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Developing a Biosensor for the Detection of Bacteria: A Comparison of Methods for Isolating Bacteria-Specific Antibodies

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DEVELOPING A BIOSENSOR FOR THE DETECTION OF BACTERIA: A COMPARISON OF METHODS FOR ISOLATING BACTERIA-SPECIFIC ANTIBODIES

by Scott Allen Walper

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ABSTRACT

DEVELOPING A BIOSENSOR FOR THE DETECTION OF BACTERIA:

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The antigen-antibody interaction is known to be a high affinity and highly specific interaction that can readily be used for the detection and identification of biological and chemical agents. These studies were conducted to develop an efficient and cost-effective method of obtaining bacteria-specific antibody molecules for integration into a field-deployable biosensor. Antigen-binding molecules were obtained both as full-length IgG molecules from a hybridoma cell line and as recombinant single-chain Fv (scFv) antibodies isolated from naïve and immunize libraries. Monoclonal and recombinant antibody systems were compared on the effectiveness of producing new, target-specific molecules; the efficiency of production and purification of these molecules; and the success/failure of integrating the molecules into the QCM biosensor for bacterial detection. Although selection of individual hybridoma cell lines was not conducted, monoclonal antibodies (mAbs) were obtained from an established cell line maintained in a hollow-fiber bioreactor. Recombinant antibodies, scFvs, capable of binding bacterial targets were isolated from libraries using a high-throughput phage display method of selection. Protocols were established for the purification of monoclonal antibodies from the bioreactor serum and scFvs from bacterial cell cultures to assess the efficiency of readily obtaining antibodies for integration into the biosensor. Finally, methods of

immobilizing antibodies and scFvs to the gold electrode of a standard QCM crystal were explored to determine suitable procedures for consistent detection of target bacteria in aqueous samples to the lowest limit of detection.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure

LIST OF TABLES

Table

LIST OF ABBREVIATIONS

bp	Base pair
BSA	Bovine serum albumin
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
DTT	Dithioreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-inked immunosorbent assay
FBS	Fetal bovine serum
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria-Bertani media
LOD	Limit of detection
mAb	Monoclonal antibody
MPBS	PBS containing 1% triton x-100 and 5% milk proteins
MOPS	3-(N-morpholino)propanesulfonic acid
NaAc	Sodium acetate
NHS	N-hydroxysuccinimide
oePCR	Overlap extension polymerase chain reaction
OsS	Osmotic shock buffer
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfony fluoride
PTSF	*p*-toluenesulfonyl fluoride
QCM	Quartz crystal microbalance
RNA	Ribonucleic acid
SAM	Self-assembled monolayer
SB	Superbroth media
SDS	Sodium dodecyl sulfate
scFv	Single-chain Fv antibody
SOB	Super optimal broth
SOC	Super optimal broth with catabolite repression
SRS	Stanford Research Systems
TBE	Tris – boric acid – EDTA buffer
TE	Tris – EDTA buffer

CHAPTER I

INTRODUCTION

The biotechnology industry has expanded from the production of therapeutic and pharmacological agents to the development of devices that aid in the detection and identification of countless small molecules originating from chemical or biological sources. Although detection assays at one time focused on common tests for the medical field, such as glucose strip tests, pregnancy tests, etc.; the current world climate has created a new market for devices capable of detecting far more sinister elements, such as food-borne pathogens and weapons of both biological and chemical origin. Since the terrorist attacks on the United States in 2001, the federal government has significantly increased funding for the development of devices, such as sensors and analytical devices, that will provide a first line of defense against an attack from terrorist groups. Funds allocated for defense based projects has increased from a mere 50 million dollars in 2001 to nearly 6 billion for the 2009-2010 fiscal year [(1)]. Increased threats of terrorist violence and growing concern over the safety of our food network have significantly increased the demand for these instruments.

Rapid and definitive detection of bacterial and viral agents is of critical importance to the agricultural industry [(2, 3)], medical field [(4)], and for the bio-defense of the nation [(5)]. Traditional methods, such as direct plating to assess microbial contamination of samples [(6)], requires several days for bacterial growth before an accurate analysis is possible. Other methods such as polymerase chain reaction (PCR) [(7, 8)] and Enzyme-Linked Immunosorbent Assay (ELISA) [(9-11)] require transport to a laboratory facility before analysis can begin. While these methods may be effective in a hospital setting, a field-

deployable device would be better suited for agriculture and bio-defense. Samples tested at the site of concern would prevent building closures such as those associated with the 2001 anthrax scare and allow field agents to analyze products stored in warehouses, thus eliminating costly delays and needless waste observed with the *Salmonellae* outbreak of 2008.

The development of biosensors has seen a dramatic increase in the past several years [(12)]. A biosensor is defined as an analytical device that couples a biological material (i.e. an enzyme, antibody, nucleic acid, microorganism, tissue, etc.) or biomimic (imprinted polymer, synthetic catalyst, etc.) to a physiochemical transducer. Transducers can be optical (fluorescence-based or surface plasmon resonance), piezoelectric (quartz crystal microbalance), electrochemical (amperometric or potentiometric) or micromechanical (microcantilever) showcasing the diversity of the emerging field [(13)]. Although the development of biosensors that incorporate nucleic acids and enzymes is increasing, the vast majority of current approaches utilizes antibodies for detection of biological agents.

The high affinity and specificity of antibodies for their target antigens is routinely exploited for numerous scientific applications. Produced as a component of the immune response to a foreign substance, antibody molecules bind surface features of the target antigen called epitopes and contribute to the removal of the intruding entity from the host organism [(14)]. Antibody preparations are classified according to the source from which they are obtained. Polyclonal antibodies are extracted directly from the serum proteins and recognize numerous epitopes of the antigen. Enrichment of antibodies capable of antigen recognition is accomplished following a series of immunizations of a host organism that stimulate maturation of the lymphocytes In contrast, monoclonal

antibodies, which are typically produced by immortalized lymphocyte cell cultures called hybridomas, bind a single defined epitope of the antigen [15]. Binding characteristics of specificity and affinity of monoclonal antibodies, because they are produced from a single cell line, are typically well defined. As with polyclonal antibodies, generation of a hybridoma cell line is preceded by a series of host immunizations. Full-length antibodies require considerable investments of time and money to develop. Recombinant antibodies, which are produced in bacteria, can be utilized to circumvent the time and cost constraints associated with antibody production.

The antigen-recognition domain of an immunoglobulin is formed from the N-terminal 100 – 110 amino acids of the heavy and light chain [16]. Using molecular techniques, the DNA sequences encoding the variable regions can readily be isolated and combined into a single fusion gene referred to as a single chain Fv antibody (scFv) [17]. Recombinant scFvs have been constructed from the genetic material of hybridoma cells [18], spleen cells [19, 20], or from peripheral blood lymphocytes [21] to form highly diverse libraries. Selection against a target antigen can be accomplished using a variety of high-throughput techniques, however, phage display is the dominant methodology. The phage display protocol utilizes scFvs fused to the pIII protein of the M13 filamentous phage capsid. A pool of phage particles is subjected to selection to isolate those displaying an scFv with affinity for a particular antigen. Since the scFv gene is encoded in a phagemid vector that is encased within the capsid, amplification of the recombinant antibody gene and enrichment of antigen-specific scFvs are readily accomplished following each round of selection. The phage display system has been utilized for the isolation of scFvs specific for a range of targets including metabolites [21], carbohydrates [22], bacterial toxins [23], and

a variety of cellular proteins and receptors [24, 25]. Using high-throughput selection and bacterial production, target-specific, recombinant antibodies can readily be obtained for integration into a biosensor.

The microgravimetric quartz crystal microbalance (QCM) sensor is a piezoelectric device that measures minuscule changes in mass through small increases or decreases in the oscillation frequency of an A-T cut quartz crystal that lies between two electrodes, generally coated with gold or some other reactive metal [26]. A gold surface is most commonly used due to the numerous attachment chemistries to which it is amenable. Proteins can readily be immobilized to the gold electrode through either direct adsorption to the gold surface or through covalent bond formation with terminal functional groups of a self-assembled monolayer [27]. Antibodies have been successfully integrated into QCM-based sensors for the detection of food-borne pathogens such as *Listeria monocytogenes* [28], *Escherichia coli* [29, 30], and *Salmonella* spp. [26, 31-33] and have shown to be capable of detecting $10^3 – 10^8$ colony forming units (cfu)/ml.

Although IgG molecules have been successfully integrated into biosensors, significant research has not been conducted with scFvs. The scFv is appealing to biosensor applications because they can be rapidly isolated from highly diverse libraries and thereby ensure adaptability to emerging threats. Additionally, the limited complexity of the scFv allows the molecule to be produced in bacterial cell cultures in contrast to the costly mammalian cultures needed for monoclonal antibody production. For this study, the manufacture and integration of scFvs into a QCM-based biosensor were examined to compare the potential of this system to established detection methods that use polyclonal or monoclonal IgG preparations.

CHAPTER II

LITERATURE REVIEW

Antibodies

The immune system responds to the presence of a foreign antigen with a series of cell-to-cell interactions and chemical signals that stimulate an array of host defenses. One such defense mechanism is the production of target –specific antibodies. Antibody is a broad term used to describe proteins that are produced by mature lymphocytes, plasma cells, in response to a foreign antigen [(14)]. The basic antibody structure is comprised of two identical subunits, each of which is formed from a heavy and light chain that are bound through disulfide bonds. The heavy chains, which are used to classify the molecule, can further be sub-divided into five isotypes, (α - IgA; γ - IgG; ε - IgE; μ - IgM; δ - IgD) based on conserved sequences in their so-called constant domains. Although the primary structure of the constant domains does not affect antigen binding, it does play a role in the functional properties of the molecules. Similarly, the light chain subunits of antibodies are classified as either a kappa (κ) or lambda (λ) chains based on conserved amino acid sequences. As with the heavy chain gene, the light chain is a product of DNA recombination events that occur during B-cell maturation.

The antibody molecule can be divided into two distinct domains, termed Fab and Fc, which are separable by digestion with the cysteine protease papain. The Fab domain contains the antigen-binding site, which is formed from the N-terminal 100 – 110 amino acids of both heavy and light chain subunits (Figure 1). Referred to as the variable domain, this N-terminal portion is highly diverse in amino acid sequence and contributes to the ability of the immune system to respond to countless foreign antigens. This is in

contrast to the constant domains of the Fc fragment, which are relatively conserved in amino acid sequence. Antibodies interact with other cells and components of the immune system through interactions with amino acids and structural motifs of the Fc domain to facilitate antigen clearance [14, 34].

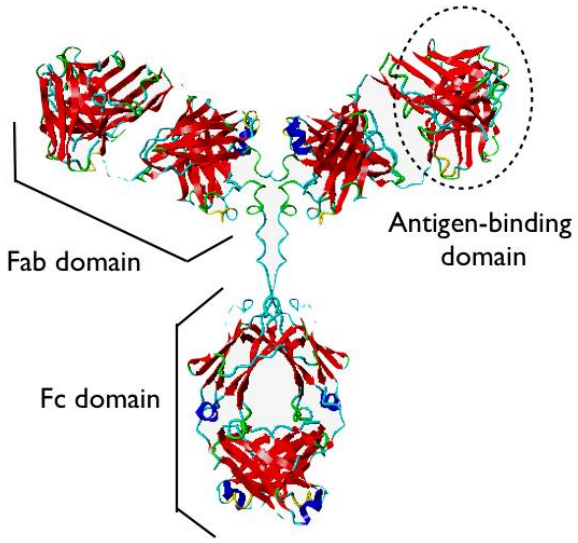


Figure 1. Structure of the IgG Antibody
The basic antibody is a homodimer comprised of identical heavy and light chain subunits. The molecule can be divided into two fragments through enzymatic cleavage. The Fab portion contains the antigen-binding domain; the Fc portion stimulates immune responses (complement, receptor binding).
Image constructed with Yasara (yasara.org) software and the anti-HIV IgG crystal structure (PDB IGG1)

The heavy and light chain genes are composed of four clusters of gene segments (V – D – J – C) that are rearranged at the gene level in countless combinations to generate the incredible diversity of antibody molecules produced by the immune system. The constant (C) gene clusters, which reside at the 3' end of the gene, rearrange to define the antibody isotype. The 5' end of the gene encodes the antigen recognition portion of the antibody and is formed from the variable (V), diversity (D), and joining (J) gene clusters. The expansive heavy chain immunoglobulin gene of humans, approximately 1 million base

pairs in length, is comprised of 95 or more V-segments, over 20 D-segments, and 9 J-segments. Both κ and λ light chains are similarly organized, although there is some variation in the number of V, D, and J-gene segments. During antibody maturation, a complex mechanism of gene recombination occurs between the V-D-J gene segments until a functional rearrangement is achieved. This process occurs independently on each of the chromosomes that carry genes for heavy and light chain subunits [(34)]. The genetic recombination events that occur within the V – D – J gene clusters result in the structural diversity of antigen-binding domains of individual immunoglobulins and form the basis for the ability of the immune system to respond to countless antigens.

In the primary immune response to an antigen, low-affinity IgM antibodies are produced as a first line of defense. The pentameric structures are quite effective at aggregating antigens and at stimulating the complement system, however, the rapidity with which they are produced suggests that little maturation of the V-D-J segments has occurred, resulting in low-affinity binding. It has been suggested by several research groups that the IgM heavy chain gene is similar to the original germ-line gene [(35, 36)]. In contrast to IgM, IgG antibodies are produced in higher concentrations during the secondary response to an antigen [(37)]. Individual plasma cells producing IgG antibodies have undergone substantial recombination of the V – D – J gene clusters. The resulting IgG antibodies have a greater diversity of structure in the variable domain. IgG antibodies produced in response to an antigen generally exhibit improved affinities and target specificities compared to their IgM counterparts, a result of the clonal expansion process [(35)].

An antibody binds a specific feature on the surface of an antigen referred to as an eptiope. Much like other protein – protein interactions, the antigen – antibody association is the product of electrostatic and van der Waals forces between the epitope and the amino acid side chains of the antigen-binding domain. Not all antibodies will bind the same epitope, and different antibodies that target the same epitope will not necessarily bind with equivalent affinity. The success of the immune system, therefore, lies in the production of a barrage of antibodies, many of which will recognize different epitopes present on the same antigen. Since a mature lymphocyte, or plasma cell, produces a single antibody molecule of defined epitope specificity and affinity, countless lymphocytes are employed by the immune system to generate the polyclonal antibody repertoire required for elimination of the foreign antigen.

Polyclonal antibodies, which are usually extracted from the serum of immunized animals, have been used for numerous applications in the scientific and medical field for well over a century. While effective, the high diversity of antibodies within the polyclonal pool can often lead to non-target interactions that complicate the interpretation of results [38, 39]. Additionally, because polyclonal antibodies are produced from individual immunized animals, lot-to-lot variation frequently skews experimental reproducibility [40]. In 1975, however, Köhler and Milstein developed a method of producing immortalized lymphocyte cell cultures capable of continuous production of an antibody clone with singular specificity [15]. These cells lines, termed hybridomas, were generated through the fusion of a plasma cell and a myeloma cell. Hybridoma cultures can be maintained indefinitely in tissue culture, overcoming the limitations imposed by

the brief life span of the progenitor plasma cell and provide a continuous source of monoclonal antibodies with defined binding characteristics.

Hybridoma cell lines are constructed through the fusion of plasma cells, generally acquired from the spleen of mice or rabbits, with cancerous myeloma cells. Maturation of lymphocytes is promoted through successive immunizations with a specific antigen to enrich for plasma cells producing antibodies with improved binding characteristics. The myeloma cells, prior to fusion, undergo selection to ensure that they are not producing antibodies themselves and are deficient in the hypoxanthine-guanine phosphoribosyltransferase gene (HGPRT) that is involved in the salvage pathway of acquiring nucleotides. The HGPRT gene deletion allows for the selection of hybridomas from the non-fused plasma and myeloma cells in HAT (hypoxanthine-aminopterin-thymidine) medium. The aminopterin in this medium inhibits *de novo* nucleotide synthesis forcing cells to utilize the salvage pathway for survival. The myeloma cells, which are $HGPRT^-$, can only survive following successful fusion with a plasma cell which recovers the HGPRT gene. Hybridoma selection is usually followed by clonal selection through limiting dilution of cells to ensure antibodies are produced from a single cell clone [(41)]. Once monoclonal cell lines are established, assays for antigen affinity and specificity allow identification of those that produce antibodies with desired binding characteristics. The development of a new hybridoma cell line can take several months to a year for each antigen of interest.

Recombinant Antibodies

In 1972, Inbar *et al*. defined the antigen-binding domain of a full-length antibody by cleaving the Fab fragment with pepsin and demonstrating that the N-terminal variable

portions of the heavy and light chains, which associate through non-covalent interactions, readily bound antigen equivalent to the full-length IgA antibody [16]. This region, because of the variability of amino acid sequence, was termed the variable fragment or Fv region. The Fv region of the antibody molecule was further subdivided into four framework regions, typically β-sheet motifs, and three complementarity-determining regions (CDRs) that form the actual antigen-binding domain. The framework regions, similar to the constant domains of the whole antibody molecule, are generally, more conserved in amino acid sequence and are thought to contribute primarily to the stability of the Fv domain [42, 43]. In contrast, the CDRs are of variable sequence, the product of the V-D-J recombination events previously described. The diversity of the antibody repertoire is credited to the sequences of the CDRs, allowing for the recognition and elimination of countless antigens encountered by the host immune system [34].

The antigen-antibody interactions occur at the antigen-binding domain, a region formed from the three CDRs of the heavy and light chain subunits. Preliminary efforts to develop recombinant antibody technologies began with experiments that demonstrated through genetic manipulation, that CDR sequences could be isolated and transferred from one antibody to the next [44, 45]. This discovery led to dramatic advances in therapeutic antibody research that involved selection of antibodies with specific characteristics in immunized animals and subsequent transfer of their CDRs to human antibodies for medical applications [44, 46, 47].

Huston *et al.* utilized the techniques of CDR transfer to form a single fusion protein comprised of the heavy and light chain variable domain sequences joined by a short amino acid linker sequence. The recombinant protein was termed a single-chain Fv

antibody or scFv [17] (Figure 2). Tethered by the linker sequence, the two variable domains could be produced as a single protein that was capable of forming the antigen-binding domain independent of any stabilizing constant domains of the heavy and light chains. Single chain Fv antibodies offered several advantages compared to monoclonal antibodies produced in hybridoma cells. The scFvs provide an additional method of immortalizing a particular antigen-specific antibody preventing loss through contamination of the cell line [17, 48, 49]. Additionally, once inserted into a bacterial plasmid, the genetic sequences of the scFv can easily be manipulated to improve the binding characteristics of the recombinant antibody [50, 51]. Finally, recombinant antibodies can readily be produced in bacterial or yeast cultures, which significantly decreases the overall cost associated with antibody-based applications [52-54].

