid particle production during amplification of phagemid particles to ensure that redundancy of clones in the library is maintained and clones are produced in high enough titers to be useful in assays that use phagemid particles. Hyperphage (Progen) was the previous phagemid particle amplification tool. Hyperphage is an M13-based phage that contains a partial deletion of the *gIII* gene to enable production of phagemid particles that display recombinant pIII proteins but no wild-type pIII proteins. However, the commercially obtained hyperphage stock was of poor quality. The hyperphage stock was stated to contain 10^{12} particles/mL; however, it only had 10^{8} tu/mL, as determined in an infection assay. Because there was not enough Tomlinson scFv phagemid library stock for panning experiments, the library stock needed to be amplified. The Tomlinson I and J scFv libraries contain 1 x 10^{8} phagemid clones. To ensure that the Tomlinson scFv libraries are amplified to ensure that the redundancy of clones is maintained, an amplification tool needs to enable high quality and high quantity phagemid particle production. Because commercially obtained hyperphage had too low of titers to be an effective phagemid particle amplification tool, other ways of phagemid particle production were analyzed.

Escherichia coli TG1 Harboring the Hyperphage Genome Was Not an Optimal Phagemid Particle Amplification Tool

To overcome the low titers of hyperphage, the hyperphage genome was incorporated into *E. coli* and maintained in the strain as a plasmid to ensure that every phagemid-containing *E. coli* also contained the hyperphage genome to enable phagemid particle production. Log phase (OD_{600} ~0.4) *E. coli* TG1 was transduced with hyperphage at a MOI of 5, incubated in a 37°C standing water bath for 30 minutes, serially diluted in BSG, and then plated on 2xTY plates containing 40 μg/mL kanamycin. The plates were incubated overnight at 37°C. The next day the plates contained small (0.5 mm diameter) and large (1-1.5 mm diameter) colonies. Two small and large colonies were single colony passaged twice. The small colonies maintained a small phenotype (0.5 mm diameter), and the large colonies maintained a large phenotype (1-1.5 mm diameter). The two strains were named *E. coli* TG1 (HyΦ-sm) and *E. coli* (HyΦ-lg).

The infection efficiencies of phagemid particles into *E. coli* TG1, *E. coli* TG1 (HyΦ-sm), and *E. coli* (HyΦ-lg) were analyzed. The three *E. coli* strains were grown to log phase and transduced with serially diluted α-BSA and Vc86 phagemid particles in BSG at a MOI of 0.001. Transductions were incubated in a 37°C standing water bath for 30 minutes and plated on 2xTY AG plates containing 40 μg/mL kanamycin for transduced strains of *E. coli* TG1 containing hyperphage and on 2xTY AG plates for transduced *E. coli* TG1. The numbers of *E. coli* cells transduced with α-BSA phagemid particles were as follows: 1,200 transductions into *E. coli* TG1, 340 transductions into *E. coli* TG1 (HyΦ-sm), and 100 transductions into *E. coli* TG1 (HyΦ-lg). When the number of α-BSA phagemid particles transduced into *E. coli* TG1 was set at an infection efficiency of 100%, the number of α-BSA phagemid particles transduced into *E. coli* TG1 (HyΦ-sm) was 28% and the number of α-BSA phagemid particles transduced into

E. coli TG1 (HyΦ-lg) was 0.1% of the number of transduced phagemid particles into *E. coli* TG1.

The numbers of *E. coli* transduced with Vc86 phagemid particles were as follows: 4,300 transductions into *E. coli* TG1, 950 transductions into *E. coli* TG1 (HyΦ-sm), and 80 transductions into *E. coli* TG1 (HyΦ-lg). When the number of Vc86 phagemid particles transduced into *E. coli* TG1 was set at an infection efficiency of 100%, the number of Vc86 phagemid particles transduced into *E. coli* TG1 (HyΦ-sm) was 22% and the number of Vc86 phagemid particles transduced into *E. coli* TG1 (HyΦ-lg) was 2% of the number of transduced phagemid particles into *E. coli* TG1. *Escherichia coli* TG1 (HyΦ-sm) had an infection efficiency that was approximately 25% of the infection efficiency of *E. coli* TG1, and *E. coli* TG1 (HyΦ-lg) had an infection efficiency that was approximately 1% of the infection efficiency of *E. coli* TG1. The infection efficiencies of *E. coli* TG1 (HyΦ-sm/lg) were too low to for the *E. coli* strains to be useful to amplify the Tomlinson scFv libraries. However, they could still be useful as an amplification tool to amplify single clones when the transduction efficiency is not critical. Therefore, the quality and quantity of phagemid particles produced by *E. coli* TG1 (HyΦ-sm/lg) were analyzed.

Escherichia coli TG1 (HyΦ-sm) and *E. coli* TG1 (HyΦ-lg) were analyzed for their ability to produce phagemid particles. *Escherichia coli* TG1 (HyΦ-sm) and *E. coli* TG1 (HyΦ-lg) were transduced with α-BSA phagemid particles (as described above) and grown under conditions to produce phagemid particles (see Materials and Methods). *Escherichia coli* TG1 containing α-BSA phagemids was superinfected and grown under conditions to produce phagemid particles (see Materials and Methods). The superinfected *E. coli* TG1 was used as a comparison for strains of *E. coli* TG1 containing hyperphage as a phagemid particle production tool. Anti-BSA

phagemid particle yields were as follows: *E. coli* TG1 that was superinfected with hyperphage yielded 3 x 10^8 tu/mL, *E. coli* TG1 (HyΦ-sm) yielded 1 x 10^9 tu/mL, and *E. coli* TG1 (HyΦ-lg) yielded 3 x 10^8 tu/mL. These results showed that *E. coli* TG1 that contained hyperphage produced high titers of phagemid particles.

To determine the quality of α-BSA phagemid particles produced by the three *E. coli* strains, the produced α-BSA phagemid particles were analyzed in an ELISA. Microtiter plates were coated with 10 μg/mL BSA or only PBS. Standard ELISA procedures were followed (see Materials and Methods) to analyze 1 x 10^8 tu/mL of α-BSA phagemid particles as the primary antibody (Fig. 3-13). Anti-BSA phagemid particles produced a significantly higher S:N ($p=0.01$)* when produced from *E. coli* TG1 (HyΦ-sm) compared to *E. coli* TG1 (HyΦ-lg). There was not a significant difference in the S:N ($p=0.50$) from α-BSA phagemid particles produced from superinfected *E. coli* TG1 and *E. coli* TG1 (HyΦ-lg). Because superinfected *E. coli* TG1 and *E. coli* TG1 (HyΦ-lg) yielded titers (~3 x 10^8 tu/mL) of α-BSA phagemid particles that were approximately a third of that of *E. coli* TG1 (HyΦ-sm) titers (~1 x 10^9 tu/mL), *E. coli* TG1 (HyΦ-sm)-produced α-BSA phagemid particles were analyzed in the ELISA with a third less phage-containing supernatant volume. The amplified α-BSA phagemid particles were from the same clone; therefore, the differences in S:N from the produced α-BSA phagemid particles was not expected. A possible reason for the S:N differences was that a different amount of particles/mL was used in the ELISA. The ELISA compared equal tu/mL titers of the α-BSA phagemid particles produced. However, tu/mL is a measure of infectious particles and not total particles. Because an ELISA measures particles with binding activity, it is possible that superinfected *E. coli* TG1 and *E. coli* TG1 (HyΦ-lg) yielded similar particles/mL yields as *E. coli* TG1 (HyΦ-sm), which would account for the differences in the S:N. The results showed

that *E. coli* TG1 (HyΦ-sm) and *E. coli* TG1 (HyΦ-lg) had infection efficiencies that were too low for them to be effective amplification tools to amplify the Tomlinson J scFv library. However, these strains could be used as tools to amplify single phagemid particles with acceptable phagemid particle production and quality.

Helper Plasmids Were Not an Optimal Phagemid Particle Amplification Tool

Helper plasmids (Los Alamos National Laboratory, Los Alamos, NM) (64) are M13-based plasmids that are maintained in *E. coli* to provide phagemid-containing *E. coli* with all of the genes necessary to produce phagemid particles. The helper plasmids come in three forms that either have a full-length (M13cp), a deleted (M13cp-dg3), or a truncated (M13cp-CT) *gIII* gene. The helper plasmid-containing *E. coli* strains were analyzed for their infection efficiencies with phagemid particles, production yield of phagemid particles, and the quality of phagemid particles produced.

The three helper plasmids were electroporated (see Materials and Methods) into electrocompetent *E. coli* TG1. All three transformations yielded colonies between 0.5-2 mm in diameter. A small (0.5 mm) and a large (2 mm) colony from *E. coli* TG1 (M13-cp), *E. coli* TG1 (M13cp-CT), and *E. coli* TG1 (M13cp-dg3) were single colony passaged twice. *Escherichia coli* TG1 (M13cp-sm) was the only strain that maintained a consistent phenotype of 0.5 mm colonies. The other 5 strains yielded colonies with diameters of 0.5-2 mm after each passage. To determine if the different colony size phenotypes had an effect on the functionality of the helper plasmids, a small and large isolate from each strain were analyzed for their infection efficiencies with phagemid particles.

To analyze the infection efficiencies of phagemid particles into helper plasmid-containing *E. coli* TG1, an infection efficiency (see Materials and Methods) experiment was performed that compared the infection efficiencies of helper plasmid-containing *E. coli* with *E. coli* that did not

contain helper plasmids. The bacterial strains were grown to log phase and transduced with α-BSA or Vc86 phagemid particles at a MOI of 0.1 (Table 3-1). The number of *E. coli* TG1cells that were transduced with phagemid particles was set at an infection efficiency of 100%, and the number of *E. coli* TG1 cells harboring helpers that were transduced with phagemid particles was compared to the infection efficiency of *E. coli* TG1. Of the six *E. coli* TG1 strains harboring helper plasmids, *E. coli* TG1 (M13cp-dg3-sm) and *E. coli* TG1 (M13cp-CT-sm) were the only strains that yielded infection efficiencies grater than 0.3% of the infection efficiency of *E. coli* TG1. *Escherichia coli* TG1 (M13cp-dg3-sm) had infection efficiencies of 175% and 97%, while *E. coli* TG1 (M13cp-CT-sm) had infection efficiencies of 7% and 13% compared to the infection efficiency of *E. coli* TG1. Because the small and large isolates of helper plasmid-containing *E. coli* had different infection efficiencies, the colony phenotype had an effect of the functionality of the helper plasmid-containing *E. coli.* Because initial tests with *E. coli* TG1 (M13cp-dg3-sm) had an infection efficiency that was as high or higher than the infection efficiency of *E. coli* TG1, its infection efficiency was further analyzed with the transduction of additional phagemid particles.

Escherichia coli TG1 (M13cp-dg3-sm) was transduced with clone 18 (a phagemid particle isolated from the Tomlinson I scFv library that recognizes the A27L protein of vaccinia virus), α-AV20N3 (a phagemid particle isolated from the Tomlinson I scFv library that recognizes rAuto (a recombinant *L. monocytogenes* murein hydrolase)), and Vc86 phagemid particles. The various strains were analyzed further for their infection efficiencies compared to *E. coli* TG1 (Table 3-2). *Escherichia coli* TG1 (M13cp-dg3-sm) had infection efficiencies of 92%, 113%, and 120% compared to the infection efficiency of *E. coli* TG1. *Escherichia coli* TG1 (M13cp-dg3-sm) had infection efficiencies that were approximately the same as *E. coli* TG1; therefore,

this strain was used for further analysis to determine the quantity and the quality of phagemid particles produced. However, before *E. coli* TG1 (M13cp-dg3-sm) was analyzed further, M13cp-CT-sm was examined.

To test if the helper plasmid (M13-CT-sm) could yield a higher infection efficiency in another *E. coli* strain, it was extracted (see Materials and Methods) from *E. coli* TG1 (M13cp-CT-sm) and electroporated (see Materials and Methods) into *E. coli* JM109. *Escherichia coli* JM109 was chosen because it was genotypically more similar than *E. coli* TG1 to the bacterial strain that Los Alamos National Laboratory used with the helper plasmids (*E. coli* DH5α F'). *Escherichia coli* JM109 and *E. coli* DH5α F' contain *relA*1 and *recA*1, while *E. coli* TG1 does not.

Escherichia coli TG1 (M13cp-CT-sm) and *E. coli* JM109 (M13cp-CT-sm) were transduced with clone 18, α-AV20N3, and Vc86 phagemid particles and were analyzed for their infection efficiencies compared to *E. coli* TG1 and *E. coli* JM109 (Table 3-3). The average infection efficiency of *E. coli* TG1 (M13cp-CT-sm) was 47%, while the average infection efficiency of *E. coli* JM109 (M13cp-CT-sm) was 28%. Therefore, using *E. coli* JM109 to maintain M13cp-CT-sm did not increase the infection efficiency.

In analyzing the infection efficiencies, *E coli* TG1 (M13cp-dg3-sm) had an average infection efficiency of 120% with a standard deviation of 33% and *E. coli* TG1 (M13cp-CT-sm) had an average infection efficiency of 30% with a standard deviation of 16%. *Escherichia coli* TG1 (M13cp-dg3-sm) had a significantly higher infection efficiency ($p=0.01$) than *E. coli* TG1 (M13cp-CT-sm).

Escherichia coli TG1 (M13cp-dg3-sm), *E. coli* TG1 (M13cp-CT-sm), and *E. coli* TG1 that was superinfected with hyperphage were analyzed for their ability to produce phagemid

particles. To determine if the addition of 1 mM IPTG to the growth medium would increase phagemid particle production, phagemid particle amplification was performed in parallel with and without the addition of 1 mM IPTG to the growth medium. Isopropyl-beta-D-thiogalactopyranoside is a molecular mimic of a lactose metabolite that induces transcription from the lac promoter. Because the lac promoter drives transcription of the *gIII*-scFv fusion, it was hypothesized that increased expression of pIII-scFv fusions would result in higher yields of phagemid particles. Phagemid particles were amplified (see Materials and Methods) and titered by the spot-titer method (see Materials and Methods) (Table 3-4). Phagemid particles were amplified to titers of 10^6-10^8 tu/mL by *E. coli* TG1 (M13cp-dg3-sm), 10^5 -10^6 tu/mL by *E. coli* TG1 (M13cp-CT-sm), and 10^8 -10^9 tu/mL by *E. coli* TG1 superinfected with commercial hyperphage. Titers of phagemid particles produced with 1 mM IPTG were either the same or lower than titers produced without IPTG. Therefore, it was determined that the addition of 1 mM IPTG did not improve phagemid particle production. Phagemid particles produced by *E. coli* TG1 (M13cp-dg3-sm) and *E. coli* TG1 (M13cp-CT-sm) yielded low titers of phagemid particles. An overnight shaking culture for the amplification of phagemid particles contains approximately 10^9 CFU/mL. Therefore, for the helper plasmids to yield titers as low as 10^5 tu/mL means that only 1 in 10,000 bacteria produced an infectious phagemid particle. These titers were too low for either helper plasmid to be used as an effective phagemid particle production tool.

Even though helper plasmids produced phagemid particle titers too low to be used as an amplification tool, the phagemid particles produced by *E. coli* TG1 (M13cp-dg3-sm), *E. coli* TG1 (M13cp-CT-sm), and *E. coli* TG1 superinfected by hyperphage were further analyzed by ELISA. Phagemid particles were serially diluted and analyzed in a standard ELISA procedure

(see Materials and Methods). Analysis of ELISA data showed that S:N values as high as 7 when less than 5 x 10^4 tu/mL of phagemid particles were used as a primary antibody. Previous data in the laboratory showed that phagemid particles were not detectable in an ELISA unless they were used at concentrations of at least 1 x 10^6 particles/mL. Spot titering is a measure of transducing units (number of infectious units), while an ELISA is a measure of the number of particles that possess antibody activity. To determine if the measured tu/mL were underestimating the phagemid particles/mL, an anti-M13 sandwich ELISA was performed to quantify the number of phagemid particles produced by *E. coli* TG1 (M13cp-dg3-sm), *E. coli* TG1 (M13cp-CT-sm), and *E. coli* TG1 superinfected by hyperphage. A microtiter plate was coated with anti-M13 pVIII monoclonal antibody at a concentration of 10 μg/mL. Phagemid particles were incubated with the coated wells at dilutions of 1:2, 1:20, or 1:200. The phagemid particles were then detected using HRP-conjugated anti-M13 monoclonal antibody at a dilution of 1:2,500, followed by standard ELISA procedures (see Materials and Methods). A concentrated phagemid preparation, which had been amplified by hyperphage, was serially diluted and used as a standard for particles/mL. The values for particles/mL were linearly extrapolated from the standard curve of the standard phagemid particle (Table 3-4). Phagemid particles amplified by *E. coli* TG1 (M13cp-CT-sm) had particles/mL to tu/mL ratios between 740 and 23,000. Therefore, phagemid particles were being produced in high quantities; however, less than 0.1% of the phagemid particles produced were infectious. Phagemid particles amplified by *E. coli* TG1 (M13cp-dg3-sm) had particles/mL to tu/mL ratios between 1 and 190. Phagemid particles amplified by *E. coli* TG1 that was superinfected with hyperphage had particles/mL to tu/mL ratios between 6 and 24. Of the three amplification tools (M13cp-dg3-sm, M13cp-CT-sm, and commercial

hyperphage), commercial hyperphage was the only tool that could amplify phagemid particles to titers that were high enough to be used in phage display applications.

Phagemid particles amplified by helper plasmids in *E. coli* TG1 yielded phagemid particle titers between 10^5 and 10^8 tu/mL. Helper plasmids amplified phagemid particles to titers too low to be useful in biopanning. To determine if another *E. coli* strain could improve the titers of helper plasmid-amplified phagemid particles, *E. coli* MG1655 containing helper plasmid (M13cp-dg3-sm) was used to analyze phagemid particle production. *Escherichia coli* MG1655 was chosen because it has few known mutations and lacks the F plasmid. Therefore, it was hypothesized that the stress upon the *E. coli* for maintaining the helper plasmids would be lessened if it did not already have to maintain the F plasmid. Four different phagemid particles were amplified with *E. coli* MG1655 (M13cp-dg3-sm), and the resulting titers were: 2.5×10^7, 6.3×10^6, 2.5×10^7, and 3.9×10^7 tu/mL for an average of 2.4×10^7 tu/mL, which was still too low to be useful in biopanning.

Homemade Hyperphage Titers Were Increased with Amplification in *E. coli* MG1655 (pGTR203)

Commercial hyperphage was the only phagemid particle amplification tool tested that produced phagemid particle progeny in yields high enough to be used in biopanning. However, the titer of the commercial hyperphage stock was not high enough to be used to amplify the Tomlinson scFv libraries. Therefore, a way to improve homemade hyperphage production was analyzed. Because hyperphage has a partial deletion of its *gIII* gene, it needs the *gIII* gene to be provided in trans in the *E. coli* strain used to produce hyperphage particles. Homemade hyperphage was previously produced by using pGTR203, a plasmid that encodes *gIII*. *Escherichia coli* TG1 (pGTR203) or *E. coli* HB2151 (pGTR203) were infected with hyperphage, and the transduced bacteria were grown under conditions that promoted hyperphage production.

In the past, hyperphage amplified from *E. coli* TG1 (pGTR203) yielded titers between 10^7 to 10^8 tu/mL and *E. coli* HB2151 (pGTR203) yielded titers between 10^6 to 10^7 tu/mL. Both of these strains are F^+ *E. coli.* Because hyperphage infects bacteria by the F pilus, it was hypothesized that some of the produced hyperphages could have been taken up by the bacteria. Therefore, it was tested if hyperphage could be produced in higher titers when produced in an F^- *E. coli.*

Two F^- *E. coli* strains, *E. coli* MG1655 and *E. coli* EC100D, were used to amplify hyperphage. The strains were made electrocompetent (see Materials and Methods) and transformed by electroporation (see Materials and Methods) with pGTR203. *Escherichia coli* MG1655 (pGTR203) and *E. coli* EC100D (pGTR203) were made electrocompetent and transformed by electroporation with the hyperphage genome that had been extracted from *E. coli* TG1 (hyperphage) with a QIAprep Spin Miniprep kit (Qiagen) (see Materials and Methods). Five colonies from each transformation with hyperphage, *E. coli* MG1655 (pGTR203) (HyΦ) 1-5 and *E. coli* EC100D (pGTR203) (HyΦ) 1-5, were single colony passaged twice and analyzed for their ability to produce hyperphage.