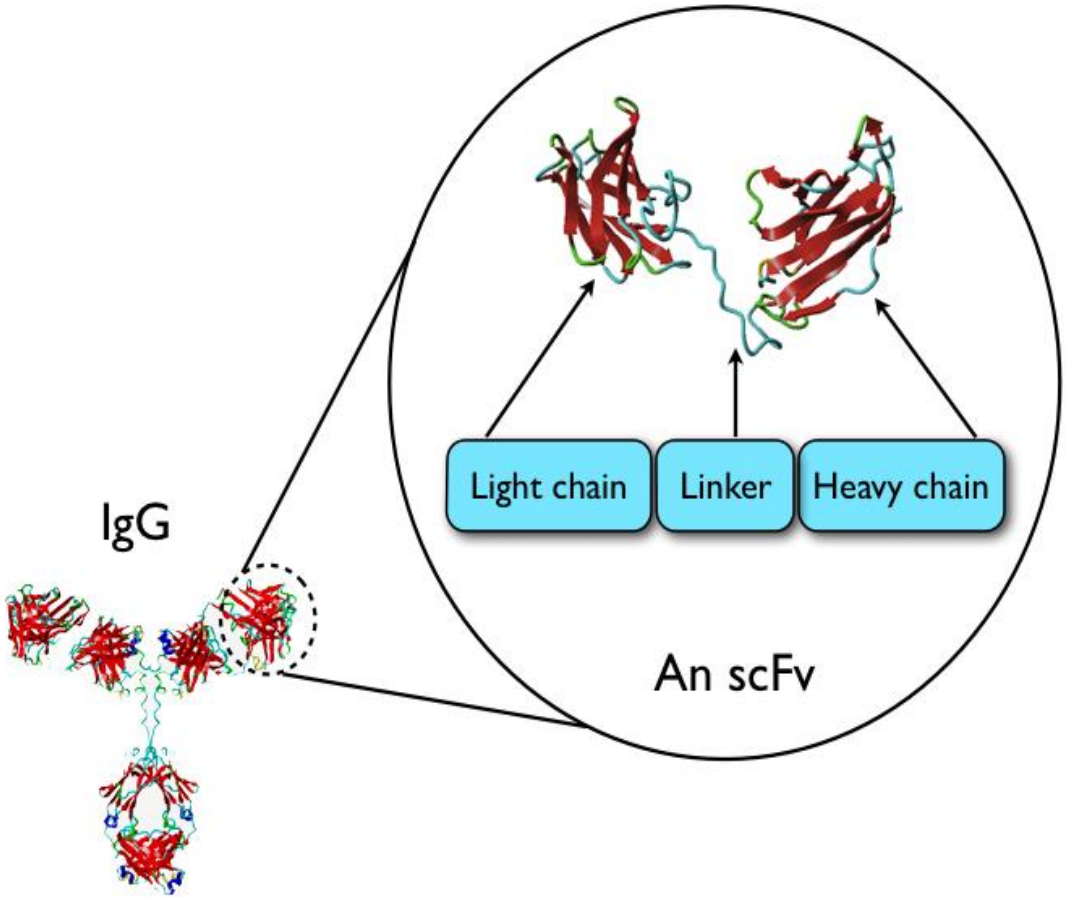


Figure 2. A Single-chain Fv Antibody (scFv) and Gene

The scFvs are constructed using molecular techniques that isolate the gene sequences of the antibody Fv domain and combine them into a single fusion gene. The heavy and light chain variable sequences are separated by a short amino acid linker sequence (12-15 amino acids) that allows non-covalent interactions between the two sequences similar to those occurring in the full-length immunoglobulin. This allows for the formation of the antigen-binding domain.

Recombinant antibodies manufactured from the genetic material of a hybridoma cell line will theoretically exhibit the same binding characteristics of the parental antibody molecule [(18, 55)]. While this offers several advantages, the true power of recombinant antibody technology lies not in the reproduction of a single antibody, but rather in the ability to mimic the incredible diversity of the immune system. Just as a single scFv can

be constructed from one hybridoma cell line, an entire library of different scFvs can be assembled from the numerous plasma cells that can be obtained from a primary lymph node such as the spleen [18, 56]. The untold number of genes for the heavy and light chain variable domains can be extracted from lymph tissues and randomly combined to form countless scFvs with varying specificities and affinities [57-60].

An scFv library can be constructed from the tissues of any number of host organisms and only requires knowledge of conserved nucleotide sequences that flank the variable domain region for PCR primer design. To date, recombinant antibody libraries have been successfully constructed from human [61-63], rabbit [19, 56, 64, 65], chicken [66], mouse [18, 67], shark [68, 69], and llama [60, 70] sources. Recombinant antibody libraries are typically classified as one of two types, either naïve or immunized. As the name implies, an immunized antibody library is constructed from a host that has been subjected to a series of immunizations and is enriched for lymphocytes with immunoglobulins genes that encode for antigen specific antibodies. In contrast, naïve libraries are assembled from lymphocytes of a host that has not purposely been exposed to a particular antigen. The naïve library, therefore, is expected to demonstrate a greater diversity of scFvs capable of binding numerous target antigens [71, 72]. Although antibody libraries are typically constructed from tissues of high lymphocyte concentration such as the spleen and bone marrow [61, 65, 73], scFv libraries have also been constructed from both peripheral blood lymphocytes [64, 74] and pools of hybridoma cell cultures [18, 75].

The success isolating scFvs from libraries is dependent upon the incredibly high diversity of antigen-binding domains present. Immunized libraries, which are enriched for antigen-specific scFvs, typically possess a clonal diversity of 10^5 or greater [76, 77]. The

naïve library attempts to simulate the clonal diversity of the host immune system; therefore clonal diversities of 10^9 or more are sought during library constructions [20, 62, 71, 78]. To select for scFvs with the desired binding affinity and specificity, a high-throughput method of selection must be employed. Although alternatives are available, the preferred method of selection is phage display.

Phage Display

George Smith first utilized the filamentous bacteriophage to isolate genes that encode the antigens for specific antibodies [79]. In his research, Smith incorporated foreign coding sequences in frame to the gene for the pIII protein of the virion capsid. The amino acids encoded by these DNA sequences were expressed as fusions to the capsid protein. To identify the target gene, antibodies known to bind an antigen of interest could be immobilized to a solid support and used to retain phages displaying peptides similar to the epitope of the target antigen. The gene encoding these peptide sequences, encoded in the phage display vector, could then be sequenced and used to identify the full-length gene that encoded the target antigen through a direct comparison of recombinant and host gene sequences. This early research led to the rapid expansion of display technologies.

Smith's research utilized filamentous bacteriophages, bacterial viruses that encapsulate a ssDNA genome within an elongated protein capsid. Filamentous bacteriophages infect a number of gram-negative bacteria and replicate within the infected host without inducing cell lysis [57, 59]. Although the f1, M13, and fd bacteriophages are all highly similar in structure and life cycle, the M13 phage, is used extensively in the phage display technique and will be the focus of this discussion.

The M13 genome is a small circular ssDNA molecule that encodes ten proteins essential to viral replication within the host bacterium [80, 81]. Five of the genes encode proteins that form the viral capsid. The most abundant capsid protein, pVIII, is present in thousands of copies and forms the main body of the virion. The pVII and pIX proteins, which interact with the membrane pore formed by pIV [82, 83], are located at one terminus of the capsid. These are the first proteins to emerge from the bacterium. The pIII and pVI proteins, located at the opposing tip of the capsid, are responsible for bacterial infection and release of the virion from the bacterium. The remaining viral proteins (pII, PX, pI, and pIV) are involved in replication and assembly of the phage particle.

Although success has been achieved with all five capsid proteins [84], display technologies have primarily utilized the pIII protein, which is well characterized in sequence and structure [85]. The pIII protein is comprised of 406 amino acids and organized into three domains. Each domain is separated by a glycine-rich linker region that, in addition to the N-terminus, can be utilized for incorporation of a foreign gene. The CT domain (residues 257 – 406) is buried within the phage particle and contributes to the release of the intact phage from the bacterium. The two N-terminal domains N1 (residues 1 – 68) and N2 (residues 87 – 217) are involved in phage infection. Binding of the N2 domain to the F pilus of *E. coli* cells induces a conformational change in the pIII protein that enables its N1 domain to interact with the bacterial protein TolA, which is located adjacent to the F-pilus, and facilitates docking of the viral particle. The actual mechanism of phage DNA entry into the bacterium is not known [59].

Since, the pIII protein is the primary protein of infection, wild-type copies of the protein are required to ensure phage propagation. Foreign genes can be cloned directly

into the phage genome; however, precautions must be taken to ensure that the protein is encoded in frame to maintain expression of downstream domains of the pIII protein. When this method is employed, all five copies of the pIII protein should contain the foreign protein. In actuality, however, proteolytic enzymes of the host bacterium will excise some number of these proteins restoring the wild-type (*wt*) protein and permitting bacterial infections [(81)]. While the direct cloning method was demonstrated successful, an alternative strategy was developed by Barbas III *et al.* that utilized a bacterial plasmid to express of viral genes [(58)]. These vectors contain a truncated portion of the pIII protein (CT domain) with upstream restriction enzyme cleavage sites that facilitates the cloning of the foreign gene in frame with this viral capsid protein [(58)]. In the presence of M13 DNA, the recombinant protein is expressed as a pIII fusion protein and incorporated into the phage capsid. Since the ssDNA phage genome contains *wt* pIII protein, which is also expressed during phage replication, only one to three copies of the capsid protein will contain the recombinant protein [(57, 58)]. This system, which relies on the expression of both recombinant fusion and *wt* pIII protein from two different DNA sources, is referred to as a 3 + 3 vector system [(81)].

The laboratory of Dr. Barbas III has produced several phagemid vectors that are applicable to phage display system. The four most commonly used vectors have been adapted to facilitate the expression of both single-chain Fab and single chain Fv antibodies with a variety of eptiope tags that facilitate detection and purification. The basic phage vector from this laboratory relies on a *lacZ* promoter to control transcription of the recombinant antibody gene, which is cloned into unique *SfiI* restriction enzyme sites. In addition to an antibiotic resistance gene for selection, the plasmid also encodes

an *ompA* leader sequence that ensures targeting of the translated protein to the periplasm, where integration into the phage capsid occurs. The pComb3 XSS vector, one of the four vectors from the Barbas labs, also includes two epitope tags, a hexa-histidine tag (6xHis) and a hemaglutinin (HA) eptiope, that can be used for purification and detection of the recombinant scFv [57]. Display on the phage capsid is accomplished through a fusion to a truncated pIII protein (aa 230 – 406). The pIII protein is preceded by an *amber* stop codon that allows for expression of the scFv gene (without the pIII fusion) and epitope tags in non-suppressor bacterial strains. A schematic of relevant domains of the pComb3 XSS vector can be seen in Figure 3.

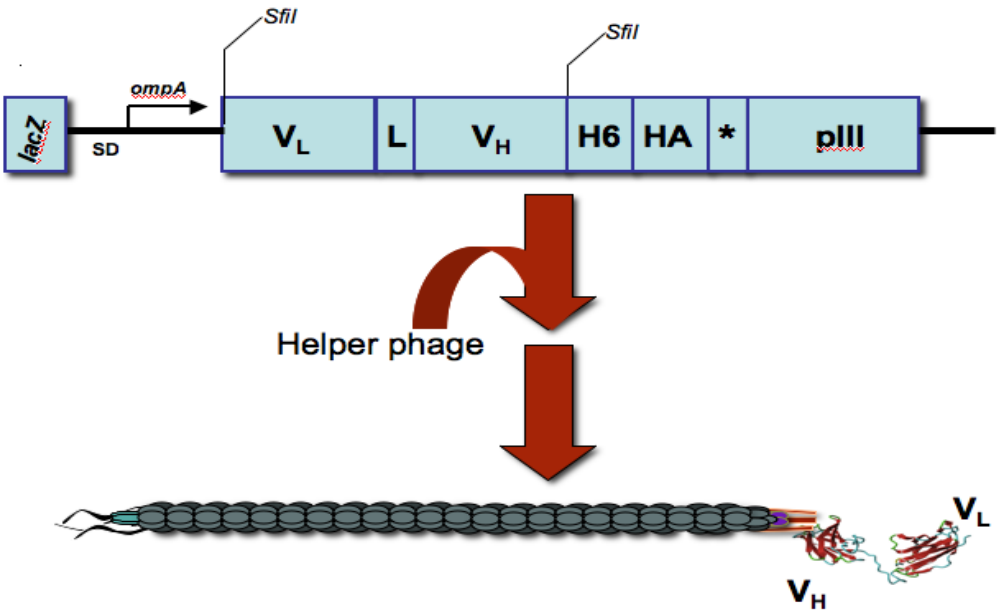


Figure 3. Schematic of the pComb3 XSS Phagemid Vector

The *lacZ* promoter regulates the transcription of the scFv gene cloned into the phage vector using the unique *SfiI* restriction sites. The scFv gene is cloned in frame with two epitope tags (6xHis and HA) and the truncated portion of the pIII protein. In the presence of a helper phage that encodes all of the *wt* proteins for phage replication, the scFv gene is expressed and incorporated into the phage capsid.
V_H/ V_L – are the scFv genes separated by the linker (L). The Shine-Delgarno (SD) and *ompA* are represented in the approximate location. The "*" represents the amber stop

Selection of ScFvs through Biopanning

The method that leads to enrichment of target-specific recombinant antibodies from the scFv library is referred to as biopanning. Through iterative rounds of selection against the target antigen and subsequent amplification of scFv-expressing phage, the large, diverse scFv repertoire is enriched to those scFvs that are able to bind the target antigen. The traditional biopanning protocol involves an antigen or ligand that is immobilized to a solid support such as a microtiter plate for selection [20, 22]. The simplicity of the protocol, however, has allowed researchers to adapt the method to satisfy experimental requirements including centrifugation of whole cell antigens [86, 87], antigen coated magnetic beads [21, 88, 89], flow cytometry with antigen coated microspheres [90, 91] and numerous other methods. Regardless of the binding and selection protocol, bound phage are eluted from the antigen and amplified in a bacterial culture. Generally, a defined number of biopanning rounds are conducted prior to the analysis of individual selected scFv clones. A diagram of a typical biopanning protocol utilizing antigen immobilized to a solid support is shown in Figure 4.

The phage display system in combination with recombinant antibody technology is a powerful tool that can readily be adapted to the isolation of scFvs with specificity for nearly any antigen. Combined with high-throughput screening methods, recombinant antibodies have been isolated against pathogenic bacteria [87] and their toxins [92], epitopes of cancer cells [93], explosives [94], and countless other small molecules. The potential applications of the recombinant antibodies and the phage display system are limited only by the creativity of the researcher.

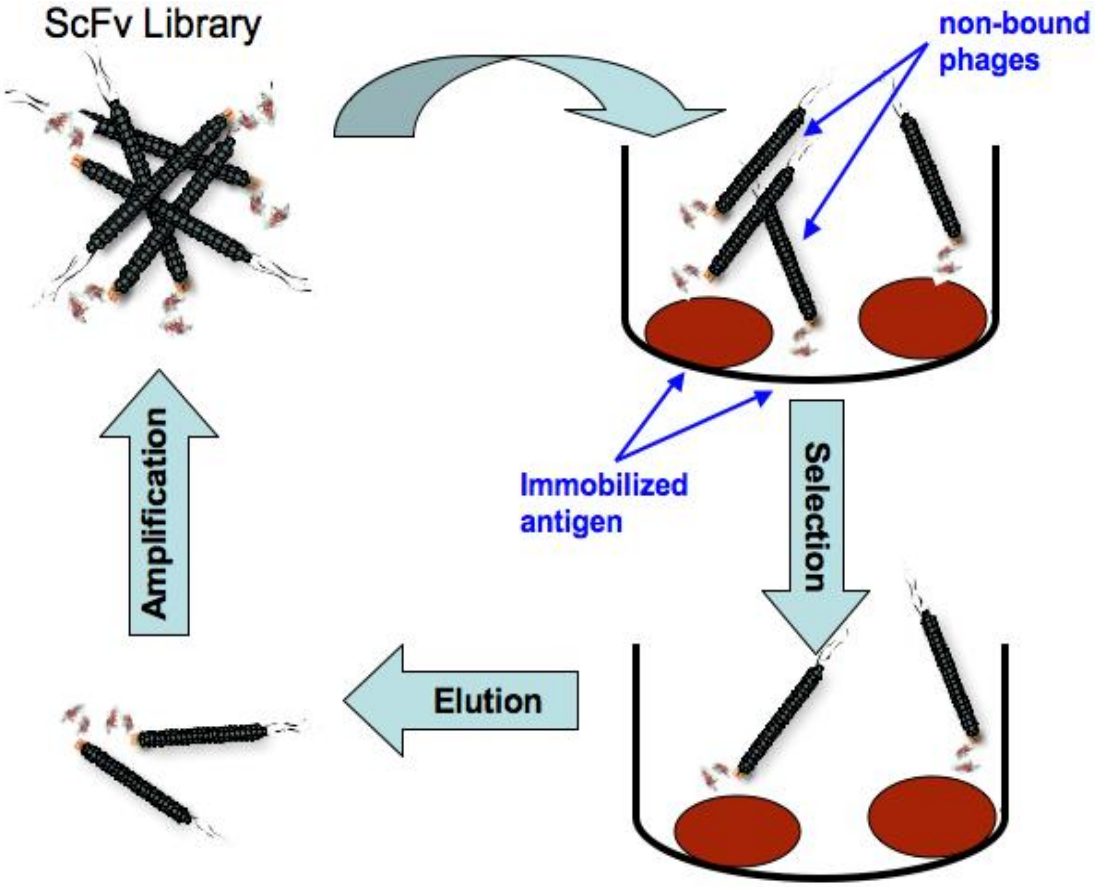


Figure 4. Selection of Antigen–Specific scFvs through Biopanning

Phages displaying the scFv library are incubated with antigen immobilized to a solid. Non-bound phages are removed through washes, leaving behind those that display scFvs with affinity for the target antigen. These molecules are eluted and amplified through infection of a bacterial culture.

Biopanning is conducted for several rounds to enrich for those scFvs that have the highest affinity for the antigen.

Biosensors and the Quartz Crystal Microbalance (QCM)

A biosensor is defined as an analytical device that integrates a biological material into a physiochemical transducing device [(95)]. The biological element can include any natural biological material (tissues, organelles, cells, antibodies, enzymes, etc.), recombinantly produced biological material (scFvs, aptamers, etc.) or biomimic (synthetic receptors or ligands, combinatorial ligands, etc.). The transducer can be based on optical, electrochemical, piezoelectric, micromechanical or a number of other suitable formats. While the range of possible biosensor principles is expansive, a biosensor is simply a device capable of detecting interactions between a target molecule and a biological material that is then relayed to an external source for interpretation. Although biosensors can utilize a diverse array of transducers, the piezoelectric quartz crystal microbalance platform, which is the transducer of the biosensor used in this study, will be discussed exclusively.

The first piezoelectric device, which converts mechanical signals such as sound waves into electrical signals, was described by Raleigh [(96)] in regards to the piezoelectricity of the quartz crystal. The quartz crystal, like all piezoelectric materials, responds to an electrical potential with a proportional internal mechanical stress or deformation [(97)]. When an alternating electrical field is applied, acoustic waves are generated as the crystal vibrates near its resonant frequency [(98)]. The direction of the acoustic wave, with respect to the crystal surface, can be controlled by the cut of the quartz in relation to the crystal axes. The AT- cut quartz crystal, which is the most conducive to QCM-based biosensors, produce an acoustic wave that travels perpendicular to the crystal surface [(99)]. The deposition or removal of mass and/or changes in viscosity

or conductivity of the solution surrounding the crystal affect the oscillation frequency of the crystal which is compared for sample analysis [100].

Sauerbrey first described QCM for *in vacuo* measurements of the rate of film deposition to the crystal surface by [101]. From his studies, the Sauerbrey equation that correlates changes in frequency to the addition of mass was derived:

$$\Delta f = -\frac{f_R}{\rho_Q dA}\Delta m = -\frac{f_R^2}{NA}\Delta m$$

where Δf is the change in resonant frequency, f_R is the resonant frequency, ρ_Q is the density of the crystal, Δm in the change in mass, and d and A are the dimensions of the crystal in regards to thickness and area, respectively. For defined crystals in which the frequency constant of the quartz (N) is known the equation can be reduced further as seen in the equation above. The Sauerbrey equation is limited to measurements of thin, rigid films and is therefore not always appropriate for analysis of aqueous solutions [102]. Modifications to the Sauerbrey equation have been made by numerous researchers to account for resistance that is attributed to the viscosity and density of the liquid surrounding the crystal surface. These modifications to the Sauerbrey equation allow QCM analysis of complex solutions [97, 103, 104].

The standard A-T cut QCM crystal is placed between two electrodes that are comprised of any conductive metal. Electrodes coated with a thin layer of gold are most often used because of the low reactivity of the element and its suitability for numerous attachment chemistries. Biomaterials, or specifically proteins, can be immobilized to the

gold electrode either through direct adsorption to the surface or through a modification of the gold surface that provides terminal functional groups for covalent attachment.

Adsorption of the proteins to the surface can involve direct interaction between the protein and the gold of the electrode [31, 32] or between the protein and a thin polymer layer such as polystyrene that is applied through spin-coating [105, 106]. Direct protein adsorption to the gold surface occurs throuhg van der Waals interaction or binding between sulfhydryl groups of amino acid side chains while binding with polystyrene is a product of hydrophobic forces. Crystal surfaces prepared using the direct adsorption are often regenerable and stable for extended periods [107].

Covalent attachment of the proteins to the surface can be accomplished following functionalization of the gold surface with silanes, thiols, sulfides, or even thin reactive films that will interact with the gold [27]. Chemistries to functionalize gold surface using this reagents are well described in the literature. In addition to these traditional chemical methods, self-assembly of monomeric subunits on the gold surface has proven quite successful. Self-assembled monolayers (SAM) consist of organized organic molecules with various functional groups (silanes, carboxyls, amines) on the gold surface [108]. In this study, a SAM with a terminal carboxyl group was utilized for the attachment of antibodies to the crystal surface. Following formation of the SAM layer, the target protein is attached through the formation of an amide bond facilitated by the chemical cross linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC). This chemical reaction activates the terminal carboxyl group of the SAM layer and facilitates bond formation with free amine groups of the target protein. As with non-covalent methods, proteins can either be attached directly through any number of chemistries or indirectly

through interaction with a second protein. The indirect method of attachment is frequently utilized with antibody molecules since orientation of the molecule is critical for their activity. For this method of attachment, the interaction between protein A and the Fc domain of immunoglobulins is generally exploited to generate a functional QCM surface [109-111]. Although more complicated to produce, the surfaces formed through covalent immobilization of proteins offer additional stability to the QCM surface, particularly if solutions of high ionic strength are analyzed which would display proteins directly absorbed to the gold surface [27].

The QCM microbalance platform has been utilized for the detection of bacteria [26, 28, 30, 112], viruses [113, 114], DNA [115, 116] and countless small –molecule chemical and biological agents. The QCM-platform is a robust system that can easily be adapted to different experimental conditions. Sample analysis is based on a simple change in frequency that can easily be interpreted by end-users. Finally, the device is of limited complexity, requiring only a crystal, oscillator, and frequency counter and is therefore amenable to filed-deployment.

Objective of this Study

In recent years there has been a considerable increase in the demand for devices capable of detecting a range of biological and chemical agents. Although a number of methods have been developed that are used in a laboratory environment, there is a need for a device that can easily be deployed and utilized by field agents and first responders. To be suitable for use by such a diverse group of end-users, a biosensor will have to be of limited complexity, produce results that are easily interpretable, and is adaptable to an array of potential targets and applications.

To satisfy these requirements, a prototype biosensor was constructed that is based on a quartz crystal microbalance platform. The device was designed to provide an instrument of limited complexity that can readily withstand the rigors of field conditions and provide a simple yes/no decision following sample analysis. The detection portion of the biosensor is based on antigen-antibody interaction to provide high specificity for the target. Two systems, monoclonal antibody and recombinant antibody production were examined to determine which method was most suitable to commercialization and able to satisfy the adaptability requirement of the biosensor.

CHAPTER III

EXPERIMENTAL PROCEDURES

Routine Apparatus

Solutions and media were prepared from chemicals obtained primarily from Thermo Fisher Scientific, Sigma Aldrich, or VWR. Chemicals and reagents were weighed using either a Mettler Toledo Model Ab54-S or PB303-S digital balance. An Orion 720A+ meter and Orion 8102BUWP electrode were used for pH measurements. The meter was calibrated using a minimum of two control buffers (pH 4.0, pH 7.0, or pH 10.0). Deionized water was obtained from a Barnstead NANOpure Diamond ultra-filtration system at measured resistivity of 18.2 MΩ/cm.

Sterilization of media and other solutions was performed using either a Steris Amsco Lab 250 or a 3031-S autoclave. All bacterial cultures, unless otherwise specified, were grown at 37°C in either a New Brunswick G24 environmental incubator shaker or a Series 25 incubator shaker. Bacterial plates were incubated at 37°C in a Fisher Scientific Isotherm incubator. Short-term storage of plates and cell lysates was accomplished using a General Electric Hotpoint refrigerator. Precision Microprocessor controlled 280 Series water bathes were maintained at 37°C and 42°C for restriction enzyme digests and transformations. A VWR Digital Heatblock was used for higher temperature restriction digests and protocols requiring elevated temperatures.

Centrifugation of small liquid samples was performed using an Eppendorf tabletop centrifuge model 5417c. Centrifugation of larger culture volumes, phage preparations, and other separations were accomplished using Beckman Avanti Model J-26 XP or J-301centrifuges and either JA 25.50 or JLA 16.250 rotors. Separation of the *Pseudomonas*

aeruginosa outer membrane proteins was accomplished using a Beckman L7-65 ultracentrifuge and JS 24.38 rotor. Low speed centrifugation of intermediate scale cell cultures and solutions was executed using a Damon Sorvall IEC HN-SII clinical centrifuge.

Buffers and Solutions

Antibiotics

Ampicillin

Prepared at stock concentrations of 100 mg/ml in sterile water.
Working concentration - 100 μg/ml

Carbenicillin

Prepared at stock concentrations of 100 mg/ml in sterile water.
Working concentration – 100 μg/ml

Kanamycin

Prepared at stock concentrations of 50 mg/ml in sterile water.
Working concentration – 50 μg/ml

Tetracycline

Prepared at stock concentrations of 5 mg/ml in 70% ethanol.
Working concentration – 10 μg/ml

Gentamicin

Obtained as a solution at a concentration of 50 mg/ml

Chromatography Buffers

DEAE Affi-gel blue

Buffer A

20 mM Tris-HCl (pH 7.2)
28 mM NaCl

Buffer B

20 mM Tris-HCl (pH 7.2)
0.6 M NaCl

Buffer C

20 mM Tris-HCl (pH 8.0)
1.4 M NaCl

Protein G

Column buffer

20 mM Na_2HPO_4 / NaH_2PO_4 (pH 7.0)

Elution buffer

0.2 M glycine-HCl (pH 2.5)

Nickel – nitrilotriacetic acid (Ni^{2+}-NTA)

Lysis buffer

50 mM Na_2HPO_4 / NaH_2PO_4 (pH 6.0)
100 mM NaCl
10% glycerol
1 mg/ml hen egg white lysozyme
1:20 dilution of B-PER II bacterial protein extraction reagent

Buffer A

50 mM Na_2HPO_4 / NaH_2PO_4 (pH 6.0)
300 mM NaCl
10% glycerol
25 mM imidazole
1 mM PMSF/PTSF

Buffer B

50 mM Na_2HPO_4 / NaH_2PO_4 (pH 4.0)
1.5 M NaCl
10% glycerol
25 mM imidazole
1 mM PMSF/PTSF

Elution buffer

50 mM Na_2HPO_4 / NaH_2PO_4 (pH 6.0)
300 mM NaCl
10% glycerol
250 mM imidazole
1 mM PMSF/PTSF

Denaturing Ni^{2+}-NTA chromatography

Denaturing buffer

6 M guanidinium hydrochloride
20 mM sodium phosphate (pH 7.8)
500 mM NaCl

Buffer A

8 M urea
20 mM sodium phosphate (pH 7.8)
500 mM NaCl

Buffer B

8 M urea
20 mM sodium phosphate (pH 6.0)
500 mM NaCl

Buffer C

8 M urea
20 mM sodium phosphate (pH 4.0)
500 mM NaCl
250 mM imidazole

Colony Lysis Buffer

0.2 % (v/v) Triton X-100
1 mg/ml RNAse A

Plasmid DNA Extraction (Alkaline Lysis)

Solution I
- 50 mM glucose
- 25 mM Tris-HCl (pH 8.0)
- 10 mM EDTA (pH 8.0)

Solution II
- 0.2 N NaOH
- 1 % w/v Sodium dodecyl sulfate
- Always prepare fresh

Solution III
- 4 M sodium acetate
- Add glacial acetic acid to a pH of 4.8

Resuspension solution
- 0.25 M sodium acetate pH 5.2
- 1 mM EDTA

RNA Extraction

DEPC water for RNA extraction
- 0.2 ml of diethyl pyrocarbonate
- 100 ml deionized water
- Shake rigorously then autoclave

Mammalian cell lysis solution
- 50 mM Tris pH 8.0
- 100 mM NaCl
- 5 mM $MgCl_2$
- 0.5% (v/v) Nonidet P40

Osmotic Shock Buffer

500 mM sucrose
100 mM Tris-HCl (pH 8.0)
1 mM EDTA

Phosphate Buffer Saline (PBS)

137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.4 mM KH_2PO_4

5X Phage Precipitation Solution

20% (w/v) PEG 8000
15% (w/v) NaCl

SDS-PAGE Gel Preparation Solutions

1.5 M Tris-HCl (pH 8.8)
0.5 M Tris-HCl (pH 6.8)
10% (w/v) SDS
40% acrylamide: Bisacrylamide, 29:1
TEMED (N,N,N',N'-Tetramethylethylenediamine)
10% (w/v) ammonium persulfate

4X SDS-PAGE Loading Buffer

200 mM Tris-HCl (pH 6.8)
40% (v/v) glycerol
8% (w/v) SDS
10% (v/v) 2-mercaptoethanol

SDS-PAGE Running Buffer

25 mM Tris base
192 mM glycine
1% (w/v) SDS

Tris Borate EDTA Buffer (TBE)

89 mM Tris base
89 mM boric acid
2 mM EDTA (pH 8.0)

Tris EDTA Buffer (TE)

10 mM Tris-HCl (pH 8.0)
1 mM EDTA
autoclave prior to use

Transfer Buffer (Immunoblotting)

25 mM Tris base
192 mM glycine
20% (v/v) methanol

Media

Dulbecco's Modified Eagle Medium Modified for Bioreactors (DMEM)

1x DMEM high glucose
- 4.00 mM L-glutamine
- 4500 mg/L glucose
- without sodium pyruvate

10% low IgG Fetal bovine serum
Gentamicin diluted to a final concentration of 100 μg/ml

Luria-Bertani (LB)

10 g/L bacto tryptone
5 g/L yeast extract
10 g/L NaCl

for solid media 15 g/L of agar (1.5% w/v) is added prior to autoclaving
for top agar (phage titering) 7.5 g/L of agar (0.75% w/v) is added prior to autoclaving

*Superbroth (SB***)**

30 g/L bacto tryptone
20 g/L yeast extract
10 g/L MOPS (pH 7.0)

Super Optimal Broth (SOB)

20 g/L bacto tryptone
5 g/L yeast extract
0.5 g/L NaCl
After autoclaving add:
10 ml of filter-sterilized 1M $MgCl_2$
10 ml of filter-sterilized 1M $MgSO_4$

Super Optimal Broth with Catabolite Repression (SOC)

20 g/L bacto tryptone
5 g/L yeast extract
0.5 g/L NaCl
After autoclaving add:
10 ml of filter-sterilized 1M $MgCl_2$
10 ml of filter-sterilized 1M $MgSO_4$
10 ml of filter-sterilized 40% glucose

Standard Protocols

Unless otherwise stated, the following methods were employed during standard experiments. Deviations from these protocols will be described in the appropriate sections.

Isolation of plasmid DNA

Plasmid DNA was isolated from *Escherichia coli* TOP10 cells (Invitrogen) or c2925 (*dcm-/dam-*) cells (NEB) primarily with the Zippy Plasmid Miniprep kit (Zymo Research) according to the manufacturer's protocol.

Large-scale plasmid isolation for construction of the phage display libraries utilized an alkaline lysis protocol [117]. A 50 ml *E. coli* culture grown in Luria Bertani media (LB) containing the appropriate antibiotics was transferred to a 250 ml polypropylene bottle and centrifuged at 5,000 x g in a JLA16.25 rotor. The supernatant was decanted and the pellet resuspended in 2 ml of alkaline lysis solution I. A 3 ml volume of solution II was added to the resuspension to facilitate cell lysis. The bottle was gently rolled to mix the contents and then incubated at room temperature for 5 min. Lysis was neutralized with 2.5 ml of solution III. The sample was incubated for 5 min on ice then centrifuged at 15,000 x g for 15 min at 12 – 13°C in the JLA16.25 rotor. The supernatant was then transferred to a 30 ml polypropylene screw-top tube and placed on ice. The nucleic acids were extracted from the supernatant with an equivalent volume of a phenol: chloroform mixture (1:1). The solution was vortexed, then centrifuged at 7,700 x g in a JA25.50 rotor for 10 min. The upper aqueous phase was transferred to a 15 ml conical tube. A half volume of isopropanol (3.5 ml) was added to the solution, mixed gently by inversion, and then incubated for a minimum of 10 min at room temperature. Precipitated nucleic acids were pelleted by centrifugation at 7,700 x g for 10 min at 4°C in a JA 25.50 rotor. The supernatant was discarded and the pellet air-dried. To remove RNA, the pellet was dissolved in 1 ml of resuspension solution containing RNase A at a final concentration of 25 μg/ml. The solution was incubated for 30 min at 37°C in a water bath. Remaining protein contaminants were removed with a second phenol: chloroform extraction, followed by centrifugation at 1,000 x g in a Damon-Sorvall IEC HN-SII clinical centrifuge for 10 min. The aqueous phase was transferred to a 1.5 ml microcentrifuge tube, 0.5 ml of isopropanol was added and the sample was incubated for 10 min at room

temperature. Precipitated plasmid DNA was pelleted by centrifugation at 10,600 x g and 4°C in an Eppendorf centrifuge for 10 min. The supernatant was decanted, the pellet air dried and then resuspended in 100 μl of sterile water. Plasmid DNA was quantitated using a Nanodrop ND-1000 spectrophotometer.

Resolution of DNA via Electrophoresis

For large DNA molecules, agarose gels (0.8 – 1.0% (w/v)) were prepared using certified molecular biology grade agarose (Bio-Rad) dissolved in 1 x Tris Borate EDTA (TBE) buffer using a Sharp Carousel microwave. An International Biotechnologies Model MPH apparatus with a 120 cm^3 gel slab or a Continental Lab Products, Inc. Model 75.710 apparatus with a 60 cm^3 gel slab were routinely used for gel electrophoresis. A Bethesda Research Laboratories Life Sciences, Inc. Model 250 power supply was used for all experiments. Diagnostic gels were resolved at 150 V for 60 min unless otherwise stated. A standard 10x endostop solution was diluted to a final 1x concentration for gel loading. DNA standards were either Promega 1Kb DNA ladder (G5711), New England Biolabs (NEB) 1 Kb ladder (N3232), NEB 100 bp ladder (N3231), or NEB PCR marker (N3234). Gels were stained for 10 min in ethidium bromide (0.5 μg/ml), washed briefly in deionized water then visualized using either an Alpha Innotech alphaimager or a Versadoc 4000 MP system (Bio-Rad).

Restriction Enzyme Digestion

DNA was digested with an assortment of restriction endonucleases for either cloning or diagnostic purposes. Enzymes *BamHI, EcoRI, KpnI, HindIII, SacI, SpeI, XbaI,* and *XhoI* were acquired from New England Biolabs. Digests were generally conducted in 0.5 ml tubes for volumes of 20 - 50 μl. Reactions consisted of 500 ng of plasmid DNA, the

appropriate buffer supplied by the manufacturer, BSA diluted to a final concentration of 0.1 mg/ml, and 10 U of enzyme. The aforementioned enzymes were incubated for 2 - 3 h at 37°C in a water bath. For the restriction enzyme *SfiI* (Roche Diagnostic Laboratories), the 50°C incubation was maintained for 3 - 5 h.

Isolation of DNA Fragments Using Agarose Gel Extraction

Restriction digest and PCR products were resolved by agarose gel electrophoresis. The ethidium bromide stained gel was transferred to a Vilber Lourmat Model TFP-M/WL transilluminator where the appropriate band was excised using a razor blade. The gel slice was transferred to a 1.5 ml microfuge tube and weighed using a Mettler Toledo digital balance. The Zymoclean Gel DNA Recovery Kit (Zymo Research) was used according to the manufacturer's protocol.

DNA Ligation and Transformation of Chemically Competent E. coli

DNA ligations were conducted in 0.5 ml tubes at room temperature for 3 h unless otherwise stated. The 10 μl reaction contained T4 DNA ligase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, and 10 mM dithioreitol), 100 ng of DNA, and 20 U of T4 DNA ligase (NEB). The pCR Blunt II TOPO ligation reaction was assembled according to the manufacturer's protocol with 1 μl of vector DNA, 4 μl of PCR product, and 1 μl of the manufacturer supplied salt solution (1.2 M NaCl, 0.06 M $MgCl_2$). The reaction was incubated at room temperature for 20 min prior to transformation of chemically competent *E. coli* cells.