Standing overnight cultures of all ten isolates were diluted 1:400 in broth and grown under conditions that promote phage production (see Materials and Methods). The hyperphage produced were analyzed for tu/mL by the whole plate titer method (see Materials and Methods) and for particles/mL by an anti-M13 sandwich ELISA (described above) (Table 3-5). The titers of hyperphage produced from the five *E. coli* EC100D (pGTR203) (HyΦ) isolates $2.1 \times 10^8 \pm 2.5 \times 10^8$ tu/mL were significantly lower than the hyperphage produced from the five *E. coli* MG1655 (pGTR203) (HyΦ), $3.0 \times 10^9 \pm 2.0 \times 10^9$ tu/mL ($p=0.02$). The ratios of particles/mL to tu/mL of hyperphages produced from *E. coli* EC100D (pGTR203) (HyΦ) were between 0.4 and 7.8, and the ratios of particles/mL to tu/mL of hyperphages produced from *E. coli* MG1655

(pGTR203) (HyΦ) were between 0.3 and 1.0. Therefore the amount of particles and transducing units of hyperphage produced from *E. coli* EC100D (pGTR203) (HyΦ) and *E. coli* MG1655 (pGTR203) (HyΦ) were approximately the same, meaning that the hyperphages produced were infectious. Both F^- *E. coli* strains generated higher titers of hyperphage than did the two F^+ *E. coli* strains. Concentration of hyperphage produced from *E. coli* MG1655 (pGTR203) (HyΦ) should generate a high enough titer of hyperphage to enable high quality and high quantity production of the Tomlinson scFv libraries.

Escherichia coli MG1655 (pGTR203) (HyΦ) isolate 5 yielded the highest titer of hyperphage (5.4×10^9 tu/mL); therefore, it was used to produce a large quantity of hyperphage to be used to amplify the Tomlinson J scFv phagemid library. One liter of hyperphage was produced from *E. coli* MG1655 (pGTR203) (HyΦ) 5 with a titer of 2.0×10^9 tu/mL, and the phages were concentrated by PEG precipitation (see Materials and Methods) to a volume of 2.8 mL with a final titer of 3×10^{11} tu/mL. This concentrated hyperphage was used to amplify the Tomlinson J scFv phagemid library.

The original Tomlinson J scFv library was amplified to ensure high quality and high quantity of phagemid particles produced. The Tomlinson J scFv phagemid library was originally received in *E. coli* TG1. This stock was grown in batch culture and frozen. Therefore, clones were not lost due to poor phagemid particle amplification with the low titer hyperphage because we grew the original bacterial stock of the library and not the previously made phagemid particle stock of the library that most likely lost redundancy of clones because it was amplified with low titer commercial hyperphage. A frozen stock of *E. coli* TG1 (Tomlinson J scFv phagemid library) was thawed on ice and inoculated into one liter of 2xTY AG broth. This culture was grown in a 37°C shaking incubator until the culture reached log phase. Thirty minutes into the

incubation an aliquot of the growing culture was removed and titered to ensure that the library was initially in a concentration high enough that redundancy of clones was present. The titer of the *E. coli* TG1 (Tomlinson J scFv phagemid library) 30 minutes into incubation was 1.3×10^7 CFU/mL, and because the culture volume was one liter there were 1.3×10^{10} CFU. The Tomlinson J phagemid library contains ~1×10^8 phagemid clones; therefore, there was initially a 100-fold redundancy of clones in the library. Approximately 2.3×10^{10} CFU of log phase *E. coli* TG1 (Tomlinson J scFv phagemid library) was superinfected with homemade hyperphage at a MOI of 10 and incubated for 30 minutes in a 37°C standing incubation. The superinfected bacteria were then titered on 2xTY AG plates containing 40 μg/mL kanamycin to ensure that enough bacteria were superinfected so that the redundancy of the phagemid clones was maintained. Approximately 2.9×10^{10} bacteria containing a phagemid were superinfected, which represents about 300-fold redundancy of clones. The superinfected bacteria were then grown in 1 liter of broth under conditions that promoted phagemid particle production (see Materials and Methods). The final yield Tomlinson J scFv phagemid particles in the amplified culture was 6.6×10^9 tu/mL. The liter of phagemid particles was then PEG precipitated to 15 mL of 4.4×10^{11} tu/mL, which yields 6.6×10^{12} tu. Titering throughout the Tomlinson J scFv library amplification ensured that the redundancy of the library clones was maintained at each step. The Tomlinson J scFv library that was amplified with homemade hyperphage was frozen and also used for biopanning.

Conclusion of Specific Aim 3

Escherichia coli TG1 harboring hyperphage was not an optimal phagemid particle amplification tool because of the low infection efficiency with phagemid particles. The infection efficiencies of phagemid particles into *E. coli* TG1 (HyΦ-sm) and *E. coli* TG1 (HyΦ-lg) were 28% or less than the infection efficiency of phagemid particles into *E. coli* TG1. Phagemid

particle amplification by helper plasmids was not an optimal amplification tool because it produced phagemid particle titers too low to be used in biopanning. Producing homemade hyperphage in *E. coli* MG1655 (pGTR203) enabled high yield of hyperphage particles. The improved high titer homemade hyperphage was used to successfully amplify the Tomlinson J scFv phagemid library to ensure that the redundancy of phagemid clones was maintained.

Specific Aim 4: Isolation of Specific Recombinant Phage to Stx2 Toxin of E. coli O157:H7

The Tomlinson J scFv phagemid library that was amplified with homemade hyperphage was panned against Stx2 toxin of *E. coli* O157:H7. Shiga toxins (Stx) kill mammalian cells and cause severe disease from infection with *E. coli* O157:H7 cells. *Escherichia coli* O157:H7 can produce Shiga toxins 1 and/or 2. *Escherichia coli* harboring the Stx2 toxin appears to be more virulent than *E. coli* harboring Stx1 or both Stx1 and 2 toxins (36-39). Therefore, Stx2 toxin was the target molecule chosen for panning. The Stx2 toxin preparation that was used to pan against was obtained from Toxin Technology (Sarasota, FL) and was stated to contain 50% Stx2 toxin. The other 50% of proteins in the Stx2 toxin preparation was most likely composed of contaminating *E. coli* HB101 proteins because the Stx2 toxin preparation was prepared from *E. coli* HB101 (pMJ100) lysates. pMJ100 encodes Stx2 toxoid.

A three round biopanning process was performed to pan the Tomlinson J scFv phagemid library against a Stx2 toxin preparation-coated immunotube (see Materials and Methods) with amplification between panning rounds to amplify the selected phagemid particles. Phagemid particles were eluted using trypsin treatment for 10 minutes. Phagemid particles contain a trypsin cleavage site between the scFv and the pIII. Therefore, phagemid particles that were bound to Stx2 toxin preparation by their scFv could be eluted from the Stx2 toxin preparation to be amplified for further rounds of panning. Approximately 1×10^{12} phagemid particles were added to an immunotube that was coated with 100 μg of Stx2 toxin preparation. Approximately

2.2×10^6 phagemid particles were eluted after round one of panning. The round-one-eluted phages were amplified with homemade hyperphage to 2×10^{10} tu/mL in 100 mL of broth. The amplified phagemid particles were then PEG precipitated to 9×10^{11} tu/mL in 2.5 mL PBS. Approximately 1×10^{12} phagemid particles from the PEG-precipitated phagemid particles from round one were panned against Stx2 for a second round of panning. Approximately 2.0×10^7 phagemid particles were eluted after round two of panning. The round-two-eluted phages were amplified with homemade hyperphage to 2.5×10^{10} tu/mL in 100 mL broth. The amplified phagemid particles were then PEG precipitated to 7.8×10^{11} tu/mL in 2.5 mL PBS. Approximately 1×10^{12} phagemid particles from the PEG-precipitated phagemid particles from round two were panned against Stx2 for a third round of panning. Approximately 1.5×10^{10} phagemid particles were eluted after the third round of panning. The increase in the number of phagemid particles eluted (2×10^6 for round one, 2×10^7 for round two, 2×10^{10} for round three) after every round suggests that phagemid particles that specifically bound to Stx2 toxin preparation were being selected and amplified.

Eighty-four phagemid particles from the third round of panning were individually amplified with homemade hyperphage (see Materials and Methods) to be analyzed for specificity by ELISA and Western blot experiments. Forty-four clones were initially analyzed by ELISA. Microtiter wells coated with 10 μg/mL Stx2 toxin preparation were incubated with phagemid particle-containing supernatants that were diluted 1:2 in blocking buffer, 1 μg/mL anti-Stx211E10 (α-Stx2 subunit A) monoclonal antibody (Toxin Technology), 1 μg/mL anti-Stx2-BB12 (α-Stx2 subunit B) monoclonal antibody (Toxin Technology), or 1×10^8 tu/mL α-BSA phagemid particles (negative control for determining the noise for S:N calculations) (Fig. 3-14). Of the 44 clones screened, 40 had a S:N between 9 and 18. The two anti-Stx toxin monoclonal

antibodies had a S:N less than 2 against Stx2 toxin preparation. The Stx2 toxin preparation may not have bound to the microtiter wells very efficiently, or the anti-Stx2 monoclonal antibodies may not have worked very well. Conversely, the phagemid clones that were positive may have bound to the microtiter well instead of the Stx2 toxin preparation.

To determine if the clones were binding to Stx2 or to the microtiter well, 4 of the previously screened clones along with 44 new clones were analyzed via ELISA using the same procedure as above with the addition of PBS-treated microtiter wells as a negative control (Fig. 3-15). Of the 48 clones, 44 had signals of 0.5 or higher against Stx2 toxin preparation-coated microtiter wells. Four clones (clones 1, 23, 35, and 39) had signals as high or higher against PBS-treated microtiter wells compared to Stx2 toxin preparation-coated microtiter wells. These four clones could possibly recognize polystyrene. Because the library was panned against Stx2 toxin preparation-coated immunotubes, it was possible that some clones were selected that bound to the polystyrene immunotube. Fifty-three of 59 clones (89%) had a S:N from 2 to 23, and 48 of 59 clones (81%) had a S:N from 10 to 23 against Stx2 toxin preparation. Therefore, the majority of the clones screened bound to the Stx2 toxin preparation. However, 4/59 (7%) of the clones bound to PBS-treated wells as well as Stx2-coated wells. The four clones had a signal in an ELISA between 0.31-0.87 against PBS-treated wells. These clones most likely bound to polystyrene. Isolation of plastic binding phages is fairly common (84). No matter how well the panning vessel is blocked with blocking buffer, phages can still be selected that bind to the panning vessel. Of the 59 clones screened, 2 (3%) did not generate a positive signal against Stx2-coated microtiter wells or non-coated microtiter wells. These clones had unknown binding specificity.

The anti-Stx monoclonal antibodies (α-Stx2 subunit A and α-Stx2 subunit B) were further analyzed to determine the reason why they did not react in the ELISA. Microtiter wells coated with 10 μg/mL Stx2 toxin preparation or carbonate bicarbonate buffer were incubated with α-Stx2 subunit A and α-Stx2 subunit B monoclonal antibodies at concentrations of 1, 10, or 100 μg/mL. Bound antibodies were detected with HRP-conjugated anti-mouse antibody (Cappel) or HRP-conjugated anti-mouse antibody (Sigma-Aldrich). Standard ELISA procedures followed (see Materials and Methods). All parameters generated S:N values less than two. The Stx2 toxin preparation appeared to be binding to the well because the amplified panning clones were binding to the Stx2 with high S:N values in microtiter wells. The concentrations of anti-Stx2 monoclonal antibodies were not an issue because using the monoclonal antibodies at a concentration as high as 100 μg/mL did not improve the signal of the monoclonal antibodies in an ELISA. Two different HRP-conjugated anti-mouse antibodies were used to determine if the secondary antibody was not recognizing the primary antibodies. Neither HRP-conjugated anti-mouse antibody improved the signal of the monoclonal antibodies in an ELISA. Positive controls for the anti-mouse antibodies were not performed in the ELISA to determine whether or not the anti-mouse antibodies were functional. However, the two HRP-conjugated anti-mouse antibodies were used within three days of these experiments and generated positive signals in an ELISA against murine antibodies. The reason why the anti-Stx monoclonal antibodies gave low signals with Stx2 toxin preparation in an ELISA was not determined.

Analysis by ELISA shows that the majority of clones isolated from the Stx2 panning bound to the Stx2 toxin preparation in an ELISA. Because the Stx2 toxin preparation was only 50% pure for Stx2 toxin, the panning clones could have been binding to Stx2 toxin or the 50% of impurities in the preparation. To analyze if the Stx2 toxin preparation panning clones recognized

the Stx2 toxin or the impurities, three clones were selected and examined for reactivity with the Stx2 toxin preparation and *E. coli* supernatants or periplasmic break fractions. The strains examined were *E. coli* O157:H7 933 (expresses Stx1 and 2 toxins), *E. coli* DH5α (pNR100) (expresses Stx2 toxoid), and *E. coli* DH5α. Microtiter wells were coated with 0.01 mg/mL Stx2 toxin preparation, 0.07 mg/mL *E. coli* O157:H7 933 periplasmic break fraction, 2 mg/mL *E. coli* O157:H7 933 supernatant, 1 mg/mL *E. coli* DH5α (pNR100) periplasmic break fraction, 1 mg/mL *E. coli* DH5α periplasmic break fraction, or carbonate coating buffer. If the clones bound to Stx2 toxin, they were expected to generate positive signals to all the antigens except for *E. coli* DH5α and carbonate coating buffer. If the clones bound to impurities in the Stx2 toxin preparation, the clones were expected to generate positive signals for all the antigens except carbonate coating buffer. Coated wells were incubated with 10 μg/mL of α-Stx2 subunit A monoclonal antibody (11E10), 10 μg/mL of α-Stx2 subunit B monoclonal antibody (BB12), 1 x 10^9 tu/mL of phagemid particles of Stx2 clones (46, 48, and 49), or α-BSA phagemids. Standard ELISA procedures were followed (see Materials and Methods) (Fig. 3-16). All parameters generated S:N values of less than 1.5 except for clones 46, 48, and 49 that were analyzed with Stx2 toxin preparation-coated microtiter wells. Because the monoclonal antibodies to Stx2 toxin preparation did not generate positive signals in an ELISA against any of the parameters, it was not determined if the clones from the Stx2 toxin preparation panning were reacting to Stx2 toxin or impurities in the commercially obtained Stx2 toxin preparation.

Clones that were selected from the Stx2 toxin preparation panning and had a positive signal by ELISA to Stx2 toxin preparation were further analyzed by Western blot to determine if the clones were binding to Stx2 toxin or to impurities in the Stx2 toxin preparation. Six clones were screened in a Western blot against Stx2 toxin preparation, *E. coli* O157:H7 933 (expresses

Stx1 and 2 toxins) supernatant, and *E. coli* O157:H7 87-23 (Stx phage cured). Because the Stx2 toxin is composed of A and B subunits that are approximately 32 and 10-kDa proteins, a clone that bound to Stx2 toxin was expected to recognize a 32 or 10-kDa protein in the Stx2 toxin preparation and *E. coli* O157:H7 933 supernatant but not in *E. coli* O157:H7 87-23 supernatant. A clone that bound to impurities was expected to recognize proteins in all three antigens.

A 4-20% polyacrylamide gel was loaded with 1.5 μg/well of Stx2 toxin preparation, 120 mg/well of *E. coli* O157:H7 933 supernatant, and 120 mg/well of *E. coli* O157:H7 87-23 supernatant. The resolved proteins were transferred to nitrocellulose and analyzed by Western blot (see Materials and Methods). Phagemid particles (α-BSA and Stx2 toxin preparation panning clones 46, 48, 80, 81, and 83) were used as a primary antibody at approximately 10^9 tu/mL, and α-Stx2 subunit A monoclonal antibody was used at 4 μg/mL. Anti-Stx2 subunit B monoclonal antibody failed to generate a positive signal by Western blot. Standard Western blot procedures were performed (see Materials and Methods) (Fig. 3-17). The α-Stx2 subunit A monoclonal antibody recognized a 32-kDa (the size of the A subunit) protein in the Stx2 toxin preparation and in the *E. coli* O157:H7 933 supernatant, thereby proving that Stx2 toxin subunit A was present in both preparations but not in the *E. coli* O157:H7 87-23 supernatant as was expected. Anti-BSA failed to recognize any of the antigens, as was expected. Clone 46 failed to recognize any of the three antigens. Clones 48 recognized 35, 45, and 55-kDa proteins in the Stx2 toxin preparation and a 35-kDa protein in both *E. coli* O157:H7 supernatants. This result means that clone 48 probably bound to bacterial protein impurities in the Stx2 toxin preparation. Clones 49 and 80 bound to a 35-kDa protein in all three antigens and a 20-kDa protein in the Stx2 toxin preparation and in *E. coli* O157:H7 87-23 supernatant. Therefore, clones 49 and 80 probably bound to bacterial protein impurities in the Stx2 toxin preparation. Clones 81 and 83

appeared to recognize the same bacterial protein impurities as clones 49 and 80, except that their band intensities were much lighter.

Western blot and ELISA analyses showed that clones obtained from panning against the Stx2 toxin preparation recognized the Stx2 toxin preparation. The clones that have been screened so far appeared to bind to impurities in the Stx2 toxin preparation. To determine if the Stx2 toxin preparation contained 50% Stx2 toxin as stated by the producer, the toxin preparation was resolved and analyzed by SDS-PAGE (Fig. 3-18). If the Stx2 toxin preparation was comprised of 50% Stx2 toxin, 10 and 32-kDa protein bands should have been the predominant protein bands. However, a protein band at approximately 32 kDa was a minor protein band. A protein band at 10 kDa was a major protein band; however, whether these protein bands were actually the Stx2 toxin subunits was not determined. The 10 and 32-kDa protein bands comprised less than 10% of the Stx2 toxin preparation. Because the concentration of Stx2 toxin was so low in the commercially obtained Stx2 toxin preparation, it was probable that the majority of the phagemid particles isolated from the Stx2 panning would recognize impurities instead of Stx2 toxin.

Conclusion of Specific Aim 4

Panning against commercially obtained Stx2 toxin preparation was successful in isolating phagemid particles that bound to the Stx2 toxin preparation. Initial screening of clones by Western blot showed that the clones bound to impurities in the commercially obtained Stx2 toxin preparation and not Stx2 toxin. The commercially obtained Stx2 toxin preparation was supposed to contain 50% Stx2 toxin; however, SDS-PAGE analysis showed that less no more than 10% of the proteins present in the preparation may have been Stx2 toxin. Therefore, it was likely that the majority of clones selected from the panning would recognize impurities in the Stx2 toxin preparation and not Stx2 toxin. However, this panning exercise showed that the Tomlinson J

library amplified with the homemade hyperphage was a more effective tool than the previously amplified libraries.

Table 3-1. Infection efficiencies of *E. coli* TG1 containing various helper plasmids with phagemid particles. Bacterial strains were transduced with anti-BSA (α-BSA) and Vc86 phagemid particles at a MOI of 0.1, and transduced bacteria were enumerated. Infection efficiency is the number of transductions into a bacterial strain divided by the number of transductions into *E. coli* TG1 and is represented as a percentage.

Strain	Transductions with α-BSA	Infection efficiency with α-BSA (%)	Transductions with Vc86	Infection efficiency with Vc86 (%)	Average infection efficiency (%)
E. coli TG1	1.2×10^6	100	3.6×10^6	100	100
E. coli TG1 (M13cp-sm)	9.3×10^2	0.1	6.9×10^3	0.3	0.2
E. coli TG1 (M13cp-lg)	8.3×10^2	0.1	9.6×10^3	0.3	0.2
E. coli TG1 (M13cp-CT-sm)	7.9×10^4	7	4.8×10^5	13	10
E. coli TG1 (M13cp-CT-lg)	$<1.3 \times 10^2$	<0.01	$<1.3 \times 10^2$	<0.01	<0.01
E. coli TG1 (M13cp-dg3-sm)	2.1×10^6	175	3.6×10^6	97	136
E. coli TG1 (M13cp-dg3-lg)	$<1.3 \times 10^2$	<0.01	$<1.3 \times 10^2$	<0.01	<0.01

Table 3-2. Infection efficiencies of *E. coli* TG1 (M13cp-dg3-sm) with phagemid particles. Bacterial strains were transduced with phagemid particles at a MOI of 0.1, and transduced bacteria were enumerated. Infection efficiency is the number of transductions with a phagemid into a bacterial strain divided by the number of transductions with the same phagemid into *E. coli* TG1.

Strain	Phagemid	Transductions	Infection efficiency (%)
E. coli TG1	Vc86	5.3×10^6	100
E. coli TG1 (M13cp-dg3-sm)	Vc86	4.9×10^6	92
E. coli TG1	Clone 18	2.4×10^6	100
E. coli TG1 (M13cp-dg3-sm)	Clone 18	2.7×10^6	113
E. coli TG1	α-AV20N3	1.5×10^6	100
E. coli TG1 (M13cp-dg3-sm)	α-AV20N3	1.8×10^6	120

Table 3-3. Infection efficiencies of *E. coli* TG1 (M13cp-CT-sm) and *E. coli* JM109 (M13cp-CT-sm) with phagemid particles. Bacterial strains were transduced with phagemid particles at a MOI of 0.1, and transduced bacteria were enumerated. Infection efficiency is the number of transductions with a phagemid particle into a helper plasmid-containing bacterial strain divided by the number of transductions with the same phagemid particle into the bacterial strain without the helper plasmid.

Strain	Phagemid	Transductions	Infection efficiency (%)
E. coli TG1	Vc86	2.4×10^5	100
E. coli TG1 (M13cp-CT-sm)	Vc86	1.3×10^5	54
E. coli TG1	Clone 18	1.4×10^5	100
E. coli TG1 (M13cp-CT-sm)	Clone 18	5.5×10^4	39
E. coli TG1	α-AV20N3	9.3×10^4	100
E. coli TG1 (M13cp-CT-sm)	α-AV20N3	4.4×10^4	47
E. coli JM109	Vc86	6.8×10^5	100
E. coli JM109 (M13cp-CT-sm)	Vc86	1.5×10^5	22
E. coli JM109	Clone 18	1.7×10^5	100
E. coli JM109 (M13cp-CT-sm)	Clone 18	6.1×10^4	36
E. coli JM109	α-AV20N3	1.4×10^5	100
E. coli JM109 (M13cp-CT-sm)	α-AV20N3	3.7×10^4	26

Table 3-4. Comparison of transducing units to particles per milliliter of amplified phagemid particles. The spot-titer method was used to determine tu/mL. Particles/mL was determined via a sandwich ELISA with anti-M13 antibodies. (n = 2 wells)

Phagemid particle amplified	Amplification tool used	tu/mL	Particles/mL	(Particles/mL)/ (tu/mL)
Vc86	M13cp-CT-sm	8.7×10^5	2.0×10^{10}	23000
Vc86	M13cp-CT-sm	4.6×10^6	3.4×10^9	740
clone 18	M13cp-dg3-sm	2.4×10^8	2.2×10^8	1
α-AV20N3	M13cp-dg3-sm	4.4×10^7	7.0×10^7	2
α-BSA	M13cp-dg3-sm	1.3×10^8	1.8×10^8	1
Vc86	M13cp-dg3-sm	8.7×10^6	1.7×10^9	190
clone 18	Hyperphage	7.1×10^8	1.1×10^{10}	15
α-AV20N3	Hyperphage	2.0×10^9	1.1×10^{10}	6
α-BSA	Hyperphage	5.8×10^8	1.4×10^{10}	24

Table 3-5. Comparison of transducing units to particles per milliliter of amplified homemade hyperphage produced from F- *E. coli* strains. The spot-titer method was used to determine tu/mL. Particles/mL was determined via a sandwich ELISA with anti-M13 antibodies. (n = 2 wells).

Hyperphage production strain	tu/mL	Particles/mL	(Particles/mL)/ (tu/mL)
E.coli EC100D (pGTR203) (HyΦ) 1	5.3×10^7	4.2×10^8	7.8
E.coli EC100D (pGTR203) (HyΦ) 2	9.4×10^7	3.5×10^8	3.7
E.coli EC100D (pGTR203) (HyΦ) 3	3.2×10^8	1.1×10^8	0.4
E.coli EC100D (pGTR203) (HyΦ) 4	2.9×10^6	$< 1.0 \times 10^7$	<3
E.coli EC100D (pGTR203) (HyΦ) 5	6.0×10^8	2.1×10^8	0.4
E.coli MG1655 (pGTR203) (HyΦ) 1	1.0×10^9	1.0×10^9	1.0
E.coli MG1655 (pGTR203) (HyΦ) 2	8.0×10^8	7.3×10^8	0.9
E.coli MG1655 (pGTR203) (HyΦ) 3	3.0×10^9	1.3×10^9	0.4
E.coli MG1655 (pGTR203) (HyΦ) 4	4.3×10^9	1.4×10^9	0.3
E.coli MG1655 (pGTR203) (HyΦ) 5	5.4×10^9	1.5×10^9	0.3

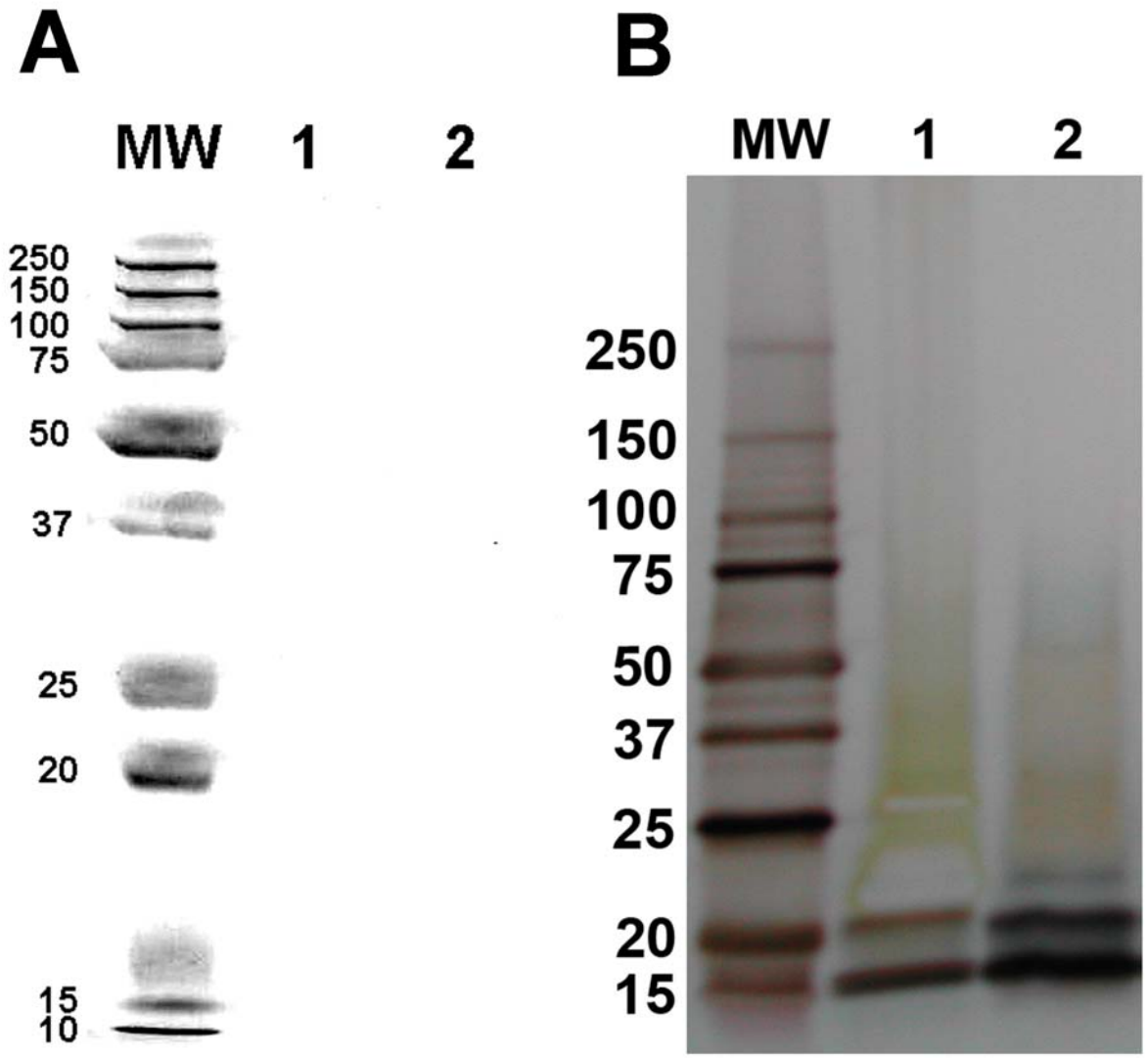


Figure 3-1. Analysis of TRIzol Reagent-extracted *V. cholerae* N16961 LPS by SDS-PAGE. Samples were resolved on a 12% (w/v) polyacrylamide gel and stained with A) Coomassie blue stain or B) Tsai-Frasch silver stain. (1) TRIzol-extracted *V. cholerae* N16961 LPS. (2) *V. cholerae* 569B LPS (Sigma-Aldrich).

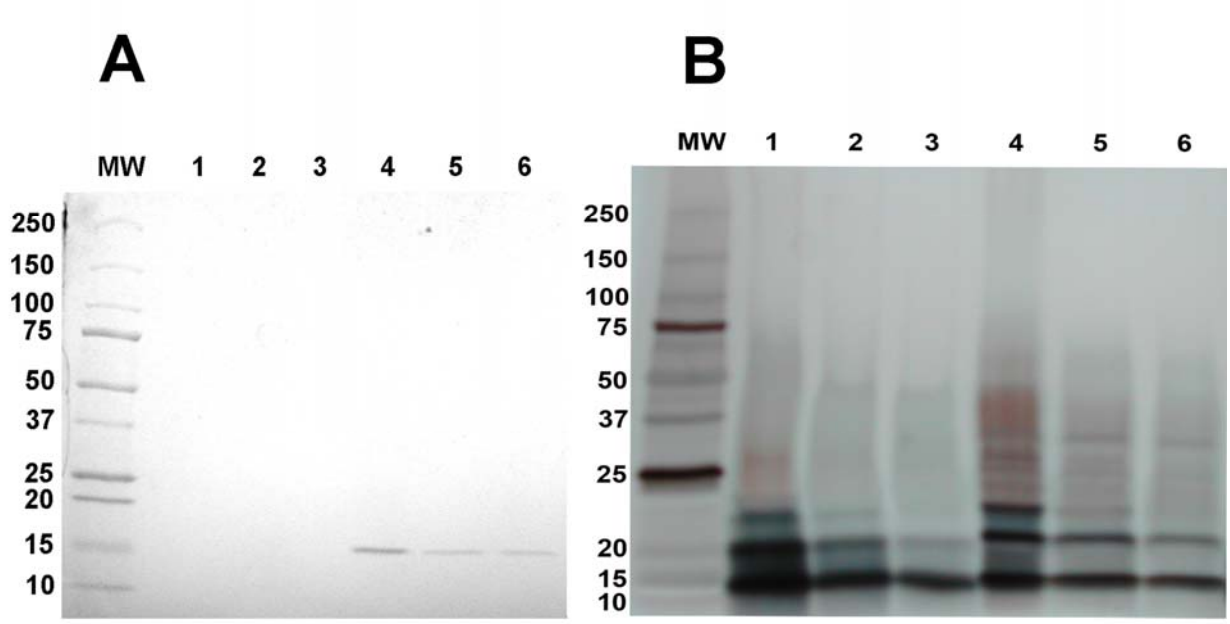


Figure 3-2. Analysis of phenol-water-extracted *V. cholerae* 569B LPS by SDS-PAGE. Samples were resolved on a 4-20% (w/v) polyacrylamide gel and stained with A) Coomassie blue stain or B) Tsai-Frasch silver stain. (1-3) *V. cholerae* 569B LPS (Sigma-Aldrich) at dilutions of 1:4 (1), 1:8 (2), and 1:16 (3). (4-6) Phenol-water-extracted *V. cholerae* 569B LPS at dilutions of 1:2 (4), 1:4 (5), and 1:8 (6).

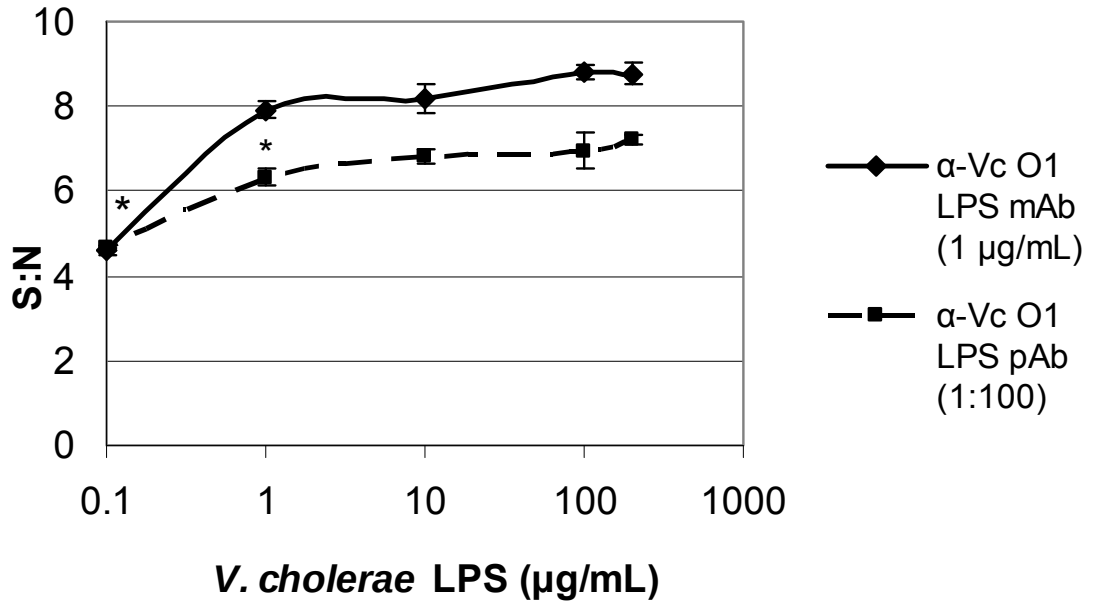


Figure 3-3. Analysis of the saturation limit of *V. cholerae* 569B LPS to microtiter wells by ELISA. Signal to noise (S:N) ratios were calculated for the reactivity of mouse anti-*V. cholerae* O1 LPS monoclonal antibody (α-Vc O1 LPS mAb) (1 µg/mL) and rabbit anti-*V. cholerae* O1 LPS polyclonal antibody (α-Vc O1 LPS pAb) (1:100) to *V. cholerae* 569B LPS (0.1-200 µg/mL)-coated microtiter wells. Bound primary antibodies were detected with either HRP-conjugated goat anti-rabbit antibody or HRP-conjugated goat anti-mouse antibody. Wells were developed with TMB substrate, and their absorbances read at 630 nm. (n=3 wells). There was a significant decrease (*p=0.01) in S:N when the concentration of LPS decreased from 1 to 0.1 µg/mL when using the α-Vc O1 LPS mAb. There was a significant decrease (*p=0.01) in the S:N when the concentration of LPS decreased from 10 to 1 µg/mL when using the α-Vc O1 LPS pAb.

A

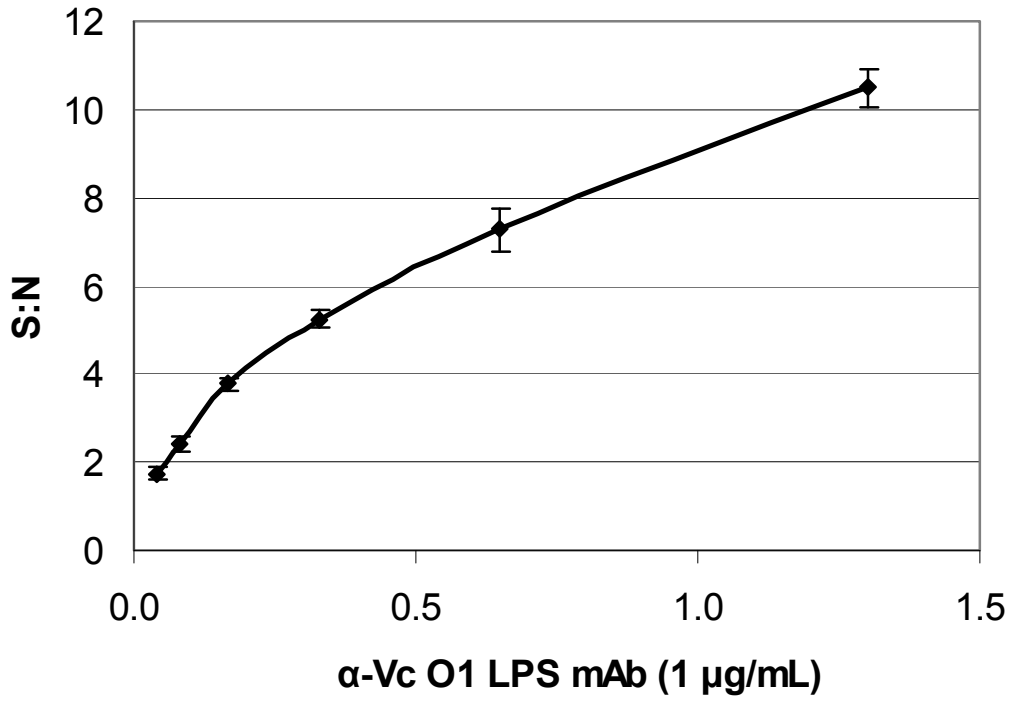
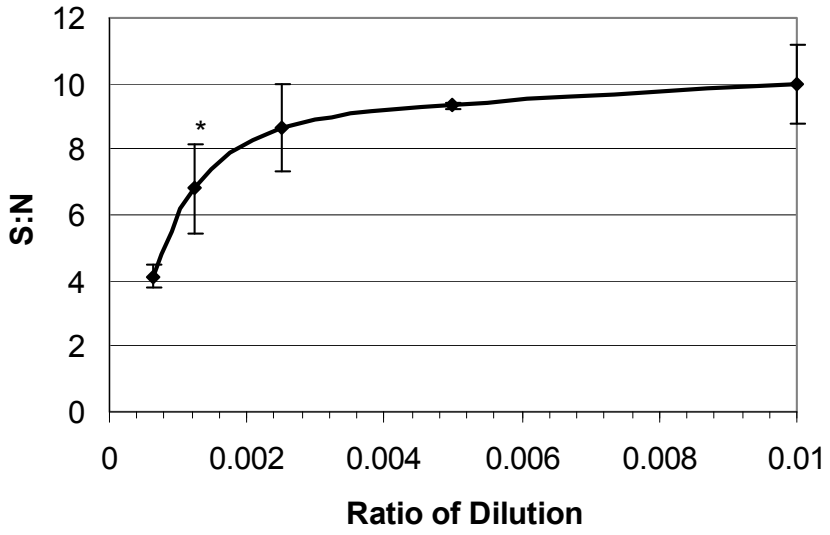


Figure 3-4. Analysis of the saturation limits of primary antibodies to *V. cholerae* 569B LPS-coated microtiter wells by ELISA. Primary antibodies: A) mouse anti-*V. cholerae* O1 LPS monoclonal antibody (α-Vc O1 LPS mAb) (0.04-1.3 µg/mL) and B) rabbit anti-*V. cholerae* O1 LPS polyclonal antibody (α-Vc O1 LPS pAb) (1:100-1:1,600) were reacted with *V. cholerae* 569B LPS (1 µg/mL)-coated microtiter wells. Bound primary antibodies were detected with either HRP-conjugated goat anti-rabbit antibody or HRP-conjugated goat anti-mouse antibody. Wells were developed with TMB substrate and their absorbances read at 630 nm. The S:N were calculated for every primary antibody concentration or dilution (n=3 wells). In (A) comparisons of S:N between every monoclonal antibody concentration used yielded significant differences ($p<0.03$) in S:N; therefore, the saturation limit of the monoclonal antibody could not be determined. In (B) a significant decrease (*$p=0.01$) in the S:N was not reached until the polyclonal antibody dilution decreased from 1:400 (ratio dilution of 0.003) to 1:800(ratio dilution of 0.001); therefore, the saturation limit of the polyclonal antibody was reached at a dilution of 1:400.

B



Ratio of Dilution, Dilution
0.01, 1:100
0.005, 1:200
0.003, 1:400
0.001, 1:800
<0.001, 1:1,600

Figure 3-4. Continued.

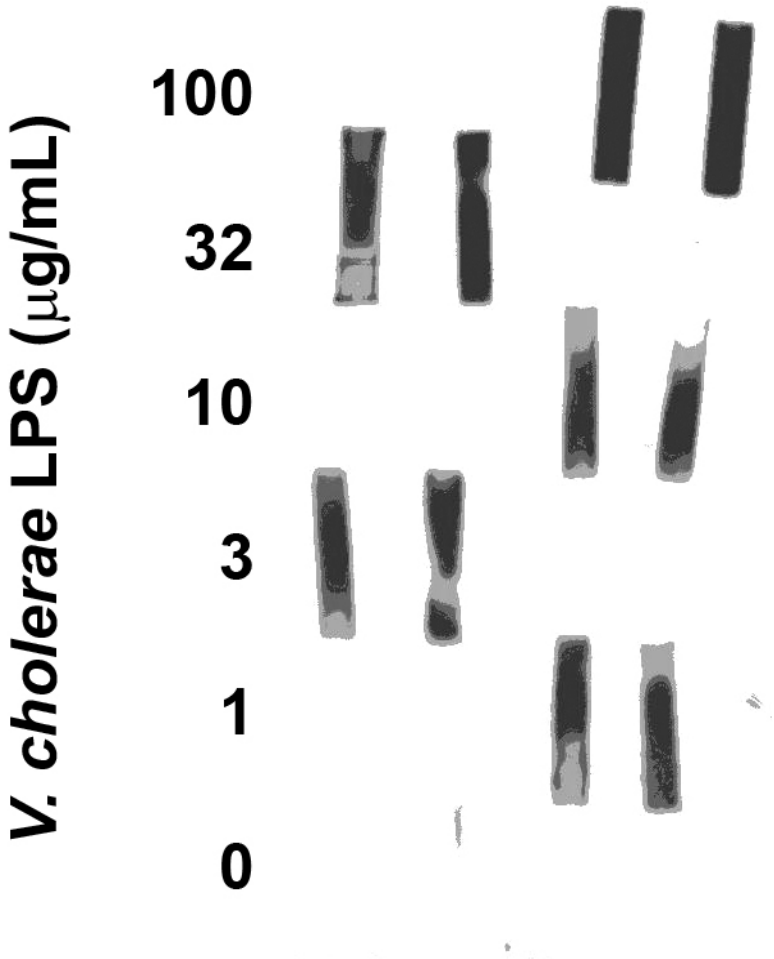


Figure 3-5. Saturation of *V. cholerae* 569B LPS to nitrocellulose paper. *Vibrio cholerae* 569B LPS was incubated with nitrocellulose paper at concentrations from 1-100 μg/mL. The primary antibody used to detect the LPS was rabbit anti-*V. cholerae* O1 LPS polyclonal antibody (1:400). Bound primary antibody was detected with HRP-conjugated goat anti-rabbit antiserum and detected with ECL substrate. The nitrocellulose paper became saturated with *V. cholerae* 569B LPS at a LPS concentration of 100 μg/mL. The strips incubated with 0 μg/mL LPS failed to stain significantly.