A variety of chemically competent *E. coli* cells were used for transformation of ligation products (see Appendix). Typically, 5 μl of the T4 ligation reaction or 3 μl of the pCR Blunt II TOPO ligation reaction were combined with 12.5 - 25 μl of chemically

competent cells in a 3 ml polypropylene tube. The suspension was placed on ice for 30 min then subjected to a 30 - 45 s heat shock at 42°C. The tubes were immediately returned to ice for 2 min. A 300 μl aliquot of room temperature Super Optimal with Catabolite-repressor (SOC) media was added to the cells, which were then transferred to a 37°C shaker incubator for 1 h. Aliquots of this culture were then plated to selective media to identify clones containing the ligation product.

Rapid Analysis of Transformants (Colony Lysis)

A small portion of an individual colony was transferred to a 0.5 ml microfuge tube containing 3 μl of sterile water and 8 μl of colony lysis buffer using a sterile 20 - 200 μl pipette tip. The cells were suspended in the lysis solution by vigorous mixing with the pipette tip. The microfuge tubes were then heated at 100°C for 30 – 60 s to lyse the cells. The samples were cooled on ice for 2 min prior to the addition of 4 μl of restriction enzyme mix (1.4 μl of appropriate NEB buffer, 0.14 μl of 100X BSA, 10 U of enzyme (0.5 μl), 2 μl of sterile water). The reactions were incubated at 37°C for 1 h prior to separation of digestion products using agarose gel electrophoresis.

SDS-PAGE and Immunoblotting

Protein concentration was determined using a bicinchoninic acid (BCA) assay [(118)]. Absorbance measurements at 562 nm were made using a Beckman Coulter DU 800 spectrophotometer. Protein samples were separated electrophoretically by sodium-dodecyl sulfate polyacrylamide gels (SDS-PAGE) using a 12% acrylamide 1.0 mm gel and a Mini-protean 3 system (Bio-Rad). Electrophoresis was conducted at 100 V for 90 min. For visualization of the protein bands, the gel was first washed for 1 h in distilled water then stained for an additional 1 h using Gel Code Blue reagent (Thermo Fisher

Scientific). The staining solution was removed and replaced with an excess of distilled water to destain the gel overnight. The Versadoc 4000MP was used for image capture.

Immunoblot analysis of separated protein samples was accomplished following initial transfer from the acrylamide gel to a 0.45 μm nitrocellulose membrane using a Mini Transfer-Blot cell (Bio-Rad). Transfer was conducted at 4°C and 100 V for 90 min in transfer buffer. The nitrocellulose membrane was blocked with a PBS-0.1% triton x-100 (v/v) containing 5% low fat dried milk solution for a minimum of 1 h. The blocking solution was decanted and replaced with a solution of the same composition that contained the primary antibody of appropriate dilution. After an hour incubation with the primary antibody, the membrane was subjected to wash steps consisting of an initial wash with PBS containing 0.1% triton x-100, then with the blocking solution, and then followed again with the PBS-triton solution. The secondary antibody was diluted in blocking solution and incubated with the membrane for 1 h. The secondary antibody was then decanted and the wash steps were repeated. Antibody binding was visualized either with a Nitro-Blue Tetrazolium Chloride and 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP Pierce) substrate for alkaline phosphatase-conjugated secondary antibodies, or a Supersignal WestPico chemiluminescent substrate (Thermo Fisher Scientific) for secondary antibodies with a horseradish peroxidase (HRP) conjugate.

Some immunoblots were visualized by exposing X-ray film to light emitted from HRP-conjugated antibodies and chemiluminescent substrate. Following a standard immunoblotting protocol, the nitrocellulose membrane was sealed in Saran wrap then placed, protein side up, in a light-sealed exposure cassette. A piece of Kodak Scientific

Imaging Film was placed on top of the nitrocellulose membrane and exposed for 30 s to 10 min. The film was removed and developed in Kodak developer and fixer reagents. Images of the developed film were scanned using either the Alpha Innotech or Versadoc instruments.

Live and heat-killed bacterial samples were immobilized to nitrocellulose accomplished using a Minifold 96 well dot blot apparatus (Schleicher & Schuell, Inc.) or a 48 well slot blot apparatus (Bio-Rad). This protocol was employed for assays that examined monoclonal antibody or scFv activity against bacterial targets. Bacteria were suspended in PBS and immobilized on the nitrocellulose membrane using a vacuum aspiration connection to a laboratory sink. Following complete aspiration of liquids from individual wells, the membrane was removed and allowed to air dry. The membrane was then developed as described for the immunoblot protocol. Visualization of monoclonal antibody or scFv binding was accomplished with alkaline phosphatase or HRP-conjugated secondary antibodies as detailed above.

Isolation of Outer Membrane Proteins from Pseudomonas Species

The method for isolation of outer membrane proteins was modified from the work of Hancock *et al.* [119-121] and Schnaitman *et al* [122]. A 50 ml overnight culture of *Pseudomonas aeruginosa* in LB media was transferred to a 250 ml polypropylene bottle and centrifuged at 15,000 x g and 4°C for 15 min to pellet the cells. The cells were washed three times in 30 mM Tris-HCl (pH 8.0) buffer to remove residual media. The final cell pellet was resuspended in 20 ml of 30 mM Tris-HCl (pH 8.0) buffer, 20% sucrose (w/v), 1 mg/ml DNase I and 1 mg/ml RNase A. The cells were broken by three passes of the suspension through a French pressure cell at 16,000 psi. Additional lysis

was ensured with the addition of 2 mg of chicken egg white lysozyme and a 10 min incubation on ice. The lysate was transferred to a 50 ml conical tube and the cell debris pelleted at 1,000 x g for 20 min.

The supernatant was transferred to a new tube and diluted with a 14 ml volume of the 30 mM Tris-HCl (PH 8.0), 20%sucrose buffer. A step gradient was prepared with a 14 ml layer of 70% (w/v) sucrose and 14 ml of 60% (w/v) sucrose in a Beckman 40 ml polycarbonate ultracentrifuge tube. A 10 ml volume of the cell lysate was carefully layered atop the sucrose gradient and fractionated by centrifugation using a Beckman L7 ultracentrifuge with a JS 24.38 rotor. Centrifugation was conducted for 5 h at 104,000 x g and 4°C. Distinct regions of separated material were identified, based on color and turbidity, and transferred and to new tubes.

Hybridoma Maintenance

Murine hybridoma cultures, Ma1-6 (ATCC CRL-1783) and Ma7-4 (Mansel Griffith, the University of Toronto), were continuously maintained in Falcon tissue culture flasks in a Sheldon Manufacturing Reach-In dry CO_2 incubator at 37°C in an atmosphere supplemented with 8% CO_2. The cultures were grown in Dulbecco's Modified Eagle Medium (Hyclone) supplemented with 100 μg / ml of gentamicin (Gibco), 2 mM L-glutamine (Gibco), and 5 - 20% low IgG fetal bovine serum (Hyclone) depending upon the health of the culture. The culture was passaged into successively larger volumes (12.5 cm^3, 75 cm^3, 150 cm^3) until a maximum cell density and viability suitable for inoculation of a bioreactor was reached.

Cell viability was determined by a direct count of viable cells using a Fisher Scientific hemacytometer and a trypan blue stain (Gibco). Confluent cells were dislodged from the

T-flask by forcibly striking the bottom of the T-flask several times with the palm of the hand. A 20 μl volume was then transferred aseptically to a 0.5 ml microcentrifuge tube that contained 20 μl of 0.4% trypan blue and 40 μl of sterile PBS. A 10 μl aliquot of this solution was transferred to the hemacytometer for a direct count of viable hybridoma cells. The ratio of live unstained to dead cells (blue) was calculated to determine the viability of the culture. The approximate cell density could be derived from the number of viable cells. Cultures were maintained at greater than 80% viability and a cell number of approximately $1x10^5$ cells/ml. Cultures that demonstrated lower than 80% viability were supplemented with a higher percentage of FBS to invigorate the culture.

In addition to growth in T-Flasks, hybridoma cultures were also maintained in a hollow-fiber bioreactor (Fibercell Systems). Hybridomas were grown in either a medium polyurethane 20 kDa MWCO cartridge capable of supporting cell cultures of approximately 10^9 cells/ml, or a large polyurethane 5 kDa MWCO cartridge capable of supporting cultures of approximately 10^{11} cells/ml. The high cell densities of the bioreactors were attained by a steady supply of nutrients provided by both a circulating medium that passed through the intercapillary space (ICS) and serum rich medium that filled the extra-capillary space (ECS) in which the cell culture was maintained.

Measurement of the glucose levels within the ICS medium guided the rate of ECS harvest and ICS medium replacement. A glucose assay reagent kit (Sigma) containing ATP, oxidized NAD, hexokinase and glucose-6-phosphate dehydrogenase was used to calculate the approximate amount of glucose in the ICS media. The enzymatic reaction produces reduced NADH, which could be quantitated by measuring the absorbance at 340 nm in a Beckman Coulter DU800 spectrophotometer. For determination of glucose

concentration, a 1 ml aliquot was aseptically removed from the ICS medium and diluted three-fold in sterile water. A 20 μl aliquot of this dilution was added to 600 μl of the glucose assay reagent and incubated at room temperature for 15 min. The absorbance value at 340 nm was determined for samples and controls consisting of the reagent only and the unused DMEM medium. The concentration of glucose in the sample medium was determined using the Beer-Lambert law. The ICS medium was exchanged when the glucose concentration dropped below 2.25 mg/ml, which is half the initial concentration in the DMEM. The antibody-containing ECS medium was exchanged every second day during periods of optimal growth.

Monoclonal Antibody Purification

DEAE Affi-gel blue purification. DEAE Affi-gel blue purification of monoclonal antibody required an initial ammonium sulfate precipitation step to remove a substantial portion of the BSA and contaminating serum proteins. A saturated ammonium sulfate solution, 5.8 M $(NH_4)_2SO_4$ (pH 7.4), was added slowly to an equal volume of ECS medium while stirring at 4°C to obtain a 50% saturated solution. After 30 - 60 min at 4°C the solution was transferred to 250 ml screw-top bottles (Nalgene) and centrifuged at 10,000 x g. The supernatant was decanted and the pellet washed 2 - 3 times with a 50% saturated $(NH_4)_2SO_4$ solution with centrifugation as above between each wash. The final pellet was resuspended in 20 mM Tris-HCl (pH 7.2) and dialyzed in 12,000 - 14,000 MWCO dialysis tubing in 4 L of the same buffer for 3 – 4 h with a minimum of two buffer exchanges. Following dialysis, the pH was adjusted to pH 7.2 using either 1 M HCl or 1 M NaOH.

The DEAE Affi-gel blue matrix was prepared following the manufacturer's protocol to remove unbound dye material. The resin was slowly transferred to a 2.5 x 10 cm Econo glass column (Bio-Rad) until a bed volume of approximately 5 ml was established. A 2.5 cm flow adapter was attached to the column and connected to a Biologics Workstation (Bio-Rad). The column matrix was equilibrated with a minimum of ten bed volumes of Buffer A prior to loading of the dialyzed sample at a constant flow rate of 1.0 ml/min. The column was washed with a minimum of ten bed volumes using Buffer A to remove non-bound proteins. To elute the monoclonal antibody, a three stage salt gradient was used. The optimized protocol is shown in Table 1.

Table 1 – DEAE Affi-Gel Blue Purification Protocol

Volume	**Salt concentration**	**Fraction size**	**Elution**
10 ml isocratic flow	0.03 M	1.25 ml	None
60 ml gradient	0.03 – 0.08 M	1.25 ml	Antibody
30 ml gradient	0.08 – 0.13 M	1.25 ml	BSA
30 ml gradient	0.13 – 0.60 M	1.25 ml	Transferrin

Protein G purification. Monoclonal antibody purification was conducted using a pre-packed Hi-Trap Protein G affinity column and Bio-Rad Econo gradient system. The Bio-Rad apparatus included a Model EP-1 Econo pump, a model EM-1 UV monitor, and a model EG-1 gradient monitor. Protein concentrations were monitored as UV absorbance that was recorded using a LKB Bromma 2210 2-channel recorder.

The Hi-Trap column was first equilibrated with 10 bed volumes of column buffer at a flow rate of 1.0 ml/min. The column was washed with a minimum of 10 bed volumes of column buffer after sample loading, until a baseline absorbance value (280 nm) was reached. The IgG antibody was eluted in the first several collected fractions with a 0.2 M glycine-HCl (pH 2.5) solution at a flow rate of 3 ml/min. Elution fractions that contained protein based on absorbance measurement were pooled together and dialyzed in 4 L of PBS (pH 8.0).

Murine scFv Construction

A 10 ml volume of cell suspension was removed from a confluent Ma1-6 T-Flask and transferred to a 15 ml Falcon screw-top tube. Cells were centrifuged at 300 x g in a Damon IEC HN-SII clinical centrifuge for 5 min at room temperature. The supernatant was decanted and the cell pellet washed three times with ice-cold PBS (pH 7.4). Following the final wash, the cells were resuspended in 375 μl of ice-cold lysis buffer and incubated 5 min on ice to promote lysis. The lysate was transferred to 1.5 ml microfuge tubes and centrifuged at 21,000 x g at 4°C for 2 min. The supernatant was transferred to a new tube and 4 μl of 20% SDS added (final concentration 0.2%). The sample was vortexed for 30 seconds. Proteinase K was added to a final concentration of 125 μg/ml and the tube was transferred to a 37°C water bath for 15 min. A phenol: chloroform: isoamyl alcohol (25:24:1) extraction was performed, followed by a chloroform: isoamyl alcohol (24:1) extraction . An ethanol precipitation using DEPC-treated 3M sodium acetate (pH 5.2) and two volumes of absolute ethanol was stored overnight at -20°C. The sample was centrifuged at maximum speed (21,000 x g) for 15 min at 4°C. The supernatant was decanted and the pellet washed with 1 ml of 75%

ethanol and 25% 0.1 M sodium acetate (pH 5.2). The sample was again centrifuged, the pellet dried, and finally resuspended in 100 μl of DEPC-treated water. The purified RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad) and the manufacturer's protocol.

Amplification of the heavy and light chain variable domain gene was accomplished using a standard reaction mixture and combinations of forward and reverse primers obtained from the literature [(67)]. The PCR reaction consisted of 1x *Pfu* turbo buffer (20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA), 0.5 μM of both the forward and reverse primer, 125 μM of each dNTP (dATP, dCTP, dTTP, and dGTP), 2 U of *Pfu* Turbo polymerase (Stratagene), 50 ng of cDNA, and sterile water to a final volume of 20 μl. Reactions were performed in a 0.2 ml thin-walled PCR tube in a Bio-Rad MyCycler thermocycler with gradient capabilities. Annealing temperatures varied greatly for the heavy chain reactions, therefore the gradient function of the thermocycler was employed to ensure optimal temperature for each primer combination. The standard reaction consisted of 30 cycles; 95°C for 30 s, 48-59°C for 30 s, and 72°C for 30 s. A final elongation cycle of 72°C for 10 min and a hold at 4°C was also included in each thermocycler program.

Heavy and light chain variable domain genes were combined into a single fusion gene using an overlap extension PCR (oePCR) (Figure 5). The scFv template was generated in the first five rounds of PCR in which no primers were present and the complementary regions of the linker region served to initiate amplification. Exponential amplification began in cycle six, after primers for the 5' and 3' ends were added. The reaction was assembled as previously described with an equivalent quantity, 25 ng, of the heavy and

light chain DNA included as template. An initial hot start cycle of 95°C for 3 min was followed by 5 cycles of 95°C for 60 s, 70°C for 60 s, and 72°C for 90 s. The forward and reverse primers *heavy1-6F* and *kappa1-6R* were added to a final concentration of 0.5 μM each. An additional 25 cycles of 95°C for 60 s, 65°C for 60 s, and 72°C for 90 s were conducted. The final elongation cycle and temperature hold were as previously described.

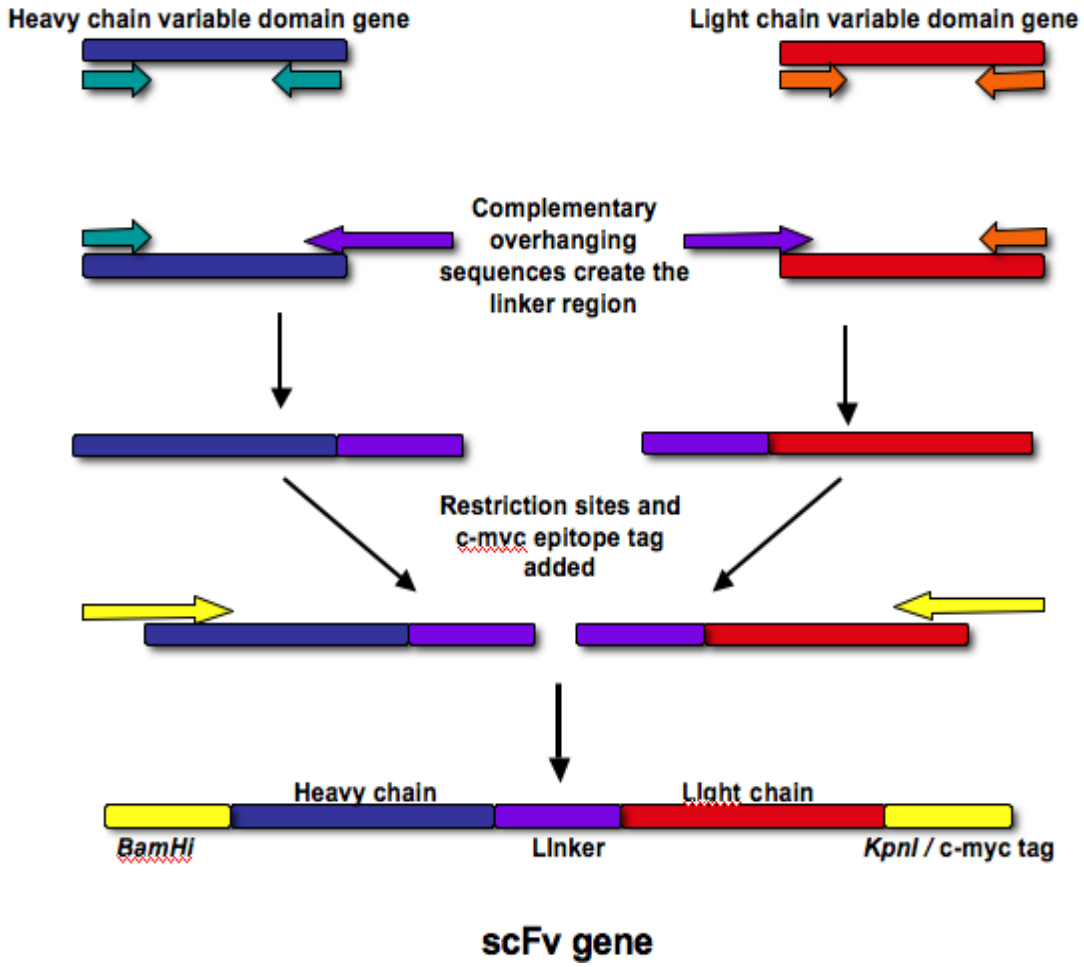


Figure 5. Single Chain Fv Antibody Construction

Schematic representation of the PCR construction of an scFv molecule. The initial heavy and light variable domain genes are isolated from a cDNA pool. Primers are designed to generate complementary overhanging regions to facilitate gene fusion and to create unique restriction enzyme cleavage sites for subsequent cloning reactions.