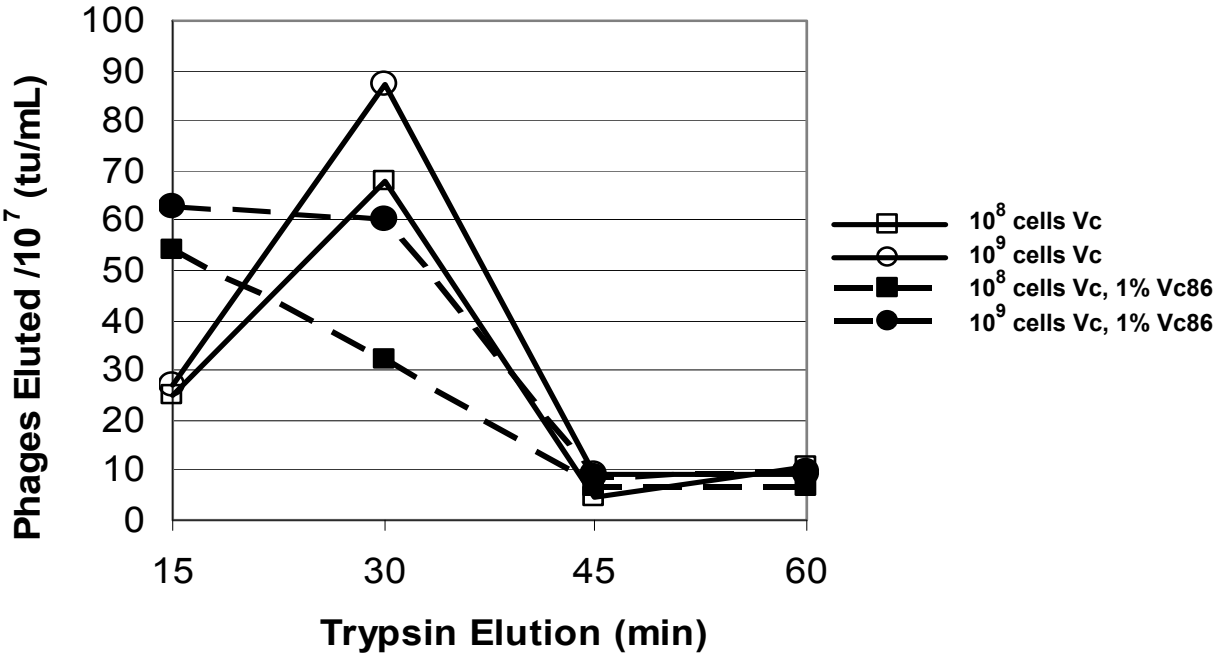
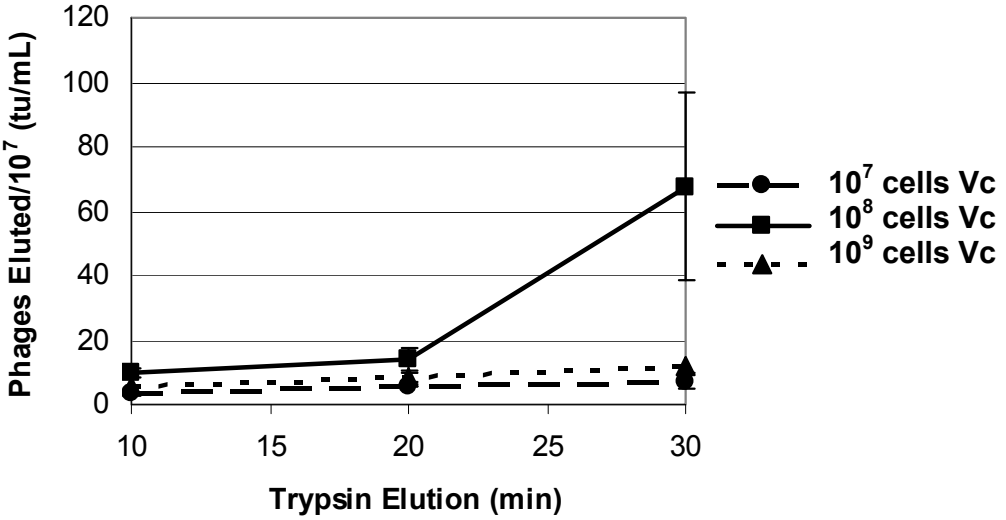


Figure 3-6. Titers of eluted phagemid particles from the first one-round panning optimization experiment. The Tomlinson J library +/- 1% Vc86 phagemid particles was panned against 10^8 and 10^9 whole cells of *V. cholerae* N16961 in suspension. Phagemid particles that were bound to the *V. cholerae* whole cells were eluted with 10% (v/v) trypsin stock (10 mg/mL trypsin, 50 mM Tris-HCl (pH 7.4), 1 mM CaCl2 in water) in PBS for 15, 30, 45, and 60 minutes. Eluted phagemid particles were enumerated via the spot-titer method.

A



B

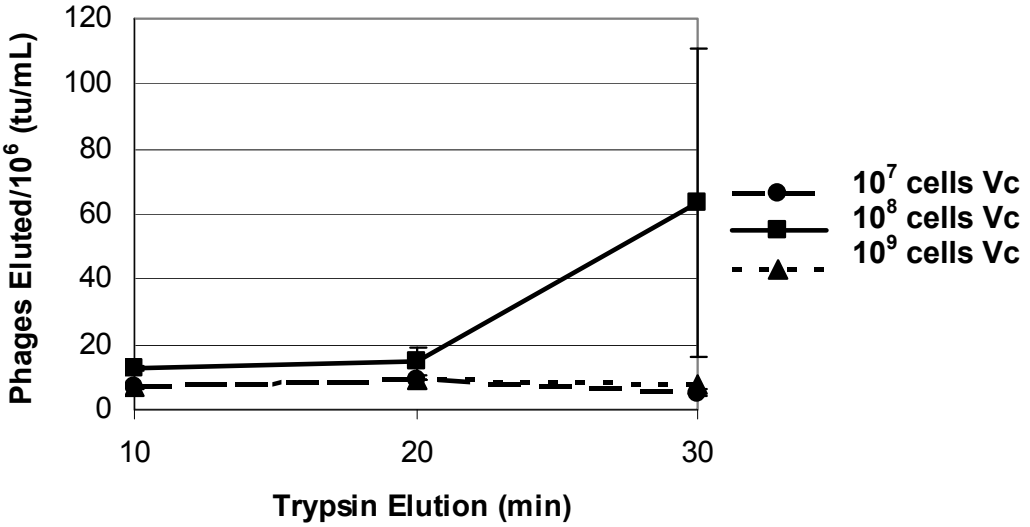


Figure 3-7. Titers of eluted phagemid particles from the second one-round panning optimization experiment. The Tomlinson J library +/- 1% Vc86 phagemid particles was panned against 10^7, 10^8, and 10^9 whole cells of *V. cholerae* N16961 in suspension. Phagemid particles that were bound to the *V. cholerae* whole cells were eluted in 10% (v/v) trypsin stock (10 mg/mL trypsin, 50 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$ in water) in PBS for 10, 20, and 30 minutes. Eluted phagemid particles were enumerated via the spot-titer method. A) Panning with the Tomlinson J library B) Panning with the Tomlinson J library (+) 1% Vc86 phagemid particles.

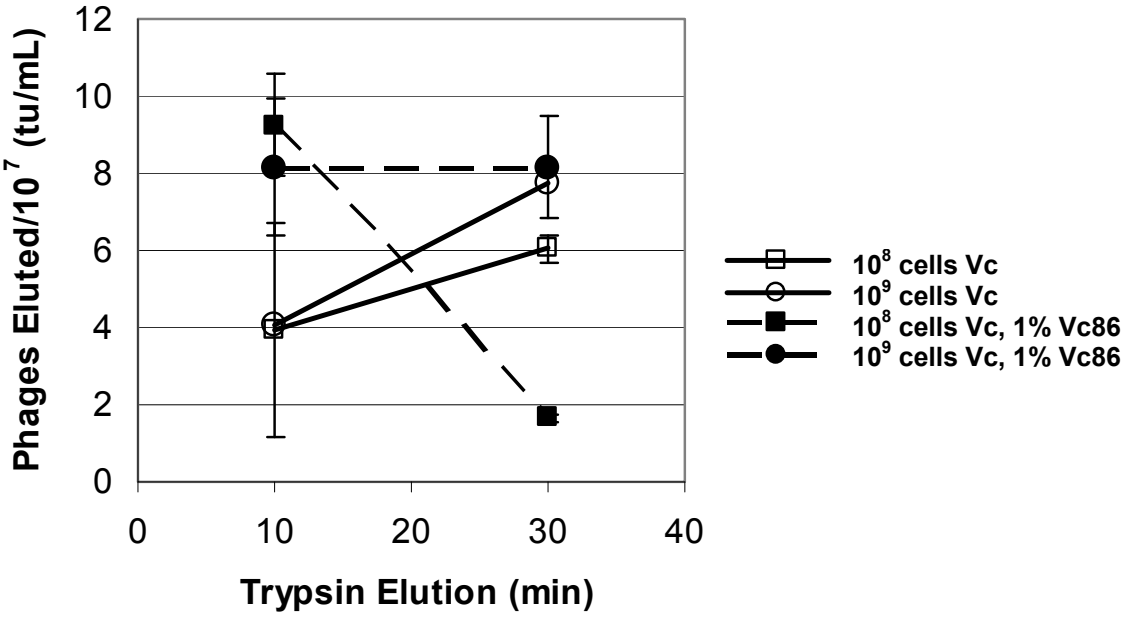


Figure 3-8. Titers of eluted phagemid particles from the third one-round panning optimization experiment. The Tomlinson J library +/- 1% Vc86 phagemid particles was panned against 10^8 and 10^9 whole cells of *V. cholerae* N16961 in suspension. Phagemid particles that were bound to the *V. cholerae* whole cells were eluted in 10% (v/v) trypsin stock (10 mg/mL trypsin, 50 mM Tris-HCl (pH 7.4), 1 mM CaCl2 in water) in PBS for 10 and 30 minutes. Eluted phagemid particles were enumerated via the spot-titer method.

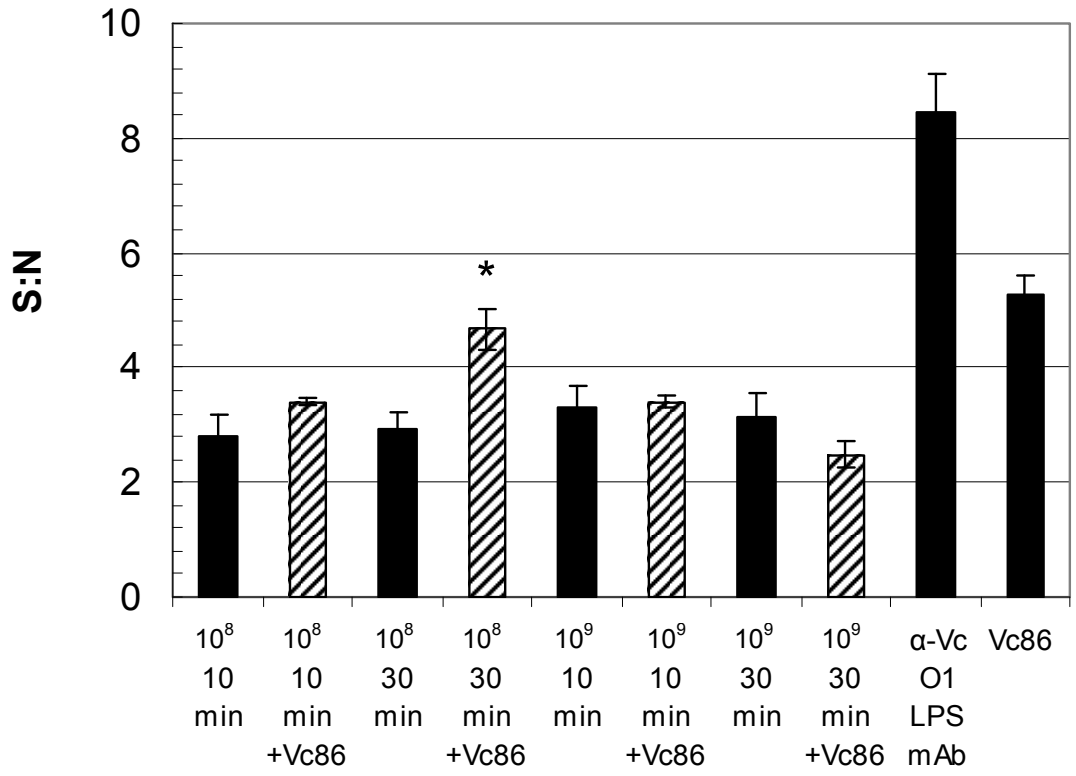


Figure 3-9. Analysis of eluted phagemid particles from the third one-round panning optimization experiment by ELISA. Microtiter wells coated with 3 x 10^8 CFU/mL *V. cholerae* whole cells or PBS and were incubated with 1 x 10^8 tu/mL of amplified eluted phagemid particles or 10 μg/mL of mouse anti-*V. cholerae* O1 LPS monoclonal antibody. Bound phagemid particles were detected with HRP-conjugated anti-M13 secondary antibody, and the monoclonal antibody was detected with HRP-conjugated goat anti-mouse antibody. Wells were developed with TMB substrate, and their absorbances were read at 630 nm (n=3 wells). There was a significantly higher S:N (*p=0.01-0.04) from the amplified eluted phagemid particles from the panning with 10^8 whole cells with a 30 minute elution using the library containing 1% Vc86 when compared to the amplified eluted phagemid particles from the other pannings.

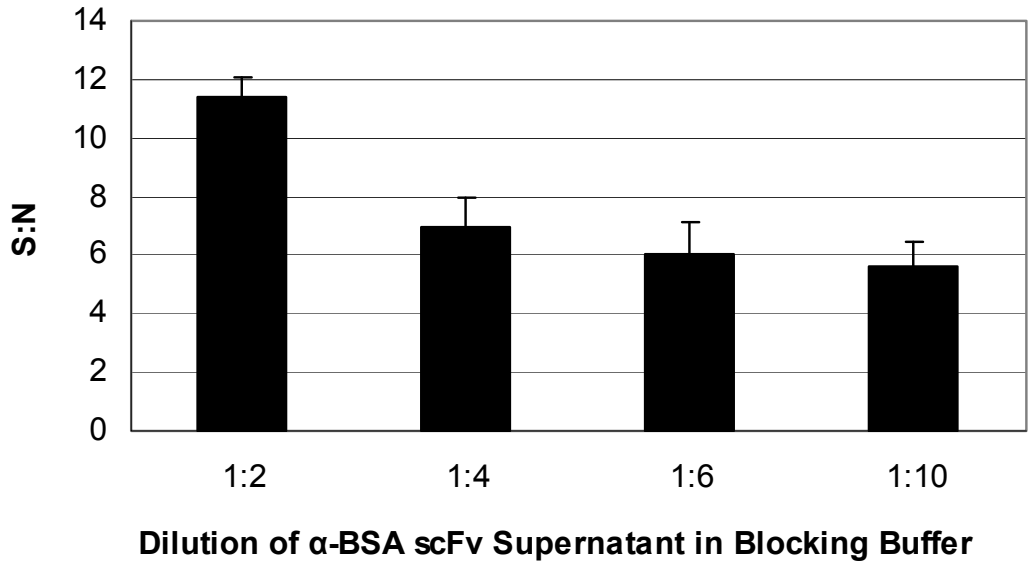
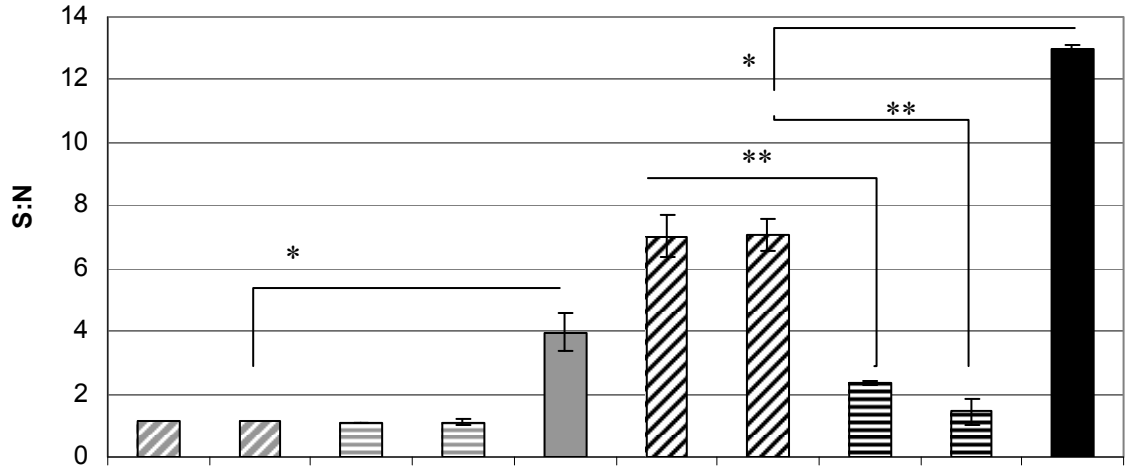


Figure 3-10. Analysis of anti-BSA (α-BSA) scFv proteins produced in microtiter wells by ELISA. Microtiter wells were coated with 10µg/mL BSA or PBS. Anti-BSA scFv particles were produced in a microtiter well and isolated by centrifugation and the scFv-containing supernatants were diluted 1:2, 1:4, 1:6, or 1:8 in blocking buffer and incubated with the coated wells. Bound scFv particles were detected with HRP-conjugated Protein L. Wells were developed with TMB substrate, and their absorbances were read at 630 nm. (n=2 wells).



Phagemid Particle	V	V	V	V	V	B	B	B	B	B
Temp. (°C)	30	30	37	37	30	30	30	37	37	30
% Glucose	0	0.1	0	0.1	0.1	0	0.1	0	0.1	0.1
Grown in	W	W	W	W	F	W	W	W	W	F

Figure 3-11. Analysis of α-BSA and Vc86 phagemid particles produced in microtiter wells by ELISA. Phagemid particles Vc86 (V) and α-BSA (B) were produced at either 30°C or 37°C in medium containing either 0% or 0.1% glucose in a microtiter well (W) or in a flask (F). Microtiter wells were coated with 10μg/mL BSA, 3 x 10^8 CFU/mL V. cholerae whole cells, or PBS. Phagemid particles were diluted 1:2 in blocking buffer and incubated with the coated wells. Bound phagemid particles were detected with HRP-conjugated anti-M13 secondary antibody. Wells were developed with TMB substrate, and their absorbances were read at 630 nm. (*) represents $p<0.05$ and (**) represents $p<0.01$. (n=2 wells)

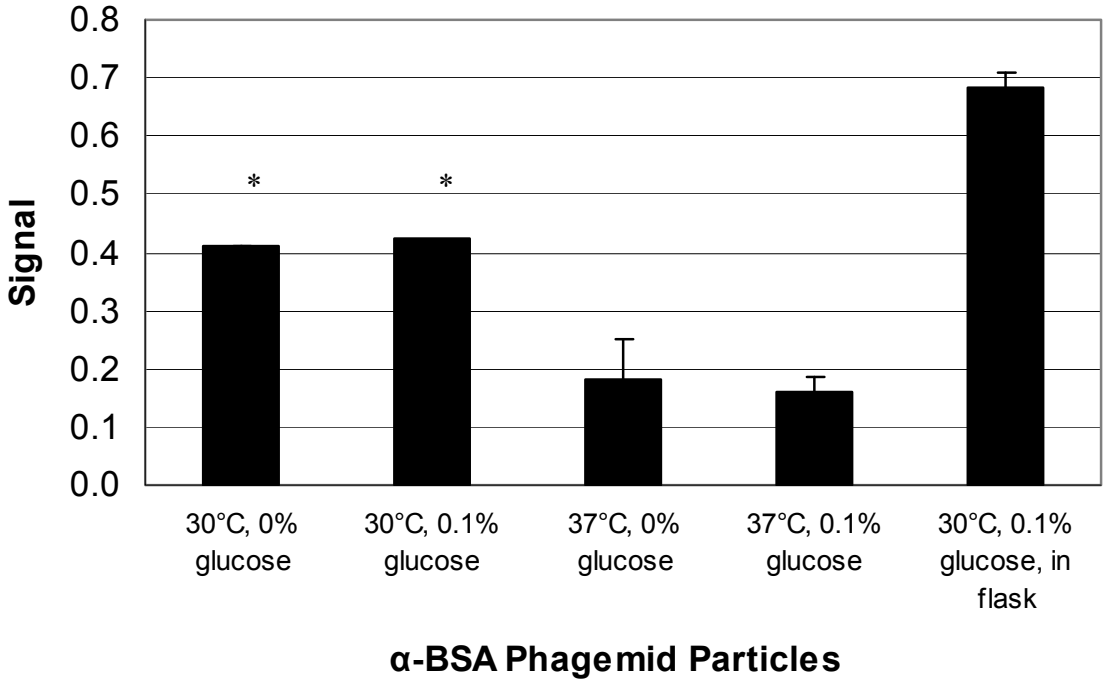


Figure 3-12. Analysis of α-BSA phagemid particles produced in microtiter wells by ELISA. Microtiter wells were coated with 10μg/mL BSA. Phagemid particles were diluted 1:2 in blocking buffer and incubated with the coated wells. Bound phagemid particles were detected with HRP-conjugated anti-M13 secondary antibody. Wells were developed with TMB substrate, and their absorbances were read at 630 nm (n=2 wells). Phagemid particles produced in a microtiter well at 30°C produced significantly higher (*p=0.03) S:N values than phagemid particles produced in a microtiter well at 37°C at the same glucose concentration.

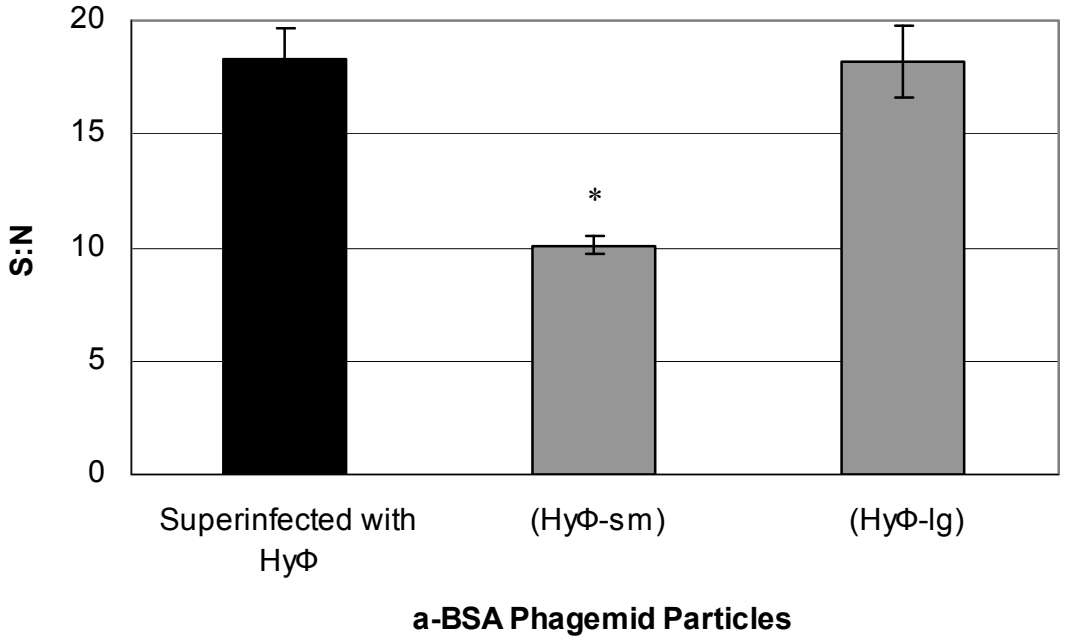


Figure 3-13. Analysis of anti-BSA (α-BSA) phagemid particles produced by hyperphage by ELISA. Microtiter wells were coated with 10 μg/mL BSA or PBS and incubated with 1 x 10^8 tu/mL α-BSA phagemid particles produced from *E. coli* TG1 strains already infected with hyperphage (HyΦ-sm, HyΦ-lg) or from *E. coli* TG1 that was superinfected with hyperphage. Bound phagemid particles were detected with HRP-conjugated anti-M13 secondary antibody. Wells were developed with TMB substrate, and their absorbances were read at 630 nm (n=3 wells). The S:N from α-BSA phagemid particles produced from (HyΦ-sm) was significantly lower (*$p<0.01$) than the S:N from α-BSA phagemid particles produced from (HyΦ-lg) or from superinfection with hyperphage.

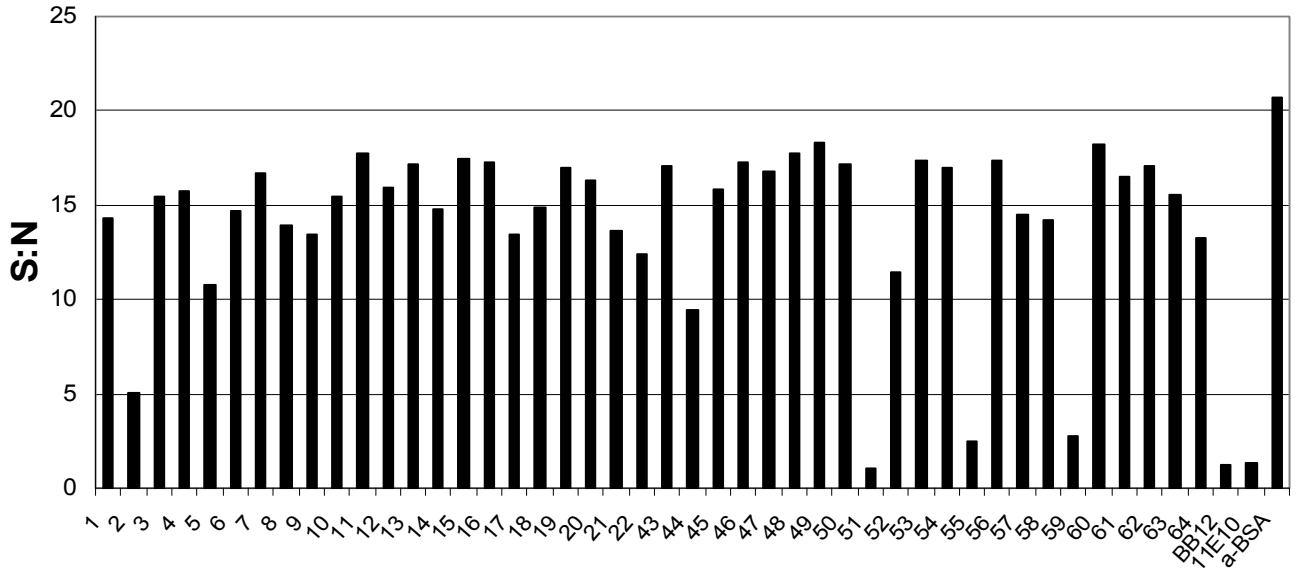


Figure 3-14. Analysis of phagemid particles (1-22, 43-64) selected from panning against Stx2 toxin preparation by ELISA. Microtiter wells were coated with 10 µg/mL of Stx2 toxin preparation (Toxin Technologies) and incubated with clones 1 through 22 and 43 through 64 diluted 1:2 in blocking buffer, 1 µg/mL anti-Stx2 subunit A monoclonal antibody (11E10), 1 µg/mL anti-Stx2 subunit B monoclonal antibody (BB12), or anti-BSA phagemid particles diluted 1:2 in blocking buffer. Microtiter wells coated with 10 µg/mL BSA were incubated with anti-BSA phagemid particles that were diluted 1:2 in blocking buffer. Bound phagemid particles were detected with HRP-conjugated anti-M13 monoclonal antibody. Bound monoclonal antibodies were detected with HRP-conjugated goat affinity purified anti-mouse antibody. Wells were developed with TMB substrate, and their absorbances were read at 630 nm (n=2 wells). The noise used to calculate S:N was the signal of anti-BSA phagemid particles that were incubated with Stx2-coated wells.

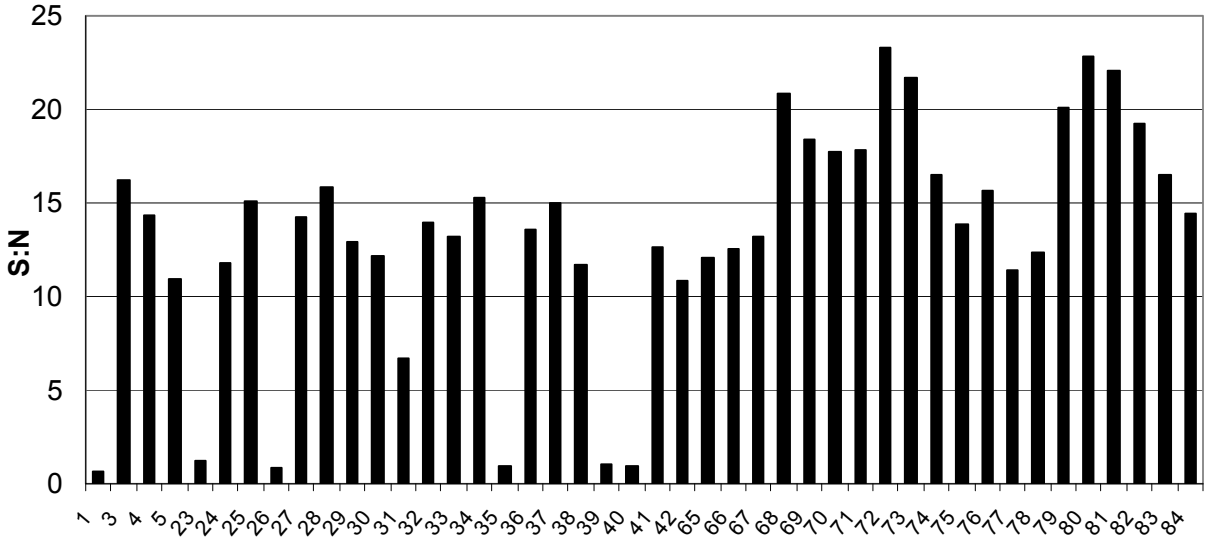


Figure 3-15. Analysis of phagemid particles (1, 3-5, 23-42, 65-84) selected from panning against Stx2 toxin preparation by ELISA. Microtiter wells were coated with 10 µg/mL of Stx2 toxin or carbonate coating buffer and incubated with clones 1, 3 through 5, 23 through 42, and 65 through 84 diluted 1:2 in blocking buffer. Bound phagemid particles were detected with HRP-conjugated anti-M13 monoclonal antibody. Wells were developed with TMB substrate, and their absorbances were read at 630 nm (n=2 wells).

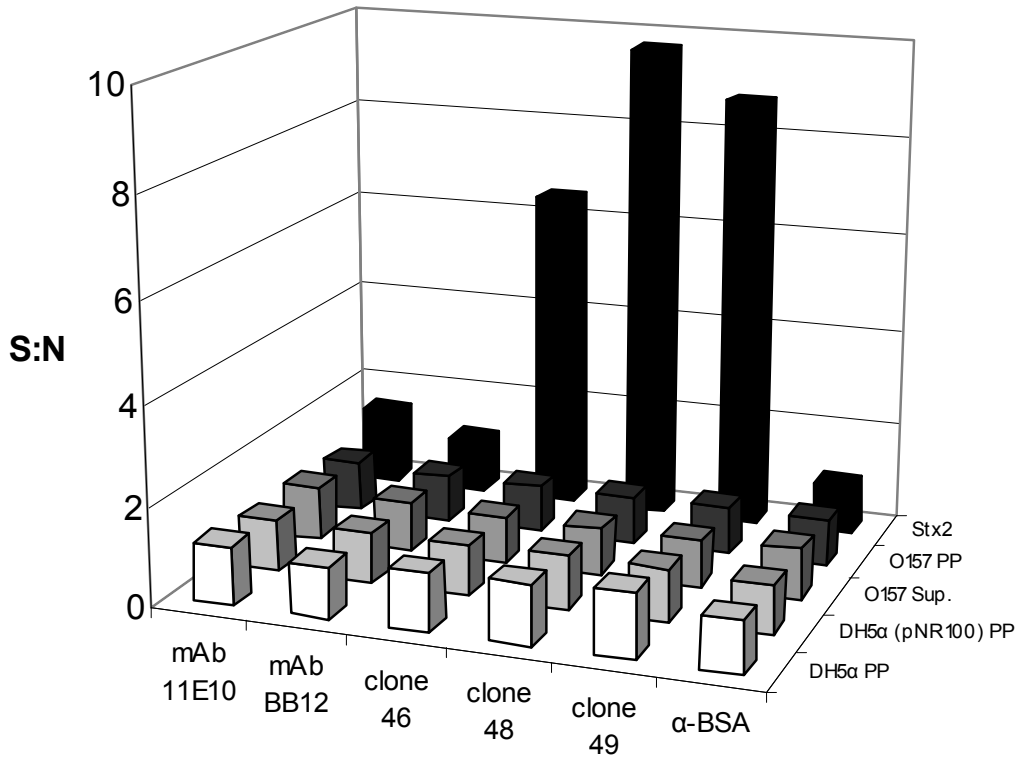


Figure 3-16. Analysis of anti-Stx2 monoclonal antibodies and clones 46, 48, and 49 by ELISA. Microtiter wells were coated with 10 µg/mL of Stx2 toxin, 70 µg/mL of E. coli O157:H7 933 periplasmic break fraction, 2 mg/mL of E. coli O157:H7 933 supernatant (O157 Sup.), 1mg/mL of *E. coli* DH5α (pNR100) periplasmic break fraction, and 1mg/mL of *E. coli* DH5α periplasmic break fraction. (PP) represents periplasmic break fractions. Bound phagemid particles were detected with HRP-conjugated anti-M13 monoclonal antibody. Bound monoclonal antibodies were detected with HRP-conjugated goat affinity purified anti-mouse antibody. Wells were developed with TMB substrate, and their absorbances were read at 630 nm (n=2 wells).

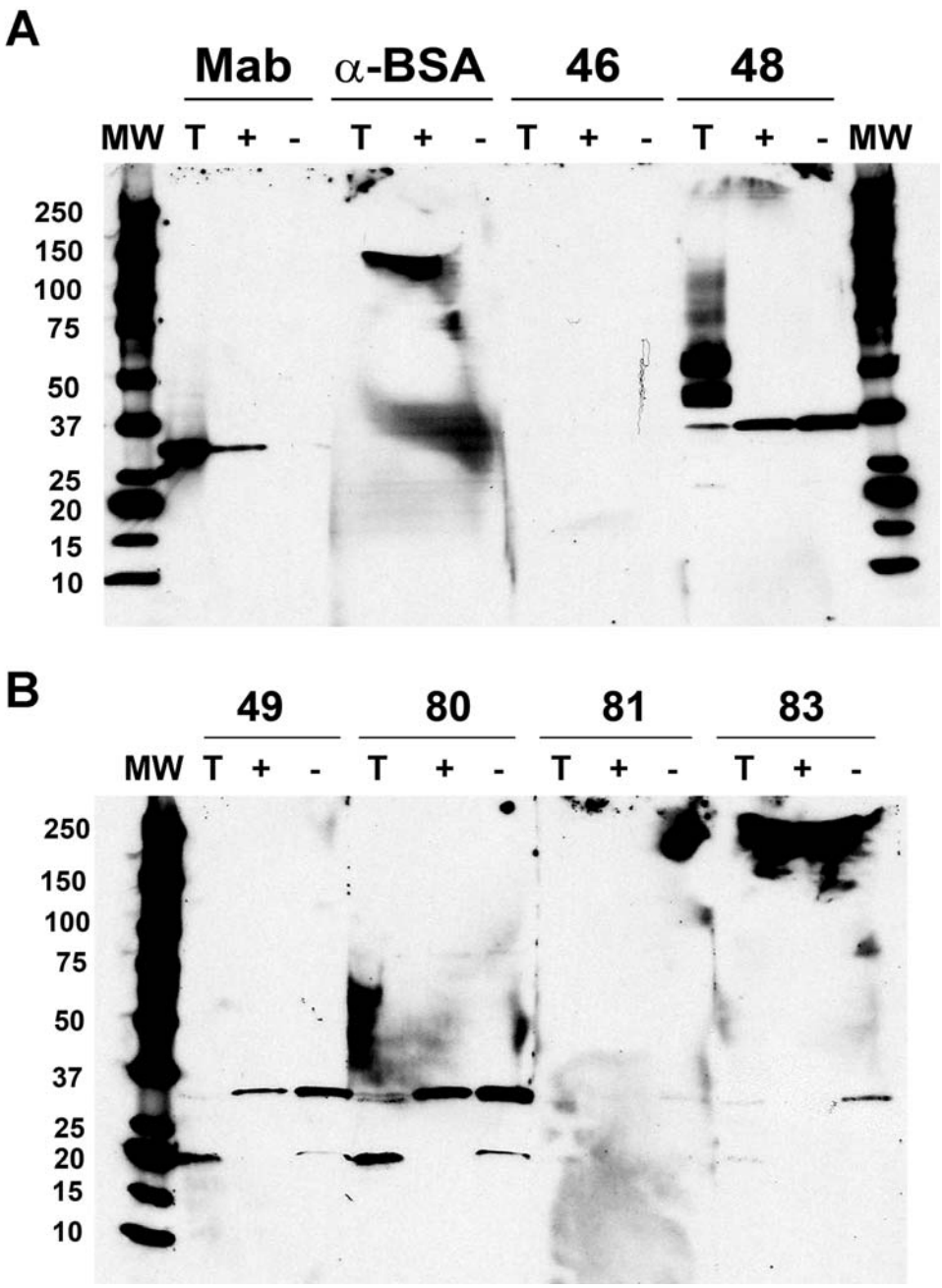


Figure 3-17. Western blot analysis of phagemid clones from panning on Stx2 toxin preparation. Samples were resolved by SDS-PAGE on a 4-20% (w/v) polyacrylamide gel and analyzed by Western blot. Samples: (T) -1.5μg/well of Stx2 toxin, (+) - 120 μg/well *E. coli* O157:H7 933 (contains Stx1 and 2 toxins) supernatant, and (-) -120 μg/well *E. coli* O157:H7 87-23 (does not produce Stx toxin) supernatant. Primary antibodies: Anti-Stx2 toxin subunit A monoclonal antibody (Mab); Stx2 phagemid clones 46, 48, 80, 81, 83. A) Western blot was developed with TMB and exposed for 30 seconds. B) Western blot was developed with TMB and exposed for 5 minutes.

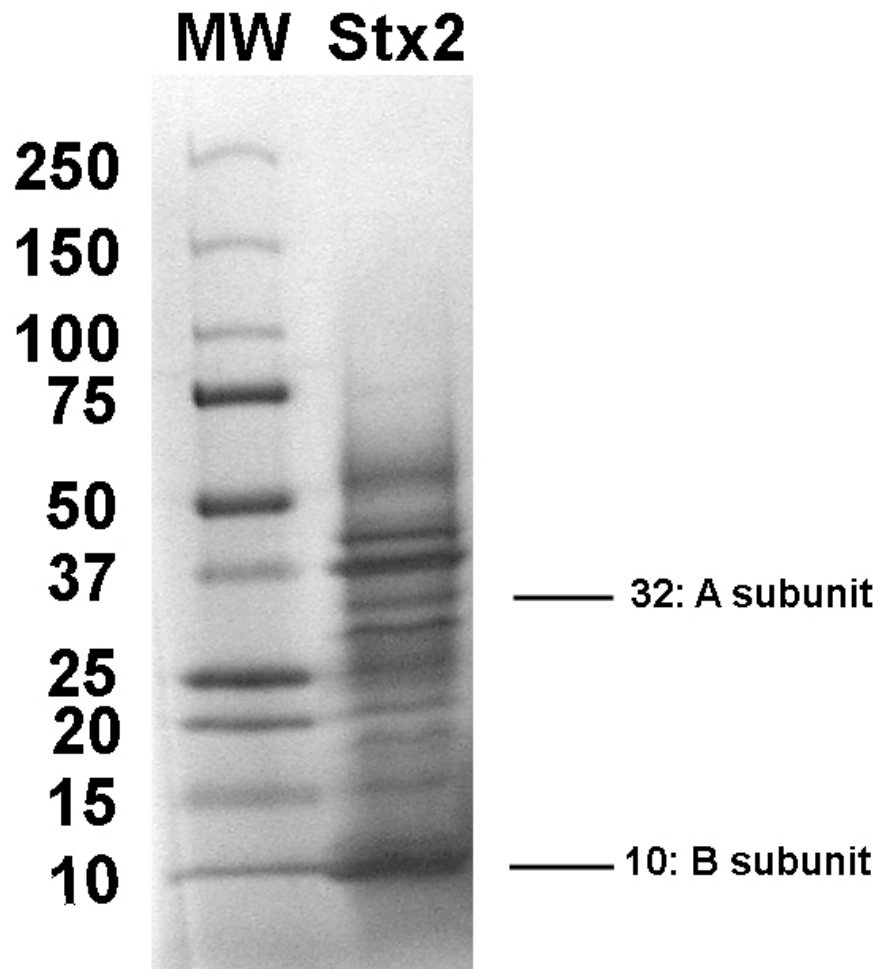


Figure 3-18. Analysis of Stx2 toxin preparation (Toxin Technologies) by SDS-PAGE. Five micrograms of Stx2 toxin preparation was resolved on a 4-20% (w/v) polyacrylamide gel and stained with Coomassie blue stain. Stx2 toxin is comprised of a 32-kDa A subunit and five 10-kDa B subunits.