Preparation of B. thuringiensis Cells for Rabbit Immunization

A 3 ml culture of *B. thuringiensis* was grown in LB media at 30°C overnight and used to inoculate a 500 ml culture. The optical density of the culture was assessed periodically until the cells reached an $OD_{600} = 0.1$. The cells were pelleted via centrifugation and resuspended in PBS (pH 7.4) three times to remove residual media. A Gram stain was performed to ensure there was no contamination of the *Bacillus* culture. A malachite green stain ensured that the cells were in a vegetative state and spore development had not begun. The cell pellet was resuspended in a minimal volume of PBS. Formalin was added to a final concentration of 1.0% and followed by an overnight incubation at 4°C. The cells were again pelleted and washed repeatedly with PBS solution to remove residual formaldehyde. Aliquots were plated onto LB media to ensure complete bactericidal activity of the formalin. Formalin-killed *B. thuringiensis* were sent to Cocalico Biologicals, Inc. (Reamstown, PA) for immunization of two rabbits.

Construction of a Rabbit scFv Library

A suitable separate work area was cleaned with RNase Zap (Ambion) to reduce RNA degradation. Using sterile technique and instruments both autoclaved and treated with RNase Zap, 200 mg of spleen material was cut and transferred to a 50 ml conical Falcon tube containing 2 ml of Trizol reagent (Invitrogen). The tissue was homogenized with a Powergene 700 homogenizer (Fisher Scientific) and the RNA purified with a subsequent chloroform extraction and isopropanol precipitation. The final pelleted material, comprised largely of RNA, was resuspended in DEPC-treated water.

Rabbit RNA was converted to cDNA using the SuperScript First Strand DNA Synthesis Kit (Invitrogen) according to the manufacture's protocol. Briefly, 2.5 μg of

RNA was combined with dNTP solution (1 mM each dNTP), and 50 ng of oligo-dT primer in 10 μl of DEPC-treated water. The reaction was heated at 65°C for 5 min then transferred to ice for 1 min. A reaction mixture comprised of buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 5 mM $MgCl_2$, 10 mM DTT, and 40 U RNaseOUT (recombinant ribonuclease inhibitor) was added. The reaction was incubated at 42°C for 2 min before 50 U SuperScript II Reverse Transcriptase was added to the reaction. The sample was incubated first at 42°C for 50 min, then at 70°C for 15 min. The reaction was allowed to cool on ice before 2 U RNase H were added and the sample incubated 20 min at 37°C to remove residual RNA.

The heavy and light chain variable domain gene sequences were amplified using all possible primer combinations obtained from the literature [(57)] and cross referenced with the known sequences within the Kabat publications [(123)]. PCR was conducted in 0.2 μl thin-walled PCR tubes using a the MyCycler thermocycler and the Expand High Fidelity PCR system (Roche). The PCR reaction consisted of 300 – 500 ng of cDNA, Expand High Fidelity Buffer 2 (proprietary), 0.2 μM of each dNTP, 0.1 μM each primer, and 3.5 U of Roche High Fidelity Enzyme Mix (*Taq* and *Tgo* DNA polymerases). Reactions were subjected to an in initial hot start cycle of 95°C for 5 min followed by amplification for 25 cycles with parameters of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s. A 10 min elongation cycle at 72°C and a 4°C cycle concluded amplification.

Gel purified heavy and light chain genes were combined using an oePCR reaction as previously described. The thermocycler parameters for gene fusion included an initial hot start cycle at 95°C for 4 min, followed by 5 cycles of 95°C for 45 s, 45°C for 30 s, and 72°C for 2 min to form the scFv template. Exponential amplification was conducted for

30 cycles with parameters of 95°C for 45 s, 55°C for 30 s, and 72°C for 2 min. The reaction was concluded with the standard elongation cycle and temperature hold.

Library Transformation (Electroporation)

Electroporation of electrocompetent *E. coli* XL1-Blue (Stratagene) was used for transformation of the scFv library prior to phage display. A Bio-Rad Gene Pulser, 0.2 mm electroporation cuvettes and parameters of 25 μF, 2.5 Kv, and 200 Ω were used for electroporation. A 100 μl aliquot of electrocompetent cells was used for each individual transformation reaction. Immediately following electroporation, 1 ml of super optimal broth with catabolite repressor (SOC) was added to the cuvette and the cells transferred to a 50 ml conical tube. The cuvette was washed two additional times with 2 ml of SOC to complete transfer of the cells. The cells were allowed to recover for 1 h at 37°C before 10 ml of SB medium containing 20 μg/ml carbenicillin and 10 μg/ml tetracycline were added. A 2 μl aliquot was removed, diluted 1:100, and plated onto selective medium to determine the ligation and transformation efficiency. The liquid culture was grown an additional h at 37°C before being transferred to a 500 ml baffled flask containing 183 ml super broth (SB) medium with the selection antibiotics. A 2 ml volume of VCSM13 helper phage was added to the culture to induce phage production, which proceeded overnight at 30°C.

Transformation of the naïve rabbit library was accomplished using the highly-electrocompetent *E. coli* strain SS320 prepared through bacterial mating of *E. coli* strains MC1061 and XL1- Blue according to the methods of Sidhu *et al*. [(124)]. Electroporation was conducted using the same parameters in a BTX Electro Cell Manipulator Model

ECM630 apparatus. Electrocompetent cells XL1-Blue and SS320 cells were prepared according to Barbas III *et al.* [57].

Production of Helper Phage

The VCSM13 helper phage (Stratagene) was used to induce production of the scFv-phage fusion during the biopanning process and for phage ELISA. Phages were prepared according to Barbas III *et al* [57] and stored as 50 ml aliquots in SB medium at 4°C. Direct counts of plaque forming units and spectrophotometric absorbance measurements at 270 nm were conducted for quantitation [125].

Library Amplification

Prolonged storage at 4°C can lead to loss of scFv activity through denaturation or cleavage of the scFv from the pIII phage protein. The scFv library was, therefore, reamplified prior to any biopanning experiment to ensure that phage displayed active scFvs. The following protocol is only slightly modified from the methods of Barbas III *et al.* [57].

An aliquot of SB medium (3 ml) containing tetracycline was inoculated with 100 μl of XL-1 Blue from glycerol stocks. The culture was grown for 3 – 5 h at 37°C until an OD_{600} = 1.0 was reached. A 2 ml volume of this culture was removed and transferred to a 50 ml conical screw-top tube and then inoculated with a 50 μl aliquot of a biopanning or library phage preparation followed by a 15 min incubation at room temperature. A total of 6 ml of SB medium containing 25 μg/ml carbenicillin and 10 μg/ml tetracycline was added to the culture. A 2 μl aliquot was removed and added to 198 μl of SB medium; of this dilution, 100 μl, 10 μl, and 1μl volumes were plated onto selective medium to determine efficiency of infection. The remaining 8 ml culture was incubated for 1 h at

37°C at which point additional carbenicillin was added to adjust the final concentration to 100 μg/ml. The culture was incubated another hour at 37°C. To induce phage production, the 8 ml culture was transferred to 91 ml of SB medium containing antibiotics and 1 ml of 10^{13} cfu/ml VCSM13 bacteriophage in a 500 ml baffled flask. The culture was grown overnight at 30°C to allow phage propagation.

The overnight culture was transferred to a 250 ml polypropylene bottle and centrifuged at 4000 x g for 15 min at 4°C. The supernatant was transferred to a new 250 ml bottle to which 25 ml of a 5X Polyethylene glycol 8000/NaCl was added. The solution was mixed for several minutes then incubated on ice for a minimum of 30 min. The precipitated phages were pelleted at 15,000 x g for 30 min at 4°C. The supernatant was decanted and the bottle inverted on paper towels for 10 min to allow excess media to drain. The sides of the bottle were gently washed with 2 ml of PBS containing 1% BSA to recover the phage. This solution was transferred to a 1.5 ml microfuge tube and centrifuged at 21,000 x g and 4°C for 5 min to pellet debris. The supernatant was transferred to a new 2 ml tube for biopanning.

Selection of Target-Specific scFv (Phage Display)

Target bacteria were pelleted from an overnight culture at 6,000 x g in an Eppendorf benchtop microcentrifuge. The cell pellet was resuspended in 1 ml of PBS (pH 7.4) then washed and centrifuged three times to remove residual media. Following the final wash step, the optical density (OD_{600}) was measured as previously described.

Bacteria were diluted from the washed overnight cells to an $OD_{600} = 1.0$ in PBS buffer. A 100 μl aliquot was transferred to a 96 well Costar high binding polystyrene microtiter plate, sealed with a mylar sheet, then incubated at 37°C for 1 h . The plate was

washed two times with water to remove non-bound bacteria, sample wells were filled with PBS containing 3% BSA for 1 h at 37°C to block any remaining binding sites of the microtiter plate and limit non-specific phage recovery. The initial round of biopanning required four such wells of target bacteria, while all subsequent rounds required only two wells.

The standard phage display protocol followed the methods of Barbas III *et al.* [57]. Briefly, 50 μl of a newly prepared phage preparation were added to each of the wells containing the immobilized bacterial target. The plate was again sealed and incubated at 37°C for 1 h. The non-bound phages were then discarded and 150 μl of PBS containing 0.5% Tween 20 were added to each of the wells. The solution was mixed using an Ovation multi-channel pipette programmed to mix 100 μl volumes five times. The plate was left at room temperature for 5 min before the wash solution was discarded. The washes were repeated five times in the first two rounds then ten times in the last two rounds of biopanning. Following the last set of washes 50 μl of 0.2 M glycine-HCl (pH 2.5) was added to each well to elute bound scFv-phage from the immobilized bacteria. The solution was mixed ten times then transferred to 2 μl of 1.5 M Tris-HCl (pH 8.8) to neutralize the solution. A 2 ml aliquot of XL1-Blue culture (OD_{600} = 1.0) was inoculated with the phage elution and incubated at room temperature for 15 min to allow phage infection. The scFv pool was then amplified overnight as described above (*library amplification).*

Specificity of scFvs for target bacteria was improved using a negative selection protocol during biopanning. These experiments were conducted as described above with a slight modification to remove those scFv that recognized conserved epitopes of non-

target bacteria. In rounds three and four of biopanning, the phage preparation was incubated first with immobilized non-target bacteria for 1 h using the aforementioned microtiter plate method. The sample was then mixed five times using a micropipetter to dislodge loosely bound phage particles. The non-bound phages were then transferred to a second well of the microtiter plate that contained the target bacteria and incubated for 1 h at 37°C. The standard protocol for plate washes and amplification of target-specific scFv-phage was applied as described. The negative selection protocol was performed a minimum of two times before examination of individual clones.

The efficiency of scFv binding during the biopanning was examined at each round of selection. Following the addition of 6 ml SB medium containing antibiotics, a 2 μl aliquot of the phage amplification culture was removed and transferred to 198 μl of SB (1:200 dilution). Aliquots of 1 μl, 10 μl, and 100 μl respectively, were plated onto selective medium to determine the phage output in terms of cfu/ml. Additionally, the approximate number of phages initially used for biopanning was determined by plating dilutions of the phage preparation. Aliquots (2 μl) of the phage preparation used for biopanning were diluted in SB medium by factors of 10^7, 10^8, and 10^9, respectively, and then used to infect a 100 μl of XL1-Blue culture (OD_{600} = 1.0). A 51 μl aliquot of each culture was plated immediately onto selective medium. The percentage of bound phage was determined from a direct count of cfu on both the biopanning output and phage input (10^7 -10^9 dilutions) plates.

Assessing Biopanning Efficiency (Phage ELISA)

The phage enzyme-linked immunosorbent assay (ELISA) protocol was used to monitor the enrichment of target-specific scFvs through successive rounds of selection.

Target bacteria were prepared from overnight cultures and immobilized on a microtiter plate as previously described. The sample wells were blocked with PBS containing 3% BSA for a minimum of 1 h at 37°C prior to the addition of scFv-phage preparations. Phage concentrations were determined for each preparation by measuring OD_{270}, then diluted to $OD_{270} = 0.5$ in PBS (pH 7.4) containing 1% BSA. A 100 μl aliquot of the diluted phage was added to the immobilized bacteria, the microtiter plate was then sealed and incubated at 37°C for 1 h. The plate was rigorously washed ten times with water to displace weak and non-specific binders. An anti-M13 HRP-conjugated (Stratagene) secondary antibody was diluted 1:1000 in PBS containing 1 % BSA then incubated for an additional h at 37°C. The plate was again washed ten times with water to remove excess secondary antibody. To visualize phage binding, 100 μl of 3,3',5,5'-tetreamethylbenzidine (TMB) substrate was added to each well. The reaction was terminated with the addition of 100 μl of 1 M HCl, which produced a yellow color. Absorbance at 450 nm was measured using a Model 680 microplate reader (Bio-Rad). The Microplate Manager software was used for data acquisition and Microsoft Excel was used for graphical representation.

Colony Screening to Identify scFv

Direct method. Individual colonies from biopanning output plates were examined for the presence of the scFv gene using a colony lysis protocol and *Sfi*I restriction enzyme digest. Colonies that contained the phagemid vector and scFv gene insert were used to inoculate 3 ml LB medium containing 100 μg/ml carbenicillin. Plasmid DNA was isolated and transformed into chemically competent *Escherichia coli* DH5α using a heat

shock protocol. Small-scale expression trials were conducted to further characterize the scFv.

Plate lift assay. A plate lift assay was performed either directly from phage output plates or from overnight patch plates using the protocol of Radosevic *et al.* [126]. A Whatman Protran BA 85 membrane was placed directly on either the output or patch plate and incubated at 37°C for 1 h to facilitate transfer of bacteria to the membrane. Concurrently, a second membrane was submerged in a Petri dish containing 10 ml of $5x10^6$ cells/ml target bacteria in PBS solution. The antigen-coated membrane was incubated for 1 h at room temperature, then allowed to air-dry. Both the antigen-coated membrane and the colony replica membrane were layered onto an LB plates containing antibiotics and 1 mM IPTG plate as demonstrated in Figure 6. The plate was incubated overnight at 37°C to allow expression of the scFv and diffusion to the antigen-containing membrane.

The replica membrane was removed and transferred to a new, antibiotic-containing LB plate to maintain individual colonies. The antigen-coated membrane was dried for 1 h, then transferred to a Petri dish containing a blocking solution of PBS plus 4% dried milk. Blocking was conducted at room temperature for 1 h. The blocking solution was discarded and replaced with the primary antibody solution of a 1:1000 dilution of a mouse anti-HA antibody (Abcam) in PBS containing 0.05% Tween-20. The membrane was incubated as above for 2 h. The primary antibody was followed by a standard set of three washes as described in the immunoblot protocol. A goat anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted 1:2000 served as the secondary antibody, which

was allowed to remain on the membrane for 1 h then followed by a repeat of the washes as described. Target bound scFvs were visualized with an NBT/BCIP substrate.

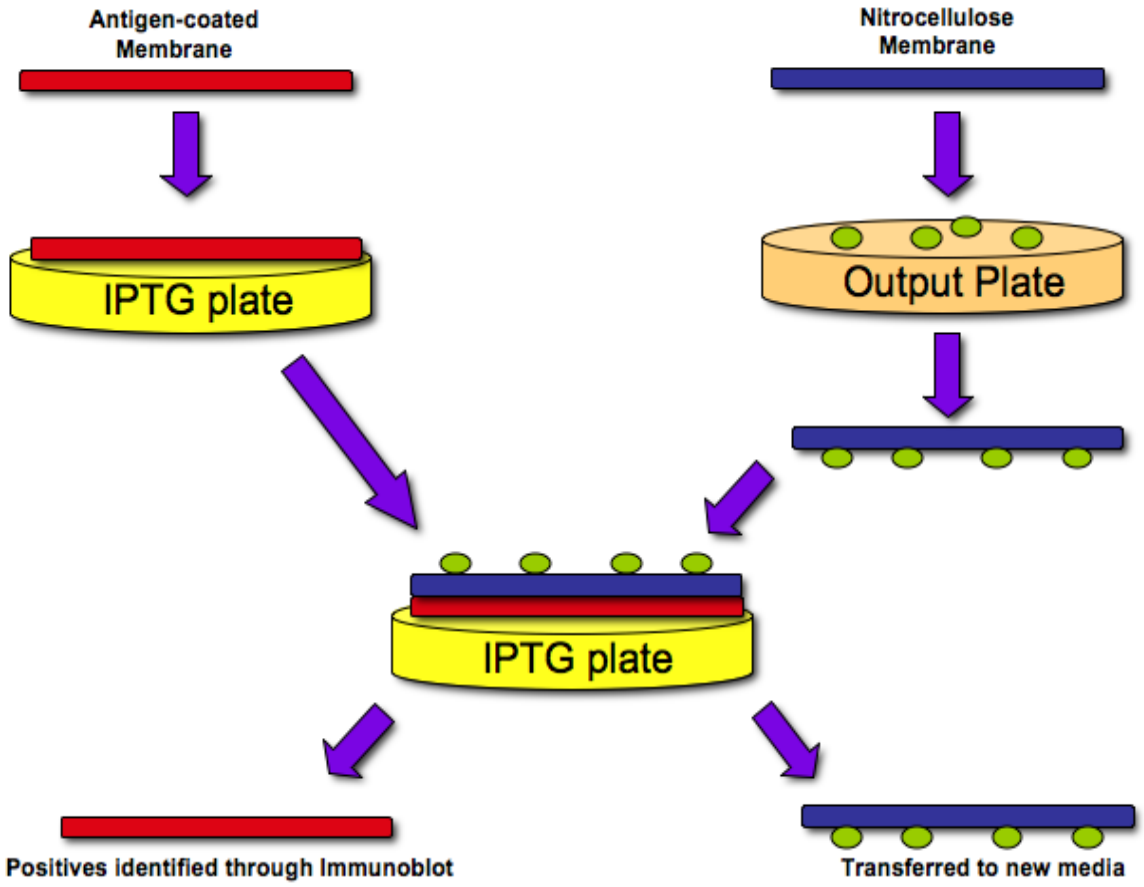


Figure 6. The Plate Lift Assay

Schematic of the plate lift assay for high throughput analysis of biopanning output plate. The protocol was utilized for the high-throughput identification of scFv specific for target bacteria.