CHAPTER 4
DISCUSSION

Detection of biological agents and their products has become a prime importance in the United States (85) since the anthrax attacks of 2001 (86). Early detection of biological agents is important to prevent or inhibit the spread of disease. Detecting biological agents in water, air, and food enables the proper course of action to be implemented to lessen or prevent the effects of the organism. Detection of biological agents or their products is also important for diagnosis of a disease. Detection of the disease-causing agent in patient samples enables the proper treatment to given.

There are vast numbers of detection assays, and a common component to most of them is a protein that binds specifically to a target antigen. Monoclonal antibodies are the default tool that scientists use today to detect target molecules; however, monoclonal antibodies have some drawbacks that are causing the use of phage display reagents to become more prevalent and take the place of monoclonal antibodies (2). First, the generation of monoclonal antibodies requires killing animals and takes months. Also, monoclonal antibodies are rarely isolated to pathogenic or self antigens, causing limitations in the scope of the target antigen for the generation of monoclonal antibodies. Phage display is advantageous because it lacks many of the drawbacks of monoclonal antibodies. In phage display, if using a non-immunized library, the killing of animals is not required and the isolation of specific recombinant phages can be done in a couple weeks instead of months. Also, the scope of antigen used for phage display is far greater than that used for monoclonal antibodies because phage display enables the selection of recombinant proteins to pathogenic and self antigens. Phage display was developed in 1985 by George P. Smith as a means to display foreign proteins on a filamentous phage (52). The sequence for the foreign peptide is encoded in frame with a coat protein gene of the phage genome. This enables

the foreign peptide to be fused to the coat protein, which effectively links the phenotype and genotype of the phage. Therefore, every phage that displays a foreign protein also encodes its peptide sequence in the genome.

This thesis describes efforts to optimize phage display methods to increase the chances of isolating recombinant antibodies or peptides for the potential use in detection assays.

Specific Aim 1: Panning to *V. cholerae* LPS

Vibrio cholerae is a human pathogen that is the causative agent of cholera. Cholera is a diarrheal disease that affects millions of people worldwide annually (9). Detecting *V. cholerae* in the environment and patient samples is the first step to preventing and treating the disease. To detect *V. cholerae* we need to isolate a recombinant phage that binds specifically and sensitively to *V. cholerae*. There are over 200 serogroups of *V.* cholerae that are classified on the basis of the O-antigen of their LPS; however, only serogroups O1 and O139 cause endemic acute diarrhea. Therefore, the LPS of these two serogroups would be excellent targets for detection.

Vibrio cholerae O1 LPS was chosen as a target for biopanning. Because *V. cholerae* O1 LPS was no longer sold commercially, it had to be extracted in the laboratory before any panning experiments could be performed. The previous method to extract LPS in the laboratory was a phenol-water extraction (76). However, a new LPS extraction was developed using a commercial RNA isolation reagent, TRIzol Reagent, that could extract LPS in a fraction of the time of the phenol-water extraction (77). The TRIzol method was even claimed to be able to extract LPS with less degradation and contamination than the phenol-water method. To determine which method was better, both methods were used to extract *V. cholerae* O1 LPS and the extracted LPS was analyzed and compared to commercially acquired *V. cholerae* O1 LPS (Sigma-Aldrich) that had been phenol extracted and purified by gel-filtration chromatography.

The criteria for the optimal LPS extraction were to have little or no protein and lipid contamination and to have similar banding patterns to the commercial *V. cholerae* O1 LPS.

Vibrio cholerae O1 LPS was extracted using the phenol-water and TRIzol methods. The LPS was resolved by SDS-PAGE and analyzed by Coomassie blue staining and Tsai-Frasch silver staining (Figs. 3-1 and 3-2). *Vibrio cholerae* O1 LPS that was extracted by the TRIzol reagent had less protein contamination than did the LPS that was extracted by the phenol-water method. The relative purity of the TRIzol-extracted LPS was less than 0.3 μg of protein/μg of LPS, while the relative purity of the phenol-water-extracted LPS was approximately 1 μg of protein/μg of LPS. However, the protein contamination in the phenol-water LPS extraction was most likely due to the RNase that was used in the extraction. The Coomassie blue stain for the phenol-water-extracted LPS showed a protein band at approximately 15 kDa. The molecular weight of RNase is 13.7 kDa. The phenol-water LPS extraction contained 0.04 mg/mL of RNase, which correlates to the band intensity of the 15-kDa protein band. Because the phenol-water LPS extraction only contained approximately 0.02 mg/mL of DNase, MW of 31kDa, the DNase might not have been concentrated enough to be seen by Coomassie blue stain. Therefore, both LPS extractions probably had less than 0.3 μg of bacterial protein contamination for every μg of LPS extracted. The TRIzol extracted LPS showed non-staining bands between 20 to 30 kDa on the silver stain (Fig. 3-1B). The Tsai-Frasch silver stain primarily stains carbohydrates and poorly stains proteins and lipids (78). Because the Coomassie blue stain for the TRIzol-extracted LPS showed no detectable proteins and due to the thickness of the non-staining bands, which correlates to concentration, the non-staining bands are most likely due to lipid contamination. Previously published silver stains of *V. cholerae* O1 LPS had bands at ~10 and 14 kDa which were stated to be the lipid A-core of LPS, and ~20-50 kDa which were stated to be

the lipid A-core plus repeating O-antigens (80,81). The non-staining bands were not the lipid A because the lipid A contains two glucosamines that are stainable by silver stain. Therefore, the TRIzol-extraction appears to have also extracted cellular lipids. Therefore, the TRIzol-extracted LPS appeared to have more lipid contamination than the phenol-water-extracted LPS.

When the resolved LPS extractions were compared by silver stain to the commercial LPS, the phenol-water-extracted LPS most closely resembled the commercial LPS. In the silver stain for TRIzol-extracted LPS and commercial LPS, the TRIzol-extracted LPS had bands at 14 and 20 kDa with non-staining bands between 20 and 30 kDa, the commercial LPS had bands at 14, 20, and 23 kDa. In the silver stain for phenol-water-extracted LPS and commercial LPS, both LPS preparations had bands at 13, 20, and 23 kDa. The phenol-water-extracted LPS had additional banding from 23-50 kDa, while the commercial LPS had more of a smear from 23-50 kDa. Previously published silver stains of *V. cholerae* O1 LPS have O-antigen banding/smears from 20-50 kDa (80,81). *Vibrio cholerae* O1 LPS has 12 to 18 O-antigen groups (83); therefore, the additional banding in the phenol-water-extracted LPS may be due to the ability of the phenol-water method to extract LPS with more O-antigens than that of the method used for the extraction of the commercial LPS which was phenol extracted and purified by gel-filtration chromatography. Because the phenol-water-extracted LPS most closely resembled the commercial LPS and appeared to have less lipid contamination than the TRIzol-extracted LPS, the phenol-water-extracted LPS was used for later experiments.

Before *V. cholerae* O1 LPS was panned, a solid support capable of binding a high quantity of *V. cholerae* O1 LPS was analyzed. In the past the Ph.D. 12mer phage library was panned against antigens that were immobilized onto a microtiter well. Previous experiments from this laboratory suggested that nitrocellulose paper could bind more LPS than a microtiter well. It was

hypothesized that having more LPS present to bind phages would enable more phages that specifically bound to the LPS to be selected. However, increasing the amount of LPS present may also increase the amount of nonspecific phages selected as well as the amount of specific phages selected. Therefore, more antigen present to pan against may not necessarily improve the chances of selecting phages specific to the antigen.

To determine which solid support could bind more LPS, the saturation limit of LPS was analyzed on a microtiter well and a piece of nitrocellulose paper with the same surface area as a microtiter well. The microtiter well became saturated with LPS when coated with 0.1-1 μg LPS, and the nitrocellulose paper became saturated with LPS when coated with ~250 μg LPS. Therefore, nitrocellulose paper could bind approximately 250-2,500 times more LPS than a microtiter well of equal area. Nitrocellulose paper has previously been used as a solid support for proteins in panning procedures involving glutathione S-transferase and hen egg white lysozyme (87). Nitrocellulose paper was chosen as a binding support because it had a high capacity to bind proteins (~100 $\mu g/cm^2$).

To determine if panning against more antigen increases the selection of phages specific for the antigen, both microtiter wells and nitrocellulose paper were used as solid supports for panning against *V. cholerae* O1 LPS. *Vibrio cholerae* O1 LPS (Sigma-Aldrich) was immobilized onto a polystyrene Maxisorp microtiter well and panned with the NEB Ph.D. 12mer phage display library. Five rounds of panning were performed with amplification of phages after the first four rounds of panning. Panning promotes the selection of phages that specifically bind to the target antigen over phages that are not specific to the antigen. Amplifying pools of eluted phages between pannings enriches the amount of specific phages. Therefore, each additional round of panning should increase the amount of specific phages in the pool of eluted phages.

One hundred phages from the fifth round of panning were individually amplified and analyzed in an ELISA against *V. cholerae* O1 LPS, carbonate coating buffer, and PBS.

Anti-Vc O1 LPS mAb generated a S:N of 15.5, proving that the LPS successfully bound to the microtiter plate. Anti-BSA phagemid particles were used in the ELISA against BSA (10μg/mL) and generated a S:N of 22, proving that the ELISA successfully detected phages. All 100 clones gave S:N <1.4, whereas a positive signal was classified as a S:N ≥2. Therefore, all of the clones screened by ELISA were negative for binding to *V. cholerae* 569B LPS.

Because panning the Ph.D. 12mer library against *V. cholerae* O1 LPS that was immobilized onto a microtiter well failed to yield phages that were positive to *V. cholerae* O1 LPS, the panning procedure was altered in an attempt to improve the chances of selecting phages that specifically bind to the antigen. The first alteration was to change the antigen binding support from a microtiter well to nitrocellulose. It was determined that nitrocellulose paper could bind more LPS than a microtiter well; therefore, with more LPS present to pan against more specific phages may be selected. Also, the mechanism of phage elution was analyzed. Previous panning procedures with the Ph.D. library used a glycine (pH 2.2) elution. There are numerous elution methods, pH, salt, pressure, temperature, antigen, none of which are optimal for every antigen-antibody complex. The key is to find a method that effectively dissociates the antigen-antibody complex without causing harm to either the antibody or the antigen (88). An acidic elution interferes with the electrostatic and hydrophobic interactions of the antigen-antibody complex and causes them to dissociate. Acidic elutions do not work for every antigen-antibody complex; sometimes the low pH of the elutant can denature the antibodies or antigens. Elution with antigen elutes the antibody from the immobilized antigen-antibody complex by competing with the immobilized antigen for binding with the antibody. While pH elutions are

the most common elutions used in phage display, elutions by antigen are not uncommon and have even been noted to be more successful than pH elutions because they decrease the elution of phages that are not specific to the antigen (89-91).

Panning the Ph.D. 12mer library against phenol-water-extracted *V. cholerae* O1 LPS that was immobilized onto nitrocellulose paper and eluted with either glycine (pH 2.2) or *V. cholerae* O1 LPS was performed. Three rounds of panning were performed with amplification of phages after rounds one and two. A subtractive panning was performed after round one. This "negative" panning was performed to decrease the amount of nonspecific phages in the library. The negative panning involved panning the library against blocked nitrocellulose paper; therefore, clones specific to the blocker, nitrocellulose paper, or polystyrene should be removed from the library. Two hundred clones from each panning were individually amplified and analyzed by ELISA. The 400 phage clones and α-Vc O1 LPS mAb were screened in an ELISA against *V. cholerae* 569B LPS. Anti-Vc O1 LPS mAb generated a S:N of 10, proving that the LPS successfully bound to the microtiter plate. Anti-BSA phagemid particles were used in the ELISA against BSA (10μg/mL) and generated a S:N of 15, proving that the ELISA was a successful assay for the detection of phages. All 400 clones gave a S:N less than 1.7; therefore, all of the clones screened were negative for binding to *V. cholerae* O1 LPS.

Previous attempts to select recombinant phages to LPS have failed to generate highly specific recombinant phages that generally lack a consensus sequence (92-94). Also, the isolation of recombinant phages that bind to LPS is less common than for the isolation of recombinant phages that bind to protein. This is most likely because protein-protein interactions are usually stronger than protein-carbohydrate interactions. The antibody-antigen complex is held together by noncovalent interactions such as hydrogen bonds, van der Waals forces,

coulombic interactions, and hydrophobic bonds (95). The strength of the antibody-antigen complex depends on the strength of the bonds used to hold it together. Protein-protein interactions make use of all noncovalent bonds, but carbohydrate-protein interactions primarily make use of hydrogen bonds and rarely make use of hydrophobic or coulombic interactions. Also, protein-protein interactions can be held together by numerous bonds due to the conformational structure of the protein-protein interaction. The carbohydrate-protein interaction is usually only bound by a single domain due to the linearity of the carbohydrate structure. Therefore, the low density and the limited variety of bonds that occur in carbohydrate-protein interactions make LPS a difficult target to select recombinant proteins that bind to it with high affinity.

Specific Aim 2: Improve Panning and Screening Process of Biopanning

Biopanning is performed to select phagemid particles that specifically bind to a target. To increase the chances of selecting phagemid particles that are specific to a target, the optimization of the biopanning and screening process was performed. Optimization of the panning process was performed with *V. cholerae* O1 whole cells as a target, and the concentration of whole cells and the trypsin elution time were analyzed. The concentration of whole cells used in biopanning usually ranges from 10^7 to 10^9 whole cells (96). The goal was to use enough whole cells to enable capture of specific recombinant phagemid particles and keep the capture of phagemid particles not specific to the whole cells to a minimum. The trypsin elution time was also analyzed. The Tomlinson libraries have a trypsin cleavage site between the pIII protein and the scFv particle. Therefore, trypsin is used to cleave the phagemid particle from the scFv peptide that is bound to the target. The Tomlinson protocol recommends a trypsin elution of 10 minutes. The trypsin elution time was analyzed to maximize the number of eluted phagemid particles that were specific to the target. Eluting for too short of a time may fail to elute specific phagemid

particles, but eluting for too long of a time may only increase the elution of nonspecific phagemid particles or even degrade the phagemid particles.

Comparative pannings were performed to determine the optimal concentration of *V. cholerae* whole cells and optimal trypsin elution time. Parallel pannings were performed with the Tomlinson J scFv library with and without 1% Vc86 phagemid particles spiked into the library. Vc86 is a clone isolated from the Tomlinson I scFv library that recognizes an unknown antigen on *V. cholerae* whole cells. Because ~10^{11} phagemids are being used in the pannings there only exists a 100-fold redundancy of clones in the non-spiked library. Therefore, enumeration of eluted phagemid particles after one round of panning may not yield significant differences in eluted titers under various test parameters. Spiking the library with 1% phagemid particles that are specific to *V. cholerae* whole cells should enable distinction of an optimal panning parameter that selects and elutes the highest number of specific recombinant phagemid particles. We were looking for a combination of the concentration of whole cells and trypsin elution time to elute more phagemid particles from the Vc86-spiked library than the non-spiked library. Because the only difference between the pannings was that the Vc86-spiked library had a higher initial concentration of specific phagemid particles to *V. cholerae* whole cells, an increase in eluted phagemid particles with a certain parameter should correlate to an increase in specific phagemid particles being eluted.

The first one-round panning used 1 x 10^8 or 1 x 10^9 whole cells of *V. cholerae* with trypsin elutions of 15, 30, 45, and 60 minutes (Fig. 3-6). Elution of phagemid particles with trypsin was optimal between 15 and 30 minutes. The phagemid particle titer decreased by 80 to 93% from 30 to 45 minutes for the four pannings. The decrease in the titer of phagemid particles was most likely due to degradation of the phagemid particles by trypsin. Therefore, trypsin elution should

not exceed 30 minutes. More phagemid particles were eluted when panned against 1 x 10^9 whole cells for both the spiked and nonspiked libraries. However, there were not enough data to do statistical analysis to determine if using 1 x 10^9 whole cells eluted significantly more phagemid particles than using 1 x 10^8 whole cells in the biopanning process.

The best whole cell concentration and trypsin elution time were not definitively obtained from the first one-round panning; therefore, a second one-round panning was performed to further analyze the trypsin elution time and whole cell concentration. The second one-round panning used 10^7, 10^8, or 10^9 whole cells of *V. cholerae* with a trypsin elution of 10, 20, and 30 minutes (Fig. 3-7). There were no significant differences in the titers of eluted phagemid particles when comparing the concentrations of whole cells and trypsin elution times used in the second one-round panning experiment for both library pannings. This could mean that there may not be a significant advantage in using the different elution times or concentrations of whole cells for selecting specific recombinant phages.

Because using titering to analyze the differences in the amount of eluted phagemid particles under various parameters failed to definitively determine a panning condition that eluted the highest number of specific recombinant phagemid particles, another one-round panning was performed with analysis of phagemid particle specificity by ELISA. Following elution of the phagemid particles, the phagemid particles were amplified in batch and analyzed by ELISA to determine which panning parameter eluted the most phagemid particles that were specific to *V. cholerae* whole cells.

The third one-round panning used 10^8 or 10^9 whole cells of *V. cholerae* with trypsin elutions of 10 and 30 minutes (Fig. 3-8). The eluted phagemid particles were amplified in batch and analyzed by ELISA. Of the different panning variations, the amplified phagemid particles

from the panning with the library that contained 1% Vc86 phagemid particles, 10^8 whole cells *V. cholerae,* and an elution time of 30 minutes generated a significantly higher S:N (p=0.01-0.04) in an ELISA compared to the amplified phagemid particles from the other panning variations. This suggests that panning with 10^8 whole cells with a trypsin elution time of 30 minutes is optimal for selecting phagemid particles that are specific to the panned whole cells compared to using 10^9 whole cells or a trypsin elution of 10 minutes. There might have been an excess of whole cells when panning against 10^9 whole cells. This excess may have promoted the binding of nonspecific phages; therefore, fewer whole cells present may have been more efficient for panning because with fewer whole cells present the specific phagemid particles should preferentially bind the whole cells and the number of nonspecific phagemid particles would be lessened. Eluting for 10 minutes may not have been long enough for the trypsin to cleave all the specific phagemid particles from the whole cells; therefore, eluting for 30 minutes was optimal because it may have given the trypsin enough time to elute the phagemid particles from the whole cells.

After the biopanning process is complete, the selected scFvs or phages are screened for their specificity to the target molecule. The previous method to screen scFv proteins was to grow amber suppressor-free phagemid-containing bacteria under conditions to secrete scFv proteins in culture tubes or flasks. However, this process was very time consuming; therefore, a way to screen scFv proteins in a high throughput manor was investigated. High throughput screening of scFv proteins is very common. A Biacore A 100 array system is capable of screening hundreds of scFv proteins per day (97). This system screens scFv proteins from crude bacterial extracts on a sensor chip surface. The antibodies that are captured onto the chip are analyzed and ranked by the percentage of bound scFv proteins remaining on the chip after

dissociation of nonspecific scFv proteins in buffer. His-tagged scFvs can be screened in an automated high throughput approach by measure of surface plasmon resonance using a Qiagen BioRobot 3000 LS (98). A chromatography press is capable of purifying and screening hundreds of scFv proteins per day (99). This system involves His-tagged scFvs being purified by a cation exchange column and immobilized metal ion affinity chromatography. The proteins were then quantified by a bicinchoninic acid (BCA) assay and analyzed by SDS-PAGE. While there are many high throughput screens for scFv proteins, most of the methods involve expensive equipment that is not readily available to our laboratory. Therefore, our goal was to develop a high throughput screen for scFv proteins.