Target bacteria were immobilized to a nitrocellulose membrane which was transferred onto an LB plate containing IPTG. A second nitrocellulose membrane was used to replicate the colonies from biopanning output plates. The replica membrane was layered atop the antigen containing membrane and all were incubated at 37°C. ScFv expressed from individual colonies diffused to the antigen-coated membrane. The antigen-coated membrane was developed using an immunoblot protocol to identify individual clones that produced scFv with affinity for the target bacteria.

Microtiter plate assay. Microtiter plate screening of phage output plates was adapted from the protocols of Bradbury *et al*. [127] to identify colonies expressing functional scFvs. Briefly, colonies from biopanning output plates were patched onto individual wells of a 96-well polystyrene microtiter plate containing 100 μl of SB medium with 100 μg/ml carbenicillin and 2% glucose (w/v). The plates were incubated at 30°C overnight in a New Brunswich Series 25 incubator shaker at 250 rpm. The following morning, a 50 μl volume of SB medium containing 2% glucose (w/v) and 30% glycerol (v/v) was added to each well to generate a master plate that could readily be stored at -80°C. A second, expression microtiter plate containing 100 μl of SB medium plus antibiotics and 0.1% (w/v) glucose was inoculated with 2 μl of culture from the corresponding well of the master plate. The plate was covered with a mylar sheet and incubated at 37°C for 2 – 3 h. A 50 μl aliquot of SB medium with antibiotics and 3 mM isopropyl ß-D-1thiogalactopyranoside (IPTG, final concentration of 1 mM) was added to each of the wells of the microtiter plate. The plate was sealed and incubated at 30°C overnight to allow for expression of the scFvs. A third antigen microtiter plate was prepared with target bacteria immobilized using the method described in the biopanning section. The expression plate was centrifuged at 3000 rpm in a Hettich Zentrigun Rotanta 460 microtiter plate centrifuge for 15 min to pellet the bacteria. Prior to addition of the soluble scFv, the bacteria-coated microtiter plate was blocked with a solution of PBS (pH 7.4) and 4% dried milk for 1h at 37°C. A 100 μl volume of the expression plate supernatant was added to each corresponding well of the antigen plate. The microtiter plate was sealed and incubated for 2 h at 37°C. Non-binding scFvs were removed by washing each well of the plate five times with a steady stream of water. A volume of 100

μl of a rat anti-HA HRP (Roche) secondary antibody diluted 1:1000 in PBS/milk blocking solution was added to each well followed by an additional 1 h incubation at 37°C. A TMB substrate and 1 M HCl stop solution were used as previously described to identify clones expressing functional, target-recognizing scFvs.

Examination of scFv Activity and Specificity (Osmotic Shock & ELISA)

Individual positive clones identified using one of the three methods described (direct, plate lift, microtiter) were used to inoculate 3 ml of SB medium containing 100 μg/ml carbenicillin. A 30 ml SB with antibiotics culture was inoculated with 100 μl of the overnight culture and grown for 3 h at 37°C. The scFv expression was induced with 0.5 mM IPTG and allowed to proceed overnight (12 – 16 h) at 37°C. Following induction, the culture was pelleted at 3000 rpm in a clinical centrifuge at 4°C. The supernatant was discarded and the pellet resuspended in 1 ml of osmotic shock buffer as described by Kipriyanov [(128)]. The cell suspension was incubated on ice for 30 min and the centrifugation repeated. The supernatant was transferred to a 1.5 ml microfuge tube.

The cells from overnight cultures of a panel of test bacteria (*Bacillus subtilis, Bacillus thuringiensis, Escherichia coli* strain DH5α*, Citrobacter freundii, Enterococcus faecium, Listeria monocytogenes, Pseudomonas fluorescens, Pseudomonas putida, Proteus vulgaris, Serratia marcescens, Salmonella enterica,* and *Salmonella enterica* serovar *Typhimurium*) were pelleted, washed three times in PBS, and quantitated via OD_{600} as previously described. Bacterial suspensions were diluted to a value of $OD_{600} = 1.0$ in PBS (pH 7.4) solution. Aliquots (100 μl) of the bacterial dilutions were added to individual wells of a 96-well microtiter plate. Immobilization of the bacteria was accomplished either through incubation at 37°C for 1 h or overnight incubation at 4°C.

Non-bound bacteria were removed from the microtiter plate by gentle washing with distilled water. The antigen plate was blocked with PBS with3% BSA for 1 h at 37°C prior to the addition of the soluble protein fraction which was isolated via osmotic shock and diluted 1:1 in PBS containing 1% BSA. The plate was sealed and incubated at 37°C for 2 h. Non-bound proteins were removed with five washes of each well with water. The rat anti-HA HRP secondary antibody was diluted 1:3000 in PBS with1% BSA. The plate incubated as previously described and then washed ten times with water to remove non-bound secondary antibody. Binding of the scFv was detected with the colorimetric TMB substrate and 1 M HCl stop solution. Absorbance at 495 nm was measured with a Bio-Rad Model 680 microplate reader, graphical analysis of data was accomplished using Microsoft Excel to assess scFv specificity.

Expression and Purification of scFv

Overnight cultures of *E. coli* strains containing the scFv gene within the phagemid vector or an alternate expression vector were grown at 37°C in the presence of the appropriate antibiotics and used to inoculate at a ratio of 1:100 50 – 500 ml of SB medium The cultures were grown for 2 – 3 h at 37°C until they reached an OD_{600} = 0.4 – 0.6 (mid-log phase of growth). Expression of scFv was induced with 0.5 – 1 mM IPTG (final concentration) and the culture maintained for 5 – 16 h at either 25°C or 37°C.

The cell culture was transferred to 250 ml polypropylene bottles and centrifuged at 4,000 x g for 15 min and 4°C. The supernatant was discarded and the pellet either stored at -80°C or directly lysed for purification. The cell pellet was resuspended in $1/20^{th}$ volume of lysis solution containing 1 mg/ml chicken egg white lysozyme (Sigma Aldrich) and a 1:20 dilution of B-PER II bacterial protein extraction reagent (Pierce). The

suspension was agitated on a rocker plate at room temperature for 30 min to facilitate cell lysis and sonicated on ice six times for 30 seconds each with a Branson sonifer 450 sonicator at a 100% duty cycle and an output control of 7. Separation of soluble and insoluble material was accomplished via centrifugation at 48,000 x g for 30 min at 4°C. The supernatant was transferred to a new tube and the pellet resuspended in an equivalent volume of PBS buffer.

The purification of scFvs was accomplished using the terminal 6xHis tag of the scFv and a Ni^{2+} - nitrilotriacetic acid (Ni-NTA)-conjugated agarose resin (Invitrogen). A 1 – 5 ml volume of the Ni-NTA resin was transferred to 2 ml microfuge tubes and pelleted via centrifugation. The supernatant was discarded and the resin resuspended in 500 μl of column Buffer A. Five to six washes were sufficient to remove all residual ethanol of the matrix storage buffer. The supernatant fraction of the cell lysate was combined with the equilibrated resin overnight at 4°C in a 50 ml conical tube using a New Brunswick Model TC-7 roller drum. To limit binding of non-scFv proteins to the Ni-NTA resin, imidazole was added to the batch slurry to a final concentration of 20 mM. The slurry was slowly added to a 1.5 x 10 cm glass Econo column. Flow rate was controlled manually with a stop cock to an approximate rate of 1 ml/min. The column was washed with a minimum of ten bed volumes Buffer A. A second wash of ten bed volumes was performed with a high-salt, low pH Buffer B to displace loosely bound bacterial proteins. An additional ten bed volumes of Buffer A was used to reduce the salt concentration. The scFv was collected using Buffer A with 250 mM imidazole (Sigma Aldrich) in three elution fractions. Fractions 1 and 2 were equal in volume to the column bed volume while

fraction 3 was twice the bed volume. Each elution was collected following a 30 min incubation of the column matrix in the presence of the imidazole solution.

For denaturing Ni^{2+}-NTA purification, the insoluble pellet material was first resuspended in 5 ml Denaturing Buffer per 1 g of cell pellet. The solution was stirred at room temperature for 1 h then centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was transferred to a 50 ml conical tube containing 2 - 5 ml of Ni-NTA resin that had previously been equilibrated in Denaturing Buffer. The slurry was incubated at 4°C in a rotator wheel overnight. Chromatography was conducted at room temperature using a Econo glass column as previously described. Following sample loading, the column was washed with a minimum of ten bed volumes of the denaturing buffer. A second series of washes was conducted with five bed volumes of denaturing Buffer A. Washing of the column with denaturing Buffer B continued until an absorbance less than 0.01 at 280 nm was attained as measured with the Nanodrop spectrophotometer. The scFv were eluted from the column with denaturing Buffer C as described above.

Renaturation of the scFvs was accomplished by a step-wise removal of the urea from the elution fractions either through dialysis or an on-column method [(129)]. In the dialysis method, the elutions were transferred to 12,000-14,000 MWCO dialysis tubing and placed in 1 - 2 L of Buffer A solution. Following a 2 h incubation a 4 °C, enough 0.01 M Tris (pH 8.0), 0.1 M Na_2HPO_4 buffer was added to dilute the concentration of urea to 6 M. The addition of buffer lacking urea was repeated a minimum of five more times, with 2 h between each dilution, until the urea concentration was negligible. For on-column renaturation the same stepwise decrease in urea concentration was performed. Prior to elution, the column matrix was washed with 5 bed volumes of Buffer A with the urea

concentration gradually reduced (6 M, 4 M, 2 M, 1 M, 0.5 M, 0.25 M, 0 urea) as above until no urea remained. The renatured scFvs were eluted with a denaturing buffer C that contained no urea.

For some experiments, scFv recovered from the Ni^{2+}-NTA column were subjected to a second affinity purification column that targeted the HA epitope tag. The column matrix was formed from the EZview Red Anti-HA affinity gel (Sigma) agarose resin to a bed volume of 0.3 – 0.5 ml. The column was washed with PBS (pH 7.4) / 0.1 mM PMSF/PTSF prior to sample loading. Eluates from Ni^{2+}-NTA purification were pooled and dialyzed in PBS to remove the excess salt and imidazole prior to column loading. The dialyzed sample was passed over the column matrix a minimum of three times to allow for scFv immobilization to the column matrix. The column was washed with ten bed volumes of PBS (pH 7.4) to remove contaminating proteins. The scFvs were eluted with a 0.2 M glycine-HCl (pH 2.3 - 2.5) solution. Fractions containing scFvs were dialyzed in PBS (pH 7.4).

Immobilization of scFvs to a Gold Electrode

Standard 1 cm 5 MHz quartz crystal microbalance slides with gold electrodes were obtained from Stanford Research Systems, Inc. (Sunnyvale, CA). Prior to use, the gold electrode was cleaned with 500 μl of a Piranha solution (3:1 H_2SO_4 : 30% H_2O_2) for 3 min. The crystal was then rigorously washed with water then ethanol and allowed to dry. A solution of 0.1 M 3-mercaptopropionic acid (Sigma) and 0.1 M 11-mercapto-undecanoic acid (Sigma) was prepared in ethanol and applied directly to the gold electrode. Formation of the self-assembled monolayer (SAM) was conducted for a minimum of 3 h at room temperature. Non-bound SAM material was removed by

submerging the crystal in ethanol prior to use. Individual crystals were removed from the ethanol and rigorously washed with water then inserted into the crystal oscillator arm. The surface was washed an additional three times with 500 μl of degassed PBS (pH 7.4) before the baseline measurement of frequency and resistance was established.

Immobilization of antibody molecules (IgG or scFv) required the activation of the carboxyl groups of the SAM using a 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 0.1 M N-hydroxysuccinimide (NHS) solution. A 500 μl aliquot of the EDC/NHS solution was reacted with the SAM for 15 min then removed and the surface washed with a single volume (500 μl) of PBS. Monoclonal IgG and scFv were attached directly to the surface through a random, free amine group of the molecule. Monoclonal antibodies were added at a saturating concentration of 3 μg of total protein based on the research of Prusak-Sochazewski *et al.* [(33)]. For the scFvs, 3 – 7 μg of total protein from elutions of Ni^{2+}-NTA purification were directly added to the surface. The antibody solutions reacted with the surface for 30 – 60 min to facilitate the formation of the amide bond. Alternatively, an indirect method of scFv attachment utilized the rat anti-HA HRP antibody that was bound to the SAM. The anti-HA antibody was diluted to 25 U in 500 μl of PBS (pH 7.4) then added to the crystal surface for 30 - 60 min. For both methods, direct and indirect, unreacted acid groups of the SAM were inactivated with a 50 mM Tris (pH 7.4) solution. ScFv immobilization was completed for the indirect method with the addition of 5 μg of total protein obtained from Ni^{2+}-NTA elutions in 500 μl of PBS (pH 7.4). The scFv solution remained on the surface for 30 – 60 min and was then discarded. The surface washed three times with PBS (pH 7.4) and a new baseline of frequency and resistance established.

QCM Detection of Target Bacteria

Baseline frequency and resistance were determined with 500 μl of degassed PBS on the crystal surface. Target and non-target bacteria were prepared from overnight cultures after washing three times in PBS (pH 7.4) buffer as previously described. The bacteria were diluted to the desired optical density, which could be correlated with an approximate cell number per ml of solution. A 10 μl aliquot of the bacterial suspension was added directly to the 500 μl volume of PBS on the crystal surface. Frequency and resistance measurements were monitored for 15 min at which point the surface was washed three times with PBS buffer to displace non-specifically bound bacteria. The final frequency and resistance measurements were determined with 500 μl of PBS on the slide surface.

CHAPTER IV

RESULTS

Experiments described herein were conducted to develop methods of rapidly obtaining target (bacterial, viral, etc.)-specific antibodies that could readily be incorporated into a field-deployable biosensor. The study examined a system of monoclonal antibody (mAb) production and several methods of producing recombinant scFvs to determine which approach would be most amenable to commercialization and also demonstrate the versatility needed to be readily adaptable to emerging threats. Both monoclonal antibodies and recombinant scFvs were integrated into a QCM-based biosensor to facilitate the detection of bacterial targets in aqueous solutions.

Monoclonal Antibodies from Hybridoma Cell Lines

Initial studies of antibody production focused on the acquisition and use of mAbs from hybridoma cell lines. Target-specific mAbs are readily isolated from hybridoma cells, immortalized lymphatic cells that can be maintained indefinitely as a cell culture. The murine hybridoma cell line Ma1-6 (ATCC CRL-1783) produces isotype IgG1 antibodies specific for an outer membrane protein H2 of *Pseudomonas aeruginosa* PAO1 strain H103 (ATCC 47085). Specificity of Ma1-6 antibody for *Pseudomonas* spp. was demonstrated by Hancock *et al.* [(130)].

Bioreactors, such as the Fibercell system used in these studies (Figure 7), allow hybridoma cell cultures to grow to significantly higher cell density compared to static flask or shaker cultures, improving productivity [(131, 132)]. The Fibercell system allows the hybridoma cells to form confluent layers on hollow polymer fibers that supply a steady source of nutrients from a circulating medium. The fibers have a low molecular weight

cut-off, ensuring that the excreted antibodies are retained within the growth chamber while small waste products are released into the circulating medium. Cell densities of 10^9 – 10^{11} cells/ml viable cells have been reported, a density three to five orders of magnitude greater than that attainable in tissue culture flasks [(133)].

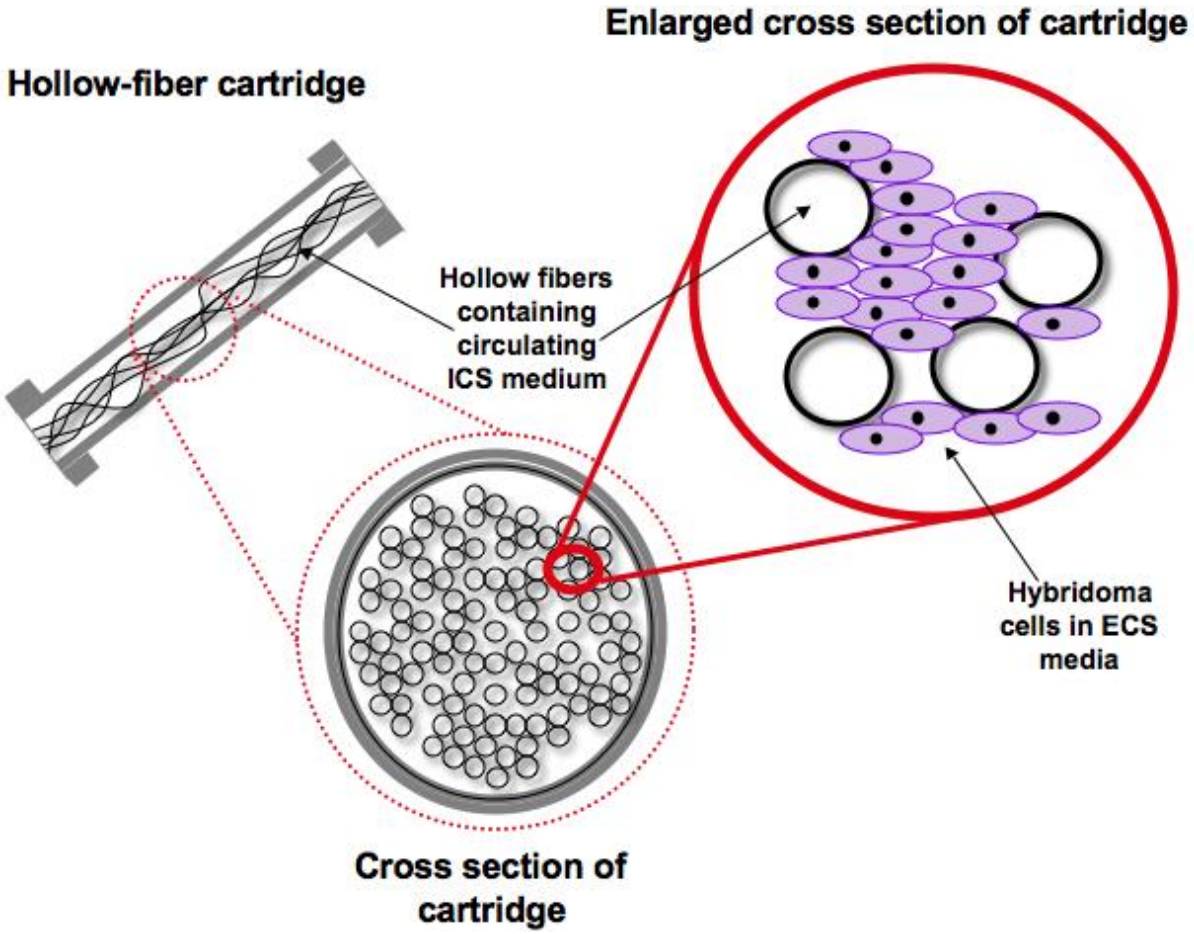


Figure 7. The Fibercell Hollow-Fiber Bioreactor
Schematic of the bioreactor hollow fiber chamber. Hybridoma cells adhere to low molecular weight cut-off polymer fibers that remove waste products and deliver nutrients via circulating ICS medium. The bioreactor chamber, in which the culture is maintained, is filled with the antibody-containing ECS medium.

Efficient monoclonal antibody production required optimization of the Fibercell system. To monitor overall health of the culture, the concentration of the primary carbon source, glucose, was measured every one to two days, which determined when both the antibody-containing extra-capillary space (ECS) medium was collected and the circulating medium replaced. As glucose levels decreased, the lactic acid concentration increased, resulting in a lower pH. As these events occurred, the overall culture viability began to wane. To ensure optimum culture vitality and mAb production, the ECS and circulating media were replaced when glucose levels decreased below 50%. Additional control of culture vitality could be exerted by adjusting the percentage of fetal bovine serum (FBS) in the ECS medium. Bioreactors were inoculated with a 10% FBS supplement of the ECS. This concentration could be gradually decreased to a final concentration of 2% FBS if the culture was expanding too rapidly, as judged by the results from the glucose assays.

Media collected from the extra-capillary space contained not only the desired monoclonal antibody, but also numerous other serum proteins including a low concentration of contaminating antibodies from the FBS and a high concentration of BSA. Initially, a purification strategy was developed that utilized a bifunctional affinity / ion exchange resin. DEAE Affi-gel Blue, an agarose resin modified with diethylaminoethyl (DEAE) functional groups and conjugated to a Cibacron Blue F3GA dye, is designed to bind the antibody through the anionic exchange capabilities of the DEAE functional groups. Serum BSA, which is retained by the Cibacron dye, requires higher stringency than the mAb to be eluted from the column.

Initial trials with the DEAE Affi-gel Blue column proved unsuccessful in removing all contaminating proteins and purifying mAbs from of the ECS medium. The high concentration of BSA saturated the Cibacron dye and was found in all fractions eluted from the column. To compensate, an ammonium sulfate precipitation was performed prior to column chromatography to remove the majority of the BSA from the serum. The precipitated proteins were dialyzed to remove excess salts then passed over the DEAE Affi-gel Blue column. This modification to the protocol improved the purity of the monoclonal antibody fractions. As shown in Figure 8**,** mAbs were eluted from the column with a low salt gradient (30 – 80 mM NaCl) over 30 fractions that were subsequently concentrated with PEG-8000 and dialyzed. The mAb eluates were free of BSA, which remained bound to the column until the salt concentration was greater than 100 mM.

The DEAE Affi-gel Blue protocol proved suitable for obtaining monoclonal IgG free of contaminating serum proteins. The initial ammonium sulfate precipitation step and the subsequent steps necessary to concentrate the antibody, however, made this method impractical for large-scale purifications. Alternative methods of concentrating the protein, such as Amicon centifugal concentrators, were not investigated but may have improved the results of this purification method.

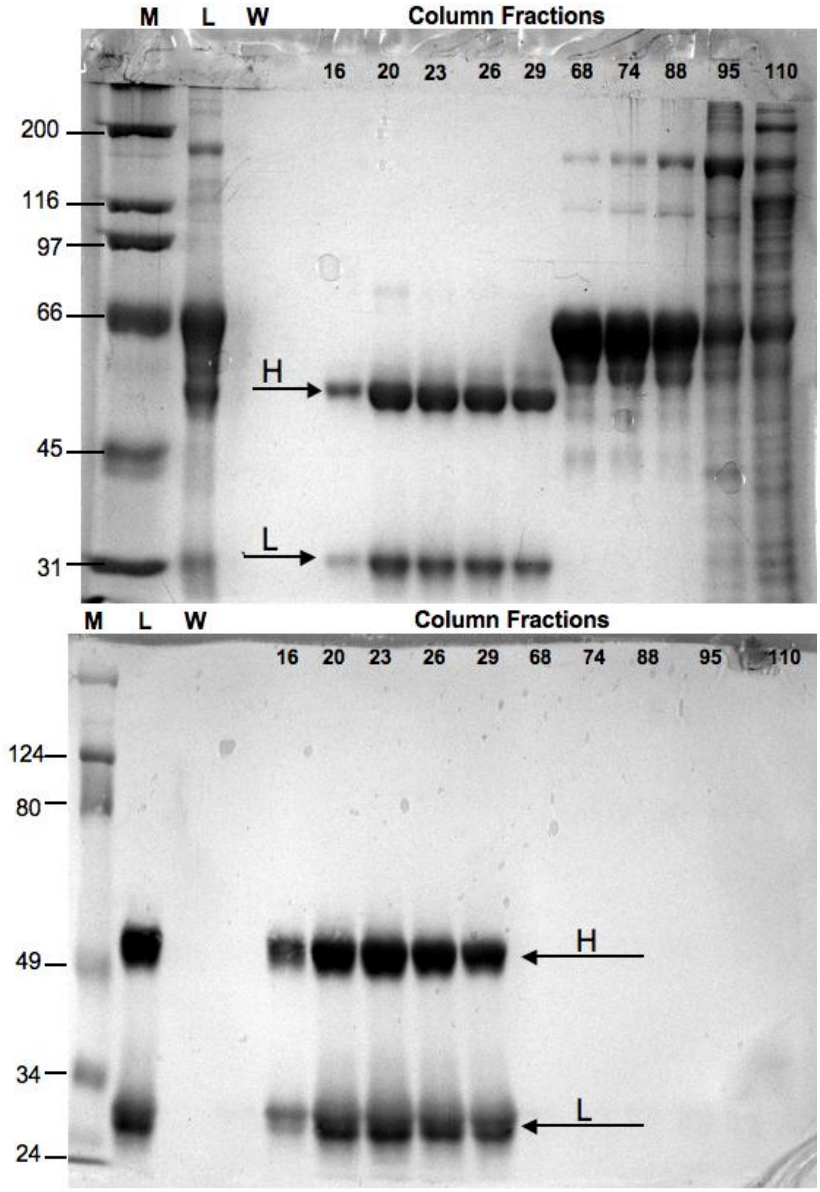


Figure 8. DEAE Affi-Gel Blue Purification of Ma1-6 Monoclonal Antibody

Antibody –containing ECS from the bioreactor was ammonium sulfate precipitated to remove excess BSA then loaded onto a 5 ml DEAE Affi-gel Blue matrix. Monoclonal antibody was separated from contaminating serum proteins using a three step NaCl gradient. Fractions from each of the gradient steps were examined using a 12% acrylamide SDS-PAGE gel. Retention of BSA (fractions 68, 74, 88) and other serum contaminants (fractions 95 and 100) is evident in the Coomassie stained gel (upper image). An immunoblot was performed to confirm the presence of mAb in the elution fractions (lower image). The heavy (H) and light (L) chains are indicated with arrows. M – Broad range molecular weight marker (Bio-Rad); L – column load; W- washes

An improved protocol that utilized a protein G–conjugated sepharose column was employed for the purification of the mAb. The Fc portion of IgG antibody molecules readily binds protein G, which is immobilized to a sepharose. This purification strategy required no initial precipitation steps to reduce the concentration of BSA; rather, the serum was diluted 1:1 with the column buffer to raise the pH of the column load. The antibody was eluted from the column with a 0.2 M glycine –HCl (pH 2.5) solution, and then dialyzed against PBS (pH 8.0) to raise the pH. The immediate neutralization of eluted fractions with 1M Tris-HCl (pH 9.0), recommended by the manufacturer, caused a significant amount of the antibody to precipitate from solution. Attempts to recover mAb from the precipitated material did not yield functional antibody. To circumvent this complication, a gradual adjustment of the pH through dialysis prevented aggregation from occurring ensuring the activity of the mAb.

Purification of the Ma1-6 antibody using the protein G column required significantly less time in sample preparation than chromatography using DEAE Affi-gel blue resin. The mAb was eluted from the Protein G column as a single fraction, therefore there was no need for concentration of the sample. As shown in the Coomassie stained gel (Figure 9), antibody could readily be obtained in a single fraction in which few contaminating proteins were apparent. Given the simplicity of this method, protein G chromatography was amenable to large-scale purification and used to obtain monoclonal antibodies for the majority of experiments.

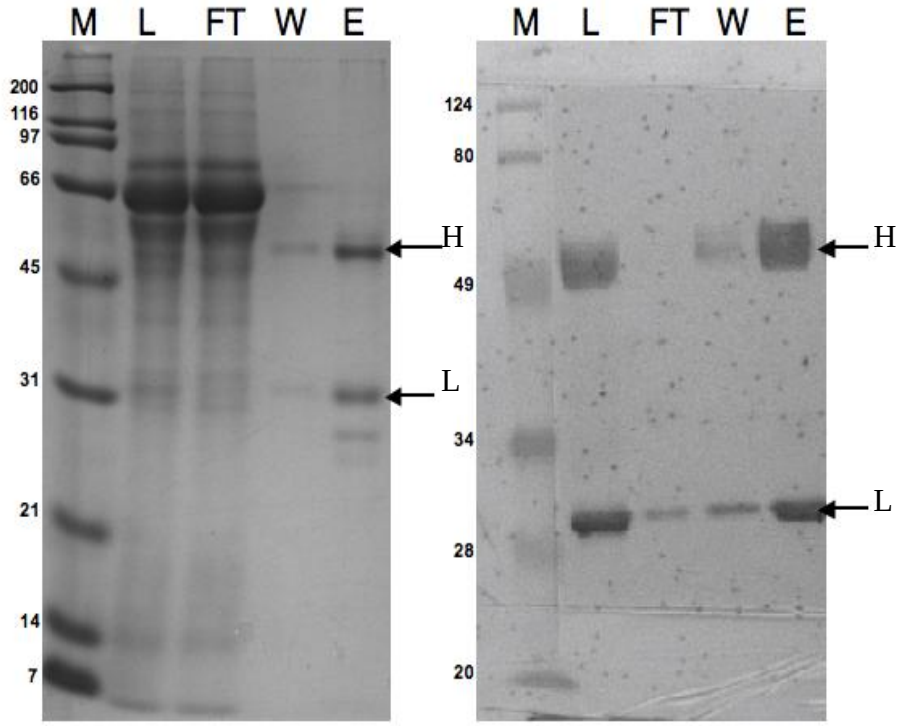


Figure 9. Protein G Purification of Ma1-6 Antibody

A 20 μl volume of the column load (L), flow-through (FT), column wash (W), and eluted fractions (E) from the Protein G column were resolved by SDS-PAGE. BSA and other serum contaminants are clearly absent in the eluted fraction as seen with Coomassie stained gel (left image). Immunoblotting using an anti-IgG antibody (right image) confirms the presence of mAb heavy (H) and light (L) chains.

Some mAb is found in the flow through and wash fractions suggesting the column was overloaded.

While maintained within the Fibercell bioreactor, the Ma1-6 hybridoma culture routinely yielded 100 mg of purified antibody per 1 L of ECS medium. A single reactor produced an average of 600 ml of ECS harvest within a one-month period. The compact size of the Duet pump and attached hollow-fiber reactors would allow several of these systems to be maintained simultaneously in a standard microbiological incubator. Cell

lines producing antibodies with varying specificities could be maintained concurrently generating sufficient quantities of mAb for integration into a biosensor.

The binding activity of the Ma1-6 antibody was examined using an immunoblot protocol in which bacteria were directly immobilized on a nitrocellulose membrane via a dot blot apparatus. Equal cell numbers of two *Pseudomonas* spp. and *E. coli* cells, as determined by optical density, were immobilized on the membrane as either live cells or after being boiled for 15 min. Defined concentrations (0.250 – 1.0 μg) of antibody purified with both chromatographic methods were evaluated for binding activity toward the *Pseudomonas* targets. Considerable affinity for both *Pseudomonas* species and limited interaction with *E. coli* was observed for antibody from both purification methods (Figure 10). The Ma1-6 antibody showed a greater affinity for heat-killed *P. aeruginosa* samples than to the live bacteria. This was attributed to the glycocalyx that surrounds *P. aeruginosa* which was likely destroyed by the heat treatment improving access to the H2 membrane protein target resulting in improved antibody binding. Antibody binding to *P. fluorescens*, which only produces a capsule during log-phase growth [(134)], is nearly equivalent for live and heat-killed samples, further evidence of interference by the capsule of *P. aeruginosa*. The interaction of Ma1-6 with live and heat-killed *E. coli* samples (Figure 10) is the product of secondary antibody interaction, which was shown in controls for other experiments. Although it allowed for the confirmation of antibody activity, the dot blot protocol could not be used for quantitation of antibody affinity via densitometric analysis of the immunoblot since excess bacteria were frequently displaced from the nitrocellulose membrane during wash steps. This phenomenon is clearly seen in the *E. coli* samples.

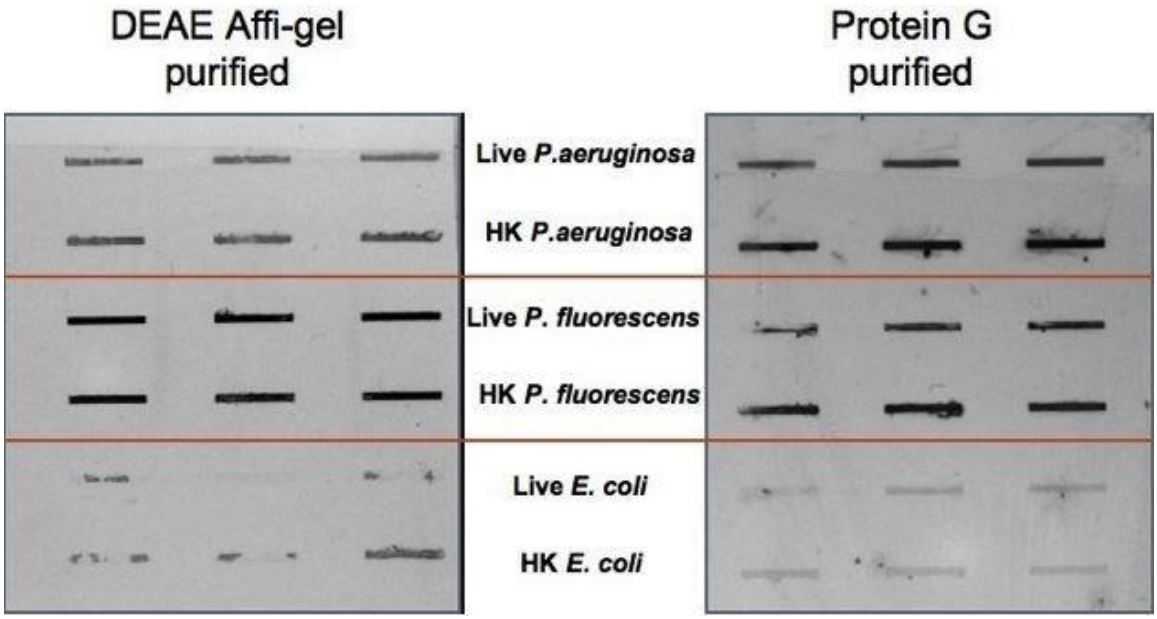


Figure 10. Dot Blot Assay to Assess Affinity of DEAE Affi-Gel Blue and Protein G Purified Ma1-6

Approximately 10^6 live or heat-killed (HK) bacteria were immobilized in triplicate to a nitrocellulose membrane using a slot blot apparatus and vacuum aspirator to assess Ma1-6 antibody affinity for bacterial targets.

Antibodies purified using both chromatography methods exhibited the expected affinity toward the *Pseudomonas* species. Quantitation of antibody binding was impossible due to the loss of immobilized antigen as evidenced by the *E. coli* controls and the variation in signal strength between replicate samples.

The Ma1-6 antibody was easily purified from the culture medium (ECS) and showed reproducible interaction with the target and non-target bacteria. Although mAb could readily be manufactured using hybridomas and Fibercell bioreactors, alternative systems of antibody production were explored for several reasons. The biosensor model proposes a device that can detect multiple bacterial targets, can readily be adapted to emerging threats, and maintains an overall low cost of production that would make the system suitable for commercialization. Generation and selection of hybridoma cell lines, even if

not performed in-house, can take from several months to a year. Since each cell line produces only one, monospecific antibody, the process would need to be repeated for each bacterial target. Additionally, once a suitable cell line has been established, the cost of maintaining mammalian cell cultures is prohibitively expensive. To circumvent these problems a system of producing recombinant proteins in bacterial cultures was investigated.

Construction of a Murine Single-Chain Fv Antibody (scFv)

The antigen-binding domain of an antibody is formed from only 100 – 110 amino acids of the N-terminal portion of the heavy and light chain genes, a region defined as the variable or Fv domain [16]. These amino acid sequences can be isolated and combined into a single fusion gene which when expressed will form a molecule capable of antigen binding [17]. The truncated molecule, referred to as an scFv, lacks the complexity of a full-length antibody and can readily be produced in bacteria [52, 54, 135].

Initial experiments to construct an scFv gene were designed to reproduce the antigen-recognizing Fv portion of the monoclonal antibody Ma1-6 and generate a recombinant antibody with similar or improved affinity for *Pseudomonas* species. Total RNA was extracted from the hybridoma cell culture using and amplified by reverse transcription to generate a cDNA pool. A pool of primers, based on conserved sequences flanking the variable domains of both the heavy and light chain genes, was designed based on information available in the literature [67]. Primer pairs were used in all possible combinations to amplify the variable domains of the target heavy and light chain genes from the cDNA pool. PCR reactions were examined by gel electrophoresis to identify those primer combinations that produced a product of approximately 300 bp (Figure 11).

Most of the light chain primer pairs yielded an amplification product of the expected size (lanes 2-7, 9, and 10), while few of the heavy chain primer pairs produced a DNA fragment of the expected size (lanes 7, 9, and 12).