A colony blot was attempted to screen scFv proteins. The colony blot involved the transfer of bacterial colonies that were grown to secrete scFv proteins onto an antigen-coated piece of nitrocellulose paper. The scFv proteins that bound to the nitrocellulose via the antigen were then detected with Protein-L peroxidase and identified with ECL. However, when this system was attempted, the colony blot was determined to be neither specific nor selective. When the α-BSA clone was screened by this method, it did not generate a positive signal on BSA-coated nitrocellulose but did generate a positive signal on PBS and *V. cholerae*-coated nitrocellulose paper. Also, when clones selected in a panning against *V. cholerae* whole cells were screened by this method, they generated positive signals to BSA, PBS, and *V. cholerae*-coated nitrocellulose papers. It appeared as if random clones bound to the nitrocellulose paper despite it being blocked with casein buffer and that all clones that produced scFvs were detected. For this problem to occur many steps or just one step could have gone wrong in the experiment. It is possible that not enough antigen was used to coat the nitrocellulose, the nitrocellulose did not capture the antigen, the nitrocellulose was poorly blocked, or the washes were not stringent

enough. The effort required to troubleshoot the colony blot was determined to not be worth the time. Therefore, a way to improve the old method for producing scFvs was developed.

To enable high throughput production of scFv proteins, a clone was grown in a 96-well plate instead of a culture tube or flask, and the scFv-containing supernatant was examined in an ELISA. When α-BSA clone was grown in a 96-well plate, its scFv-containing supernatant diluted 1:2 yielded a S:N of 11.5 against BSA in an ELISA. Anti-BSA scFv produced in a flask yielded a S:N between 10 and 15; therefore, producing scFv proteins in a microtiter well may not be optimal, but it is acceptable for the purpose of a high throughput screen. Next was to determine how far the microtiter well-produced scFvs could be diluted and still generate a sufficient signal to be detected in an ELISA. This test was to determine how many clones could be mixed together (i.e., diluted) and screened in a single microtiter well to enable even greater high throughput screening of scFvs. The α-BSA clone was grown in a microtiter well to produce scFvs. The supernatants were diluted 1:2, 1:4, 1:6, and 1:10 in casein blocking buffer and analyzed by ELISA (Fig. 3-10).

When α-BSA scFv-containing supernatant was diluted 1:10 it still yielded a S:N of 5.6 in an ELISA. This S:N is high enough for detection of positive clones. However, the α-BSA clone was a very strong clone; therefore, the majority of clones selected will have a weaker signal than the α-BSA clone. To lessen the chance of not detecting scFvs with weak affinity or low titers, it was decided to only dilute scFv clones 1:4 to 1:6 for high throughput screening by ELISA. This would still enable screening of hundreds of clones per day, a vast improvement to the previous method of screening clones in culture tubes or flasks which only enabled screening of a couple dozen clones per day.

Because the high throughput screen for scFv proteins was successful, it was examined if a similar high throughout ELISA could be applied to the screening of phagemid particles. Previous methods of screening phages involved producing phagemid particles in a culture tube or flask and screening the supernatants by ELISA. This method was time consuming; therefore, a high throughput screen was investigated. Previous high throughput ELISAs have been developed for the screening of phages; however, while their screening is rapid, the production of the phages is time consuming. These high throughput ELISAs require overnight production of phages in a culture tube or flask followed by PEG precipitation of the phages for the concentrated phages to be screened by ELISA (100,101). We hoped to develop an even more high throughput ELISA by not only screening phagemid particles in a microtiter well but also growing the phagemid particles in a microtiter well.

Phagemid particles from the α-BSA and Vc86 clones were transduced into *E. coli* TG1 (HyΦ-sm), which harbors the hyperphage genome as a plasmid. Producing phagemid particles in *E. coli* TG1 (HyΦ-sm) removes the additional step of superinfection with hyperphage; therefore, reducing the time required to produce phagemid particles. Colonies from the transduction were picked and grown in a microtiter well to produce phagemid particles. Growth temperature and glucose concentration of the medium were analyzed. The Tomlinson protocol recommends producing phagemid particles in medium that has 0.1% (w/v) glucose. However, this concentration of glucose could promote catabolite repression of the *lac* promoter, which promotes transcription of the *gIII* gene. Therefore, it was examined if phagemid particles would be produced better in medium with or without 0.1% (w/v) glucose.

The α-BSA and Vc86 phagemid particles were produced in a microtiter well at 30°C or 37°C with and without 0.1% (w/v) glucose. The phage-containing supernatants were diluted 1:2

in blocking buffer and analyzed by ELISA (Fig. 3-11). There was not a significant difference in the S:N ($p=0.07$-0.48) of phagemid particles produced in microtiter wells with and without 0.1% (w/v) glucose. Therefore, the 0.1% (w/v) glucose in the medium was probably not enough glucose to cause catabolite repression of the *lac* promoter. Vc86 phagemid particles produced in microtiter wells with or without glucose at 30°C and 37°C did not produce a S:N greater than 1, whereas Vc86 phagemid particles produced in a flask (S:N ~4) yielded a significantly higher S:N ($p=0.05$). Observation of the turbidities of the bacterial cultures that were grown in a microtiter well revealed only slightly turbid cultures compared to bacterial cultures that were grown in a flask. The growth conditions may not have been optimal for the bacteria to grow when they had the added stress of producing phagemid particles. It was possible that the bacterial cultures that were grown to produce phagemid particles in a microtiter well experienced less aeration than bacterial cultures grown in a flask or culture tube. The decreased aeration could decrease bacterial growth and reduce phagemid particle production. *Escherichia coli* is a facultative anaerobe which means it makes ATP, which drives biosynthesis, by aerobic respiration or fermentation. Aerobic respiration generates 36 ATP molecules from one molecule of glucose, while fermentation only generates 2 ATP molecules from one molecule of glucose. Because biosynthesis is fueled by ATP, aerobic respiration is more effective for bacterial growth.

When α-BSA phagemid particles were produced at 30°C, the S:N was significantly higher ($p<0.04$) than when α-BSA phagemid particles were produced at 37°C at either glucose concentration in a microtiter well. It appeared that phagemid particle production was more permissive at 30°C opposed to 37°C. The α-BSA phagemid particles produced in a flask (S:N ~12) yielded a significantly higher ($p=0.02$) S:N compared to α-BSA phagemid particles produced in microtiter wells (S:N~7) when analyzed at a 1:2 dilution in an ELISA. The reduced

S:N of phagemid particles used in an ELISA could have been due to less phagemid particles present in the phagemid particle-containing supernatant that was produced in a microtiter well as opposed to the phagemid particle-containing supernatant that was produced in a flask. This could have been due to reduced aeration in the microtiter well causing decreased bacterial growth and thus reduced quantities of phagemid particles produced in a microtiter well.

Phagemid particle production in microtiter wells was repeated a second time with α-BSA phagemid particles (Fig. 3-12). Anti-BSA phagemid particles yielded significantly higher signals ($p<0.03$) when produced at 30°C as opposed to 37°C at either glucose concentration. These data support the previous data that 30°C is a more permissive temperature than 37°C for phagemid particle production. There was not a significant difference ($p=0.34$) in the signals produced by phagemid particles that were produced with or without glucose at 30°C. These data also support the previous data that 0.1% (w/v) glucose in the medium does not provide an advantage or hindrance to phagemid particle production. While the second experiment did not yield a significant difference ($p=0.09$) in signals produced by α-BSA phagemid particles produced in a flask opposed to in a microtiter well, the microtiter well-produced phagemid particles yielded lower signals than did phagemid particles produced in a flask.

Because phagemid particles that were produced from the same clone resulted in various ELISA signals when phage-containing supernatants were analyzed at a dilution of 1:2 it was possible that the reason for the differences in ELISA signals from phage-containing supernatants were due to differences in phagemid particle titers produced under the various conditions. Therefore, the titers of α-BSA and Vc86 phagemid particles produced in a microtiter well and in a flask were analyzed. Phagemid particles produced at 30°C in a microtiter well yielded approximately 10^7 tu/mL while phagemid particles produced at 37°C in a microtiter well yielded

approximately 10^6 tu/mL. Producing phagemid particles at 30°C in microtiter wells yielded approximately 10-fold higher tu/mL titers than phagemid particles that were produced in microtiter wells at 37°C. Whether the growth medium contained 0.1% (w/v) glucose or not did not affect the titers of phagemid particles. When α-BSA and Vc86 phagemid particles were produced in flasks at 30°C with 0.1% (w/v) glucose in the medium, the titers were approximately10^8 to10^9 tu/mL. Producing phagemid particles in a microtiter well yielded higher titers and signals in an ELISA when produced at 30°C. Whether glucose was in the media or not did not affect phagemid particle titers or ELISA signals. Phagemid particles could not be produced in a microtiter well in high enough titers to make a high throughput screen acceptable. When clone Vc86, a clone of average strength, was produced in a microtiter well, it was not able to generate a positive signal. Therefore, the high throughput ELISA screen for phagemid particles was not acceptable for screening of phagemid particles.

Specific Aim 3: Improve Phagemid Particle Production

Amplification of phagemid particles is an essential step in biopanning. A phagemid contains the *gIII* gene of M13 but lacks the rest of the M13 genes needed for phagemid particle production. Therefore, a phagemid requires an amplification tool to provide the rest of the M13 genes in trans to enable phagemid particle production. It is essential that there is high quality and high quantity phagemid particle production during amplification to ensure that the redundancy of clones in the library is maintained and that the library is in high enough titers to be useful in assays.

The most commonly used amplification tool for phagemids is helper phage. There are many variations of helper phages, R408, VCSM13, and M13KO7 (63) that differ slightly. Of the helper phages, M13KO7 is the most commonly used. Because helper phages contain a wild type *gIII* gene, they are not optimal for phagemid particle amplification because the produced

phagemid particles will express non-recombinant pIII proteins along with recombinant pIII proteins. To overcome the drawback of helper phages encoding a wild type *gIII* gene, many *gIII* gene mutated variations of helper phages were engineered. Many of these strains either have a deletion of the *gIII* gene or encode amber stop codons within the *gIII* gene to lessen the expression of wild type pIII proteins (65-67). Of the *gIII* gene mutated helper phages, hyperphage is the most effective for producing high titers of phagemid particles that have multivalent display of pIII-fusion proteins (66). Hyperphage was the previous amplification tool used by our laboratory to amplify phagemid particles; however, the titer of the hyperphage stock was too low to effectively amplify every phagemid particle in the library. Therefore, a way to improve phagemid particle production was investigated.

To overcome the problem of the low titer of the stock of hyperphage from effectively infecting every phagemid-containing bacterium, the hyperphage genome was transduced into *E. coli* to be maintained as a plasmid to ensure that every phagemid-containing bacterium also contained hyperphage to enable amplification of phagemid particles. When hyperphage was transduced into *E. coli* TG1, small (0.5 mm diameter) and large (1-1.5 mm diameter) colonies resulted. When the small and large colonies were passaged, the small colonies maintained a small phenotype (0.5 mm diameter) and the large colonies maintained a large phenotype (1-1.5 mm diameter). The two strains were named *E. coli* TG1 (HyΦ-sm) and *E. coli* (HyΦ-lg). The reason for the variation in the colony size was not known. Because colonies were grown under kanamycin resistance for hyperphage, both strains contained hyperphage, or at least they contained the kanamycin resistance gene that hyperphage carries. The differences in the colony sizes suggested that the bacteria that formed the colonies were not replicating at the same rate. The smaller colonies might be small because they are under stress from replicating the

hyperphage genome along with the *E. coli* genome, and the large colonies might be larger because they are capable of growing faster because they are under less stress because they may not be replicating everything. To determine if the two strains were functionally different, their infection efficiencies were compared to *E. coli* TG1 and their ability to produce phagemid particles was compared to *E. coli* TG1 containing a phagemid that was superinfected with hyperphage.

The infection efficiencies of α-BSA and Vc86 into *E. coli* TG1, *E. coli* TG1 (HyΦ-sm), and *E. coli* (HyΦ-lg) were analyzed. When the number of α-BSA phagemid particles transduced into *E. coli* TG1 was set at an infection efficiency of 100%, the number of α-BSA phagemid particles transduced into *E. coli* TG1 (HyΦ-sm) was 28% and the number of α-BSA phagemid particles transduced into *E. coli* TG1 (HyΦ-lg) was 0.1% of the number of transduced phagemid particles into *E. coli* TG1. When the number of Vc86 phagemid particles transduced into *E. coli* TG1 was set at an infection efficiency of 100%, the number of Vc86 phagemid particles transduced into *E. coli* TG1 (HyΦ-sm) was 22% and the number of Vc86 phagemid particles transduced into *E. coli* TG1 (HyΦ-lg) was 2% of the number of transduced phagemid particles into *E. coli* TG1. *Escherichia coli* TG1 (HyΦ-sm) had an infection efficiency that was approximately 25% of the infection efficiency of *E. coli* TG1, and *E. coli* TG1 (HyΦ-lg) had an infection efficiency that was approximately 1% of the infection efficiency of *E. coli* TG1. It appears as if *E. coli* TG1 has a reduced ability to be transduced with phagemid particles when it is harboring hyperphage. The differences in the infection efficiencies between *E. coli* TG1 (HyΦ-sm) and *E. coli* TG1 (HyΦ-lg) show that their functionalities are different. The reason for *E. coli* TG1 (HyΦ-lg) generating larger colonies may have been because it shed a function required in transduction and therefore was able to grow faster. For example, the production of

the F pilus could have been compromised by the stress from *E. coli* TG1 replicating the hyperphage genome.

Both hyperphage-containing strains of *E. coli* TG1 had infection efficiencies that were too low for the strains to be used to effectively amplify clones in a library. While the hyperphage-containing *E. coli* strains have infection efficiencies too low to be used to amplify the library their infection efficiencies are still acceptable for amplifying single clones because the transduction efficiency is not critical for the amplification of a single clone. Therefore, the quality and quantity of phagemid particles produced by *E. coli* TG1 (HyΦ-sm/lg) were analyzed.

Anti-BSA phagemid particles were produced by *E. coli* TG1 (HyΦ-sm), *E. coli* TG1 (HyΦ-lg), and *E. coli* TG1 that was superinfected with hyperphage. Anti-BSA phagemid particle yields were as follows: *E. coli* TG1 that was superinfected with hyperphage yielded 3 x 10^{8} tu/mL, *E. coli* TG1 (HyΦ-sm) yielded 1 x 10^{9} tu/mL, and *E. coli* TG1 (HyΦ-lg) yielded 3 x 10^{8} tu/mL. While the ability of hyperphage-containing *E. coli* TG1 to be transduced with phagemid particles was reduced compared to the ability of *E. coli* TG1, its ability to produce phagemid particles was not reduced. Phagemid particles produced by hyperphage-containing bacteria yielded titers high enough to be used as a phagemid particle amplification tool for single clones.

The qualities of α-BSA phagemid particles produced by the three *E. coli* strains were analyzed by ELISA (Fig. 3-13). Anti-BSA phagemid particles produced a significantly higher S:N ($p=0.01$) when produced from *E. coli* TG1 (HyΦ-sm) compared to *E. coli* TG1 (HyΦ-lg). There was not a significant difference in the S:N ($p=0.50$) from α-BSA phagemid particles produced from superinfected *E. coli* TG1 and *E. coli* TG1 (HyΦ-lg). Because the titer of phagemid particles produced from *E. coli* TG1 (HyΦ-sm), ~1 x 10^{9} tu/mL, was three times higher than the titer of phagemid particles produced from *E. coli* TG1 (HyΦ-lg) or *E. coli* TG1

that was superinfected with hyperphage, ~3 x 10^8 tu/mL, a third less phage-containing supernatant was used in the ELISA for phagemid particles that were produced from *E. coli* TG1 (HyΦ-sm). The decrease in the S:N from α-BSA phagemid particles produced from *E. coli* TG1 (HyΦ-sm) could have resulted if the particles/mL of the phagemid particles produced from all three strains were the same. Because hyperphage-containing *E. coli* TG1 was not going to be used to amplify the library, further analysis of it was stopped, so that the time used to analyze it could be put to better use finding a more effective phagemid particle amplification tool. Hyperphage-containing bacteria was not acceptable as an amplification tool for the library because of their low infection efficiencies, but *E. coli* TG1 (HyΦ-sm) was acceptable for use as an amplification tool for the amplification of single clones.

Helper plasmids are another amplification tool for the production of phagemid particles (64). They are M13-based plasmids that are maintained in *E. coli* to provide phagemids with all the genes necessary to produce phagemid particles. Helper plasmids were engineered in three forms that differ in the length of their *gIII* genes. The helper plasmids contain a full-length (M13cp), a deleted (M13cp-dg3), or a truncated (M13cp-CT) *gIII* gene. These three helper plasmids were transformed into *E. coli* and analyzed for their infection efficiency with phagemid particles, their ability to produce phagemid particles, and the quality of the phagemid particles produced.

When the helper plasmids were transformed into *E. coli* TG1, the resulting colony sizes varied from a diameter of 0.5 to 2 mm. The reasons for various colony sizes are hypothesized above. A small (0.5 mm) colony and a large (2 mm) colony from each transformation were passaged to determine if the colony size phenotype would be maintained. *Escherichia coli* TG1 (M13cp-sm) was the only strain that maintained a consistent phenotype of 0.5 mm colonies, the

rest resulted in colonies that had a diameter between 0.5 and 2 mm. A small and large of each strain were further analyzed to determine if the different colony phenotypes also had different functionality.

The six helper plasmid-containing *E. coli* TG1 strains were analyzed for their infection efficiencies with two phagemid particles in relation to the infection efficiencies of *E. coli* TG1 that did not contain any helper plasmids. *Escherichia coli* TG1 (M13cp-dg3-sm) and *E. coli* TG1 (M13cp-CT-sm) were the only strains that yielded infection efficiencies greater than 0.3% of the infection efficiency of *E. coli* TG1 (Table 3-1). *Escherichia coli* TG1 (M13cp-dg3-sm) had infection efficiencies of 175% and 97%, while *E. coli* TG1 (M13cp-CT-sm) had infection efficiencies of 7% and 13% compared to the infection efficiency of *E. coli* TG1. The differences in the infection efficiencies between the small and large strains of helper plasmid-containing *E. coli* revealed that they had different functionalities. Except for *E. coli* TG1 (M13cp-dg3-sm), all of the other helper plasmid strains had reduced infection efficiencies compared to *E. coli* TG1. Therefore, the cost for the *E. coli* cells maintaining the helper plasmids resulted in a decrease in their infection efficiencies. Because *E. coli* TG1 (M13cp-dg3-sm) and *E. coli* TG1 (M13cp-CT-sm) had higher infection efficiencies compared to their large phenotypes, it suggested that the large strains had a defect in some component of the cell involved in transduction.

Escherichia coli TG1 (M13cp-dg3-sm) had the highest infection efficiencies of the helper plasmid strains; therefore, it was further tested to determine if the high infection efficiencies could be maintained when tested with additional phagemid particles. *Escherichia coli* TG1 (M13cp-dg3-sm) had infection efficiencies of 92%, 113%, and 120% compared to the infection efficiencies of *E. coli* TG1 with Vc86, clone18, and α-AV20N3 (Table 3-2). The infection

efficiency of *E. coli* TG1 (M13cp-dg3-sm) was approximately the same as *E. coli* TG1; therefore, *E. coli* TG1 did not suffer any reduction in infection efficiency due to maintaining M13cp-dg3-sm.

Escherichia coli TG1 (M13cp-CT-sm) was the only other helper plasmid strain besides *E. coli* TG1 (M13cp-dg3-sm) that had an infection efficiency close to that of *E. coli* TG1. However, its infection efficiency was still too low for it to be used as an amplification tool for the library. Therefore, M13cp-CT-sm was transformed into *E. coli* JM109 to try and increase its infection efficiency. *Escherichia coli* DH5α F' was the bacterial strain that the engineers of the helper plasmids used to work with the helper plasmids; therefore, we tested if an *E. coli* strain more similar to *E. coli* DH5α F' would generate higher infection efficiencies if it contained M13cp-CT-sm. *Escherichia coli* JM109 and *E. coli* DH5α F' contain *relA1* and *recA1* while *E. coli* TG1 does not. However, infection efficiency experiments with *E. coli* JM109 (M13cp-CT-sm) with three different phagemids yielded lower infection efficiencies than did *E. coli* TG1 (M13cp-CT-sm). The average infection efficiency of *E. coli* TG1 (M13cp-CT-sm) was 47%, while the average infection efficiency of *E. coli* JM109 (M13cp-CT-sm) was 28%. Therefore, using *E. coli* JM109 to maintain M13cp-CT-sm did not increase its infection efficiency.

When the infection efficiencies of *E coli* TG1 (M13cp-dg3-sm) were analyzed, this strain yielded an average infection efficiency of 120±33% and *E. coli* TG1 (M13cp-CT-sm) had an average infection efficiency of 30±16%. *Escherichia coli* TG1 (M13cp-dg3-sm) had a significantly higher infection efficiency ($p=0.01$) than *E. coli* TG1 (M13cp-CT-sm).

Escherichia coli TG1 (M13cp-dg3-sm), *E. coli* TG1 (M13cp-CT-sm), and *E. coli* TG1 that was superinfected with hyperphage were analyzed for their abilities to produce phagemid particles. Phagemid particles were produced with and without 1 mM IPTG in the growth

medium to analyze if IPTG had an effect on phagemid particle production yield. Because the *lac* promoter drives transcription of the *gIII*-scFv fusion it was hypothesized that the expressing more pIII-scFv fusions would result in more phagemid particles produced. However, when phagemid particles were titered, the phagemid particles produced in growth medium that contained IPTG had the same or lower titers of phagemid particles than when phagemid particles were produced without IPTG in the growth medium. Because there was not an increase in the phagemid particle yields when they were produced with IPTG, it suggests that the amount of pIII protein was not a limiting factor in phagemid particle production. For the titers that had a decrease in phagemid particle yields when produced with IPTG the increase in pIII protein may have been harmful to phage assembly because the excess of pIII protein may have been toxic. Phagemid particles were amplified to titers of 10^6-10^8 tu/mL by *E. coli* TG1 (M13cp-dg3-sm), 10^5 -10^6 tu/mL by *E. coli* TG1 (M13cp-CT-sm), and 10^8 -10^9 tu/mL by *E. coli* TG1 superinfected with commercial hyperphage. Titers of phagemid particles produced from *E. coli* TG1 that contained helper plasmids were too low for the helper plasmids to be an effective amplification tool. Phagemid particle production cultures usually contain ~10^9 CFU/mL; therefore, for the helper plasmids to only generate 10^5 tu/mL means that only 1 in every 10,000 bacteria produced an infectious phagemid particle. The use of helper plasmids as an amplification tool did not generate high enough titers of phagemid particles for them to effectively amplify a library.

Even though the helper plasmids were determined to be an ineffective amplification tool, they were further analyzed for the quality of phagemid particles produced by ELISA. Analysis by ELISA revealed that phagemid particles produced by helper plasmids resulted in a S:N of 7 when less than 5 x 10^4 tu/mL of phagemid particles were used as a primary antibody. Data obtained from our laboratory showed that phagemid particles are not detectable in an ELISA

unless they are used at concentrations of at least 1 x 10^6 particles/mL (data not shown). Because the spot titer method measures the number of infectious phagemid particles, it is possible that the total number of phagemid particles far exceeded the number of infectious phagemid particles. To determine if the total number of phagemid particles is greater than the number of infectious particles an anti-M13 sandwich ELISA was used to enumerate the particles/mL of phagemid particle-containing supernatants (Table 3-4). Phagemid particles amplified by *E. coli* TG1 (M13cp-CT-sm) had particle/mL to tu/mL ratios between 740 and 23,000, *E. coli* TG1 (M13cp-dg3-sm) had particle/mL to tu/mL ratios between 1 and 190, while phagemid particles amplified by *E. coli* TG1 that was superinfected with hyperphage had particle/mL to tu/mL ratios between 6 and 24. Phagemid particles produced by helper plasmid-containing *E. coli* had particle/mL to tu/mL ratios that were much higher than those for phagemid particles produced by *E. coli* that was superinfected hyperphage. In biopanning the number of phagemid particles is not important, the number of infectious phagemid particles is what matters because if a phagemid particle is not infectious then it can not enter a bacterial cell and be produced to eventually be selected and screened. Therefore, even though the number of particles produced by helper plasmids was high, the number of infectious particles produced was low, making the helper plasmids an inadequate amplification tool for the library.

The helper plasmids yielded phagemid particle progeny at titers between 10^5 to10^8 tu/mL. In a last attempt to get the helper plasmids to generate high titers of phagemid particles, the M13cp-dg3-sm helper plasmid was incorporated into *E. coli* MG1655 to determine if a less complex *E. coli* strain could generate higher titers of phagemid particles. *Escherichia coli* MG1655 was chosen because it has few known mutations and does not contain the F-plasmid. Four different phagemid particles were amplified with *E. coli* MG1655 (M13cp-dg3-sm), and the

resulting titers were: 2.5 x 10^7, 6.3 x 10^6, 2.5 x 10^7, and 3.9 x 10^7 tu/mL. Producing phagemid particles from *E. coli* MG1655 that contained a helper plasmid did not increase the titer of phagemid particles produced. The helper plasmids were extensively analyzed and determined to be poor amplification tools. Therefore, hyperphage was the best amplification tool, but it was available in titers too low to be used effectively.

To overcome the low titers of commercial hyperphage, we made homemade hyperphage. Homemade hyperphage was previously made by transducing F^+ *E. coli* cells that harbored a plasmid that encoded the *gIII* gene, pGTR203, with hyperphage and growing these transduced cells under conditions that promoted phage production. In the past, hyperphage amplified from *E. coli* TG1 (pGTR203) yielded titers between 10^7 to 10^8 tu/mL and *E. coli* HB2151 (pGTR203) yielded titers between 10^6 to 10^7 tu/mL. Many liters of hyperphage had to be produced and concentrated to generate hyperphage in a titer high enough to be used for a single panning experiment. Therefore, a way to increase the titer of homemade hyperphage was investigated to generate enough hyperphage to be used in multiple experiments. *Escherichia coli* TG1 and *E. coli* HB2151 are both F^+ strains. Because hyperphage infects bacteria by the F pilus, it was hypothesized that some of the hyperphage produced were being taken back into the bacteria which would decrease the titers of the hyperphage produced. Therefore, it was investigated if hyperphage could be produced in higher titers when produced from a F^- *E. coli* strain.

Escherichia coli MG1655 and *E. coli* EC100D were the two F^- strains of *E. coli* chosen to amplify hyperphage. These strains were transformed with pGTR203 encoding the *gIII* gene and the hyperphage genome, and five isolates from each strain were grown under conditions to promote phage production. The particles/mL and the tu/mL were analyzed (Table 3-5). The average titer of hyperphage produced from the five *E. coli* EC100D (pGTR203) (HyΦ) isolates

was 2.1 x 10^8±2.5 x 10^8 tu/mL. The average titer of hyperphage produced from the five *E. coli* MG1655 (pGTR203) (HyΦ) isolates was 3.0 x 10^9 ±2.0 x 10^9 tu/mL. The titer of hyperphage produced from the *E. coli* MG1655 (pGTR203) (HyΦ) isolates was significantly higher (p=0.02) than the titer of hyperphage produced from the *E. coli* EC100D (pGTR203) (HyΦ) isolates. The higher titers produced from *E. coli* MG1655 might be because it has less known mutations than *E. coli* EC100D. The ratios of particles/mL to tu/mL of hyperphages produced from *E. coli* EC100D (pGTR203) (HyΦ) were between 0.4 and 7.8. The ratios of particles/mL to tu/mL of hyperphages produced from *E. coli* MG1655 (pGTR203) (HyΦ) were between 0.3 and 1.0. The particle/mL to tu/mL ratios of hyperphage produced from the F^- strains were approximately the same, which means that approximately all of the phagemid particles produced were infectious. *Escherichia coli* MG1655 (pGTR203) (HyΦ) produced higher titers of hyperphage than did both F^+ strains. Whether or not the higher titers of hyperphage were due to being produced in an F^- strain was not determined because *E. coli* EC100D (pGTR203) (HyΦ) produced approximately the same titers of hyperphage as *E. coli* TG1 (pGTR203) (HyΦ). What was determined was that the bacterial strain used to produce hyperphage had an effect on hyperphage production.

Because *E. coli* MG1655 (pGTR203) (HyΦ) 5 produced the highest titers of hyperphage, it was used to produce a large batch culture of hyperphage. The hyperphage was then concentrated to approximately 1 x 10^{12} tu in 3 mL, which was concentrated enough to amplify the Tomlinson J scFv phagemid library. The Tomlinson J scFv phagemid library was amplified with homemade hyperphage with quality control checks by titering to ensure that the amplification of the library generated high quality and high quantity of phagemid particles in a way that the redundancy of the library was maintained.

Specific Aim 4: Isolation of Specific Recombinant Phage to Stx2 Toxin of *E. coli* O157:H7

Once the Tomlinson J scFv library was amplified and homemade hyperphage was improved to enable high quality and high quantity amplification of the library, a target molecule had to be selected for panning. *Escherichia coli* O157:H7 is an enterohemorrhagic serotype of *E. coli* that is responsible for approximately 73,000 illnesses and more than 60 deaths per year in the United States (30). Stx toxins kill mammalian cells and are the major causes of damage in *E. coli* O157:H7-infected individuals. *Escherichia coli* O157:H7 can produce Stx toxins 1 and/or 2. *Escherichia coli* that produces Stx2 toxin appears to be more virulent than *E. coli* that produces Stx1 or both Stx1 and 2 toxins (36-40). Therefore, Stx2 toxin would be a good target for detection in animal and patient samples.

A Stx2 toxin preparation was obtained that was stated to be 50% pure for Stx2 toxin. The other 50% of the preparation most likely contained bacterial impurities from *E. coli* HB101 because the Stx2 toxin preparation was made from *E. coli* HB101 (pMJ100-encodes Stx2 toxin). A 100% pure Stx2 preparation would have been ideal; however, if the preparation contains 50% Stx2 toxin then the most of the clones selected should theoretically be specific to Stx2 toxin.

The Tomlinson J scFv library that was amplified with homemade hyperphage was panned against the Stx2 toxin preparation. Three rounds of panning were performed with amplification of eluted phagemid particles by homemade hyperphage between each panning. Every panning used 1×10^{12} phagemid particles. The number of eluted phagemid particles for the three pannings were as follows: 2×10^{6} for round one, 2×10^{7} for round two, and 2×10^{10} for round three. The increase in the numbers of eluted phagemid particles suggested that phagemid particles specific to Stx2 toxin preparation were being selected and amplified at each round. Clones from the third round of panning were individually amplified and screened by ELISA. Analysis of 59 clones resulted in 53/59 clones (89%) having a S:N from 2 to 23 with 48/59

clones (81%) having a S:N from 10 to 23 against the Stx2 toxin preparation. Therefore, the panning was successful in isolating clones that bound to Stx2 toxin preparation. Not all of the clones that were selected bound to Stx2 toxin preparation; 4/59 (7%) of the clones bound to PBS-treated wells as well as Stx2-coated wells. The four clones had a signal in an ELISA between 0.31 and 0.87 against PBS-treated wells. These clones most likely bound to polystyrene. Even though the immunotube was blocked with blocking buffer, the unintentional isolation of clones that bound to the panning vessel is common (84). Of the clones screened, 2/59 (3%) did not bind to Stx2 toxin preparation or a microtiter well. These clones have unknown binding specificity. It is common to select clones that have unknown binding specificity. The fact that only approximately 3% of my clones had unknown binding specificity proves that the panning was successful in isolating specific recombinant phagemid particles.

Most of the clones selected from panning recognized the Stx2 toxin preparation; however, the Stx2 toxin preparation was only 50% pure for Stx2 toxin. Therefore, it was unknown if the clones bound to Stx2 toxin or bacterial impurities in the Stx2 toxin preparation. To determine if the selected clones bound to Stx2 toxin, two anti-Stx2 toxin monoclonal antibodies were examined. One antibody recognized the A-subunit of Stx2 toxin, while the other antibody recognized the B-subunit of Stx2 toxin. However, these monoclonal antibodies never generated a positive signal in an ELISA against the Stx2 toxin preparation. Using the monoclonal antibodies at the manufacturer recommended concentration of 1 μg/mL failed to generate a positive signal in an ELISA. It was initially reasoned that the monoclonal antibodies may need to be used at a higher concentration to increase the S:N values. Increasing the concentration of monoclonal antibodies in an ELISA to 100 μg/mL still failed to produce a positive signal in an ELISA. Therefore, it was hypothesized that the HRP-conjugated anti-mouse secondary antibody

that recognized the anti-Stx2 toxin monoclonal antibodies was not recognizing the anti-Stx2 monoclonal antibodies. However, when another HRP-conjugated anti-mouse secondary antibody was used to recognize the anti-Stx2 toxin monoclonal antibodies it also failed to produce a positive signal in an ELISA. Both anti-mouse antibodies had been tested within three days of these experiments against murine monoclonal antibodies and produced a positive signal. However, positive controls for the anti-mouse antibodies were not used in the ELISA that analyzed the two anti-mouse antibodies against Stx2 toxin preparation to determine whether or not they were functional. It was not determined why the monoclonal antibodies did not recognize the Stx2 toxin preparation in an ELISA. One reason might be that there was not enough Stx2 toxin with a correctly folded binding site in the Stx2 toxin preparation for the monoclonal antibodies to recognize. Also, it is possible that the batches of monoclonal antibodies that we received were somehow defective.

Because the anti-Stx2 toxin monoclonal antibodies could not be used to determine the binding specificity of the selected panning clones in an ELISA, other means of determining the binding specificities of the clones were analyzed. Three clones that bound to Stx2 toxin preparation were screened against Stx2 toxin, *E. coli* O157:H7 933 (expresses Stx 1 and 2 toxins) periplasmic break fraction, *E. coli* O157:H7 933 supernatant, *E. coli* DH5α (pNR100) (expresses Stx2 toxoid) periplasmic break fraction, and *E. coli* DH5α periplasmic break fraction. A clone that bound to the *E. coli* bacterial impurities in the Stx2 toxin preparation would be expected to bind to all of the antigens, but a clone that bound to Stx2 toxin would be expected to bind to all of the antigens except the *E. coli* DH5α periplasmic break fraction. However, the three selected clones only recognized the Stx2 toxin preparation and none of the other antigens; therefore, it was not determined if the clones were actually binding to Stx2 toxin.

Analysis of clones by ELISA proved that the majority of the selected clones from panning bound to the Stx2 toxin preparation but failed to determine if the clones bound to Stx2 toxin because the anti-Stx2 monoclonal antibodies and comparative analysis of +/- Stx2 bacterial preparations failed to generate positive signals in an ELISA. However, when both of the anti-Stx2 toxin monoclonal antibodies were examined in a Western blot against SDS-PAGE resolved Stx-2 toxin preparation, the anti-Stx2 A subunit toxin monoclonal antibody successfully recognized the Stx2 toxin A subunit in the Stx2 toxin preparation. Therefore, analysis by Western blot could at least determine if any of the clones recognized the A subunit of Stx2 toxin.

Six clones were screened in a Western blot against SDS-PAGE resolved Stx2 toxin preparation, *E. coli* O157:H7 933 (expresses Stx1 and 2 toxins) supernatant, and *E. coli* O157:H7 87-23 (Stx phage cured) supernatant (Fig. 3-17). Because the Stx2 toxin is composed of A and B subunits that are approximately 32 and 10-kDa proteins, a clone that recognized Stx2 toxin was expected to recognize a protein band at 32 kDa or 10 kDa on the Stx2 toxin preparation and the *E. coli* O157:H7 933 supernatant but not on the *E. coli* O157:H7 87-23 supernatant. If a clone recognized bacterial impurities then it would be expected to recognize proteins in all three antigens. The anti-Stx2 A subunit monoclonal antibody recognized a 32-kDa protein in the commercial Stx2 toxin preparation and in the *E. coli* O157:H7 933 supernatant proving that Stx2 A subunit was present in both preparations and not in *E. coli* O157:H7 87-23 supernatant, as was expected. Anti-BSA was also analyzed against the three antigens as a negative control to ensure that phagemid particles did not generate artifact bands. Anti-BSA failed to recognize any of the antigens, as was expected. Clone 46 failed to recognize any of the three antigens. It is possible that the binding site of the target that it recognized in the Stx2 toxin preparation was denatured by SDS-PAGE. Clone 48 recognized 35, 45, and 55-kDa proteins in the Stx2 toxin preparation

and a 35-kDa protein in both *E. coli* O157:H7 supernatants, which means that it probably recognizes bacterial protein impurities in the commercial Stx2 toxin. Clones 49 and 80 bound to a 35-kDa protein in all three antigens and a 20-kDa protein in the Stx2 toxin preparation and in *E. coli* O157:H7 87-23. Therefore, clones 49 and 80 probably bound to bacterial protein impurities in the commercial Stx2 toxin. Clones 81 and 83 appeared to recognize the same bacterial protein impurities as clones 49 and 80, except that their band intensities were much lighter.

Screening by ELISA showed that 89% of the clones bound to the Stx2 toxin preparation. Western blot analysis of six clones revealed that none recognized Stx2 toxin and that five of the clones most likely recognized bacterial impurities in the Stx2 toxin preparation. Because both of the anti-Stx2 monoclonal antibodies did not recognize Stx2 toxin in the Stx2 toxin preparation in an ELISA and the anti-Stx2 B subunit monoclonal antibody did not recognize Stx2 B subunit in a Western blot, we assumed that there was not a high concentration of Stx2 toxin in the Stx2 toxin preparation. The Stx2 toxin preparation was stated to be 50% pure for Stx2 toxin by SDS-PAGE analysis. To determine if this was true, the Stx2 toxin preparation was resolved by SDS-PAGE and stained by Coomassie blue (Fig. 3-18). If the Stx2 toxin preparation was 50% Stx2 toxin, 32-kDa and 10-kDa protein bands should have been the most prominent bands. However, SDS-PAGE analysis of the Stx2 toxin preparation showed that a 32-kDa protein band was a minor protein band that represented less than 5% of the protein in the preparation. There was a band at 10 kDa that had a strong band intensity. These bands might not have even represented Stx2 toxin subunits but may have just been proteins with a similar molecular weight. What is certain is the amount of Stx2 toxin in the Stx2 toxin preparation did not comprise 50% or even 10% of the proteins in the Stx2 toxin preparation. Therefore, it is probable that the majority of

the clones screened from the panning recognized bacterial impurities from *E. coli* and not Stx2 toxin. Extensive screening of additional clones may result in isolation of a clone specific to Stx2 toxin; however, it is recommended to try panning again with a better Stx2 toxin preparation. The panning was successful in selecting phagemid particles that bound specifically to the Stx2 toxin preparation, the phagemid particles selected just did not have the desired binding specificity. It is recommended to use the newly amplified Tomlinson scFv library with the aid of the improved homemade hyperphage to pan against another target with a high degree of purity.

This thesis details the optimization of phage display protocols and reagents. Analysis of extraction methods for LPS were compared and it was determined that a phenol-water method, opposed to a TRIzol method, extracted *V. cholerae* O1 LPS that most closely resembled commercially acquired *V. cholerae* O1 LPS. Also, binding platforms were analyzed to determine which platform could bind the most LPS. It was determined that nitrocellulose paper could bind approximately 250 to 2,500 times more LPS than that a microtiter well. Optimization of whole cell panning with the Tomlinson J scFv phagemid library revealed that a trypsin elution between 10 and 30 minutes eluted the highest number of phagemid particles; this may be due to phagemid particle degradation occurring with trypsin treatment after 45 minutes. Screening of phage display reagents was analyzed and a high throughput ELISA was developed for the production and screening of scFv proteins that was able to generate S:N values of produced scFv proteins from an ELISA when diluted 1:10. A similar high throughput ELISA was developed for the production and screening of phagemid particles; however, this method was not acceptable due to low titers produced of phagemid particles in a microtiter well.

The optimization of phagemid particle production was performed. Multiple phagemid particle amplification tools were analyzed for their infection efficiencies, yields of phagemid

particles produced, and the quality of phagemid particles produced. Hyperphage-containing *E. coli* resulted in unacceptable infection efficiencies and helper plasmid-containing *E. coli* resulted in unacceptable phagemid particle production titers. However, improvement of hyperphage that was produced in our laboratory was performed by use of *E. coli* MG1655 (pGTR203) which generated high titer and high quality hyperphage. This high quality hyperphage was used to successfully amplify the Tomlinson J scFv phagemid library to ensure that the redundancy of phagemid clones was maintained. This library was then panned against an Stx2 toxin preparation and resulted in 89% of phagemid particles from the third round of panning recognizing the Stx2 toxin preparation in an ELISA. The efficiency of this panning was greatly improved from previous pannings from this laboratory, which usually resulted in no better than 1% of selected phagemids being specific to the target antigen, and often no isolation of usable specific clones. Therefore, the optimization of methods and reagents in this thesis will greatly improve our laboratory and other laboratories chances of selecting recombinant phages specific to a target molecule.

CHAPTER 5
EPILOGUE

The homemade hyperphage-amplified Tomlinson J library produced in these studies was used to pan against *E. coli* O157:H7 flagella and yielded over 80% positive phagemid clones. The phagemids recognized the major flagellin protein by Western blot. The high throughput scFv screen was used in the *E. coli* O157:H7 flagellum project and proved useful in identifying rare scFv-secreting clones. Therefore, the improvements on phage display techniques and tools described in this thesis are, in fact, useful and offer promise of success for continuing studies in the laboratory.

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BIOGRAPHICAL SKETCH

Crystal Harpley (Mazur) was born in Columbus, OH; and shortly after moved to Wellington, FL, where she completed grade school. In 2006 she graduated with high honors from the University of Florida, with a major in microbiology and cell sciences and a minor in chemistry. During the process of finishing her master's thesis, Crystal Harpley got married and is now Crystal Mazur.