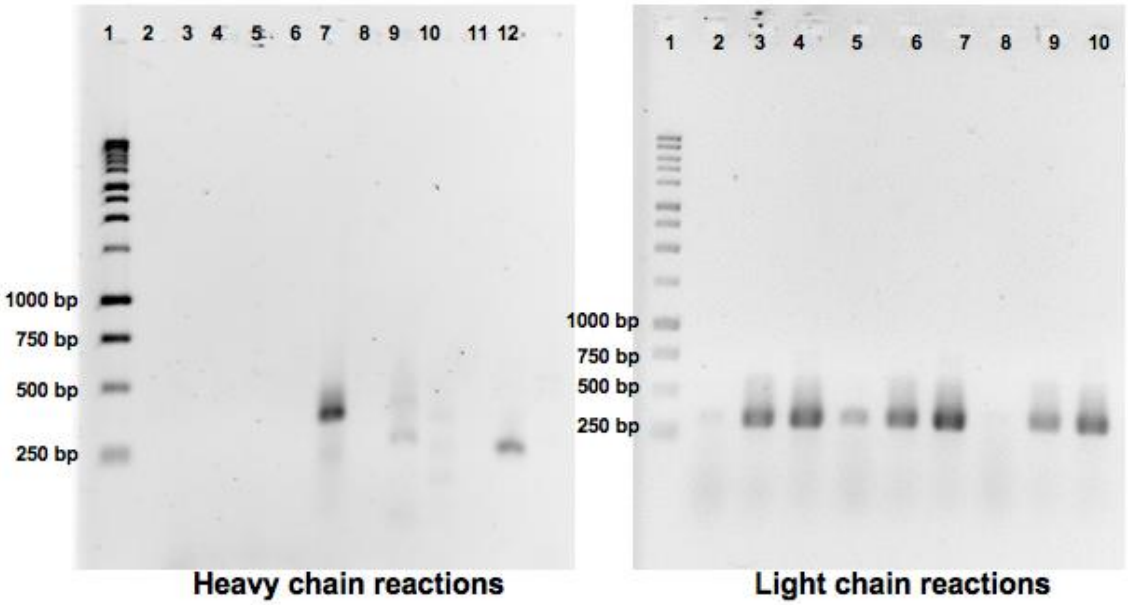


Figure 11. PCR Amplification of Murine Heavy and Light Variable Domain Genes
The heavy and light variable domain genes were isolated from the cDNA pool using numerous combinations of forward and reverse primers that were specific for the conserved regions flanking the variable domains.
PCR reactions were examined by electrophoresis on a 1% agarose TBE gel. DNA was visualized with ethidium bromide and compared to DNA standards for amplicon size estimated. Positive reactions were those that produced a product of 300 to 400 bp, the anticipated size of the variable domain sequence.

The 300 bp bands were purified from the agarose gel and cloned into the pCR Blunt II TOPO vector. Plasmid DNA containing putative gene inserts was identified using a restriction enzyme digest with *EcoRI*, which only cleaves the vector on either side of the inserted gene. The variable domain genes could not be identified strictly on size analysis of PCR products and gel electrophoresis, therefore, plasmid DNA from twelve clones was sent for sequence analysis to the University of Maine DNA Sequence Facility. Four

of the twelve heavy chain clones and all of the light chain clones except two, contained an identical gene sequence that produced an open reading frame when translated using the Transeq software (www.ebi.ac.uk/Tools/emboss/transeq/index.html). A BlastN (www.blast.ncbi.nlm.nih.gov) search of the mouse genome database was performed with the nucleotide sequences that produced an ORF to identify highly similar genes within the mouse genome. The top five genes returned by the BlastN search for both the heavy and light chain gene sequences were reported IgG variable domain genes. The DNA sequences were aligned using ClustalW (www.ebi.ac.uk/Tools/clustalw2/index.html) to determine the percent similarity between the hybridoma-derived genes and those identified by the BlastN search. ClustalX [(136)] was used for graphical representation of the alignments. The heavy chain gene sequence (clone 13) demonstrated 79 - 89% identity (Figure 12) with other known murine heavy chain variable domain sequences and the light chain (clones 4 and 7) showed 78 - 98% identity (Figure 13). The strong homology with known IgG sequences suggested that the isolated gene fragments were the variable domains of the Ma1-6 heavy and light chain antibody. In addition to *in silico* analysis, the deduced amino acid sequences of the amplified genes were further compared to murine antibody sequences of the Kabat database [(123)], confirming that highly conserved sequences of the framework regions were encoded by the isolated genes.

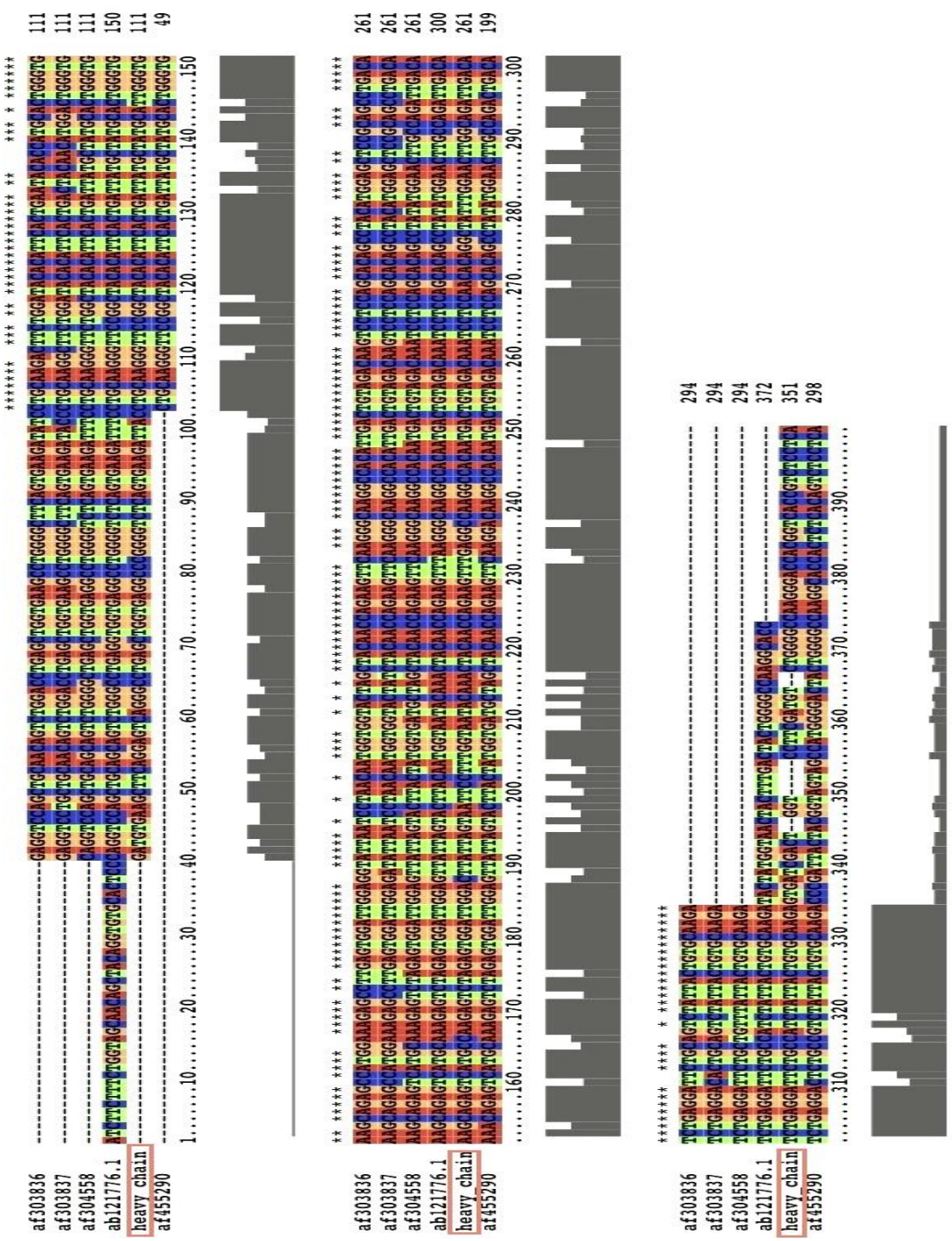


Figure 12. Alignment of Amplified Heavy Chain Variable Domain Gene Sequences

Gene sequences exhibiting high identity to the putative heavy chain variable domain sequence were identified using a BlastN search of the mouse genome. Each of the identified genes was identified as variable domain of the mouse IgG heavy chain. Multiple sequence alignments were performed using ClustalW and ClustalX programs. The amplified heavy chain gene sequence was 79 to 89% identical to other variable domain sequences.

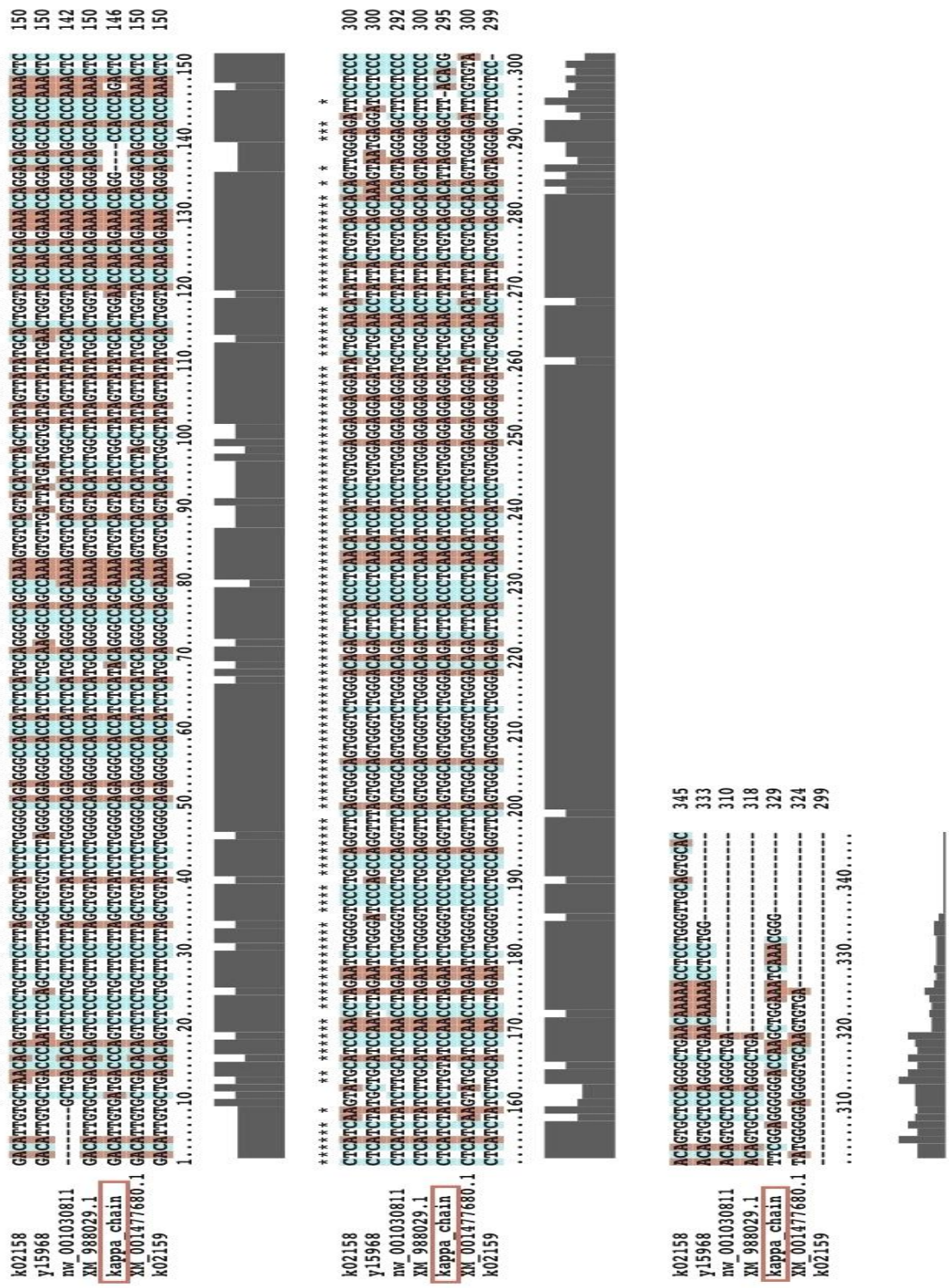


Figure 13. Alignment of Amplified Light Chain Variable Domain Gene Sequences

Gene sequences exhibiting high identity to the putative light chain variable domain sequence were identified using a BlastN search of the mouse genome. Each of the identified genes was identified as the variable domain of the mouse IgG light chain. Multiple sequence alignments were performed using ClustalW and ClustalX programs. The amplified light chain gene sequence was 78 to 98% identical to other variable domain sequences.

The scFv fusion gene was generated through overlap extension PCR (oePCR) of the single heavy chain gene and both light chain genes. Primers were designed to add a DNA sequence encoding a twelve amino acid glycine-serine repeat to the 3' end of the heavy chain coding sequence and a complementary sequence to the 5' end of the light chain coding sequence. This linker sequence served to combine the two genes through the oePCR process (Figure 5). The heavy and light chain genes were amplified from the respective plasmid DNAs and the products of these reactions purified using gel electrophoresis to ensure there were no contaminating primers that may interfere with the generation of the fusion gene template in the second round of PCR. Following the initial five rounds of amplification, which elongated the two halves of the scFv template (Figure 1), forward and reverse primers were added and the reaction continued for another 25 - 30 cycles. The heavy-light chain fusion gene (scFv) was amplified exponentially following primer addition, whereas heavy and light chain templates that did not fuse during the initial five cycles were amplified linearly with a single primer. The scFv gene of approximately 800 bp was the most prominent DNA band visible on the ethidium bromide-stained agarose gel (Figure 14, lanes 4 - 6).

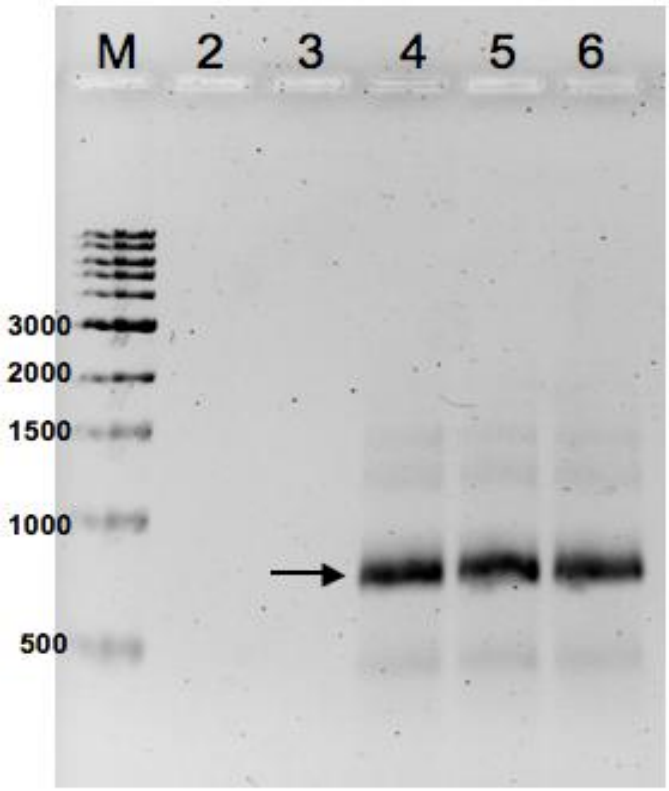


Figure 14. Generation of the scFv Gene by Overlap Extension PCR (oePCR)
The heavy and light chain variable domain genes were combined into a single fusion product using an oePCR protocol. Lanes 2 (no polymerase) and 3 (no primers) are negative control. Lanes 4 - 6 clearly depict a product of approximately 800 bp corresponding to the 767 bp, calculated size of the scFv gene (arrow).

The amplified fragment was excised from the gel, cloned into the pCR Blunt II TOPO vector, and the plasmid transformed in *E. coli* strain DH5α. Plasmid DNA from six clones was sequenced to confirm the presence of the each of the genes in the translational fusion. The terminal restrictions sites, *BamHI* and *KpnI*, and the C-terminal *c-myc* eptiope tag (EQKLISEEDL) that was added to aid in detection and purification were also confirmed. The clone scFv 4-2 was used for all future experiments.

The scFv 4-2 gene was excised from the TOPO vector by *BamHI* / *KpnI* restriction enzyme digestion and ligated into a similarly cut pProEx HTb vector. The pProEx vector system allows for inducible, cytoplasmic expression of recombinant proteins under the control of the *lac* promoter. Additionally, the pProEx vector encodes a C-terminal hexa-histidine (6xHis) sequence that can be utilized for downstream applications including immunoblot detection and purification using an immobilized metal affinity chromatography (IMAC) protocol.

Small-scale expression trials were conducted with scFv 4-2 to assess the solubility of the recombinant protein. While detectable by immunoblotting, scFv 4-2 was not visible on Coomassie stained gels, suggesting that only small quantities were produced in *E. coli*. Additionally, the vast majority of expressed scFvs was isolated as insoluble aggregates in the lysed bacterial cell pellet (Figure 15, lanes labeled P). Several different expression conditions, which included lower induction temperatures and IPTG concentrations, were attempted to improve solubility and scFv expression. These conditions, however, were not successful in improving the quantity of proteins expressed or their solubility.

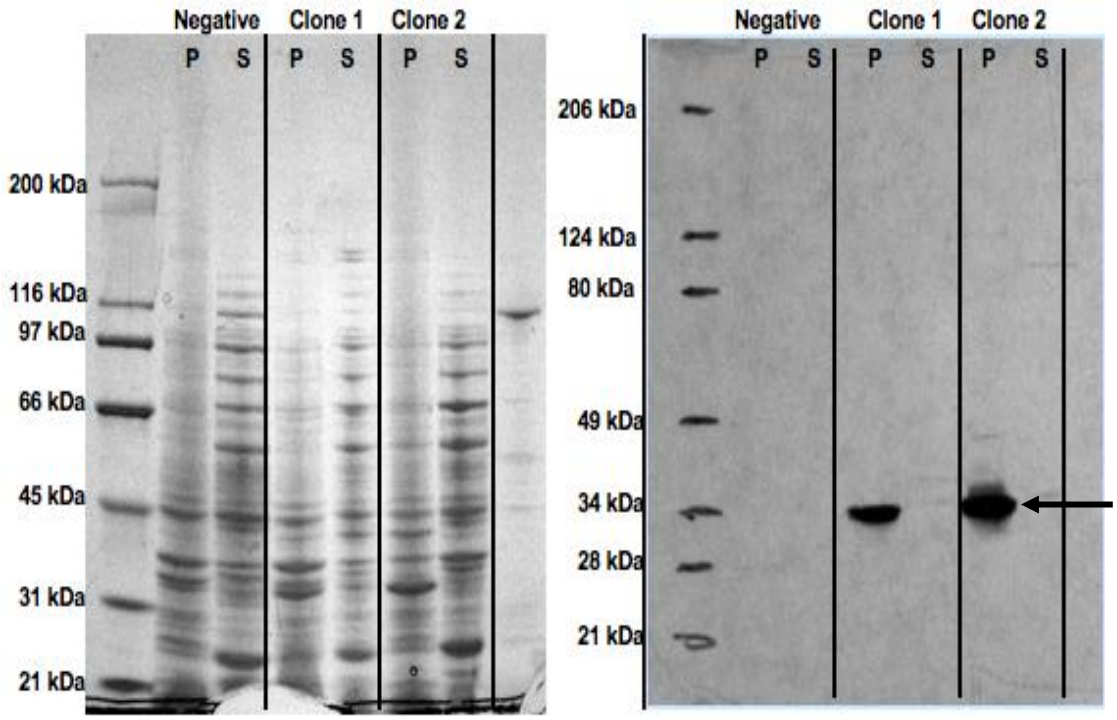


Figure 15. Small-Scale Expression of Murine scFv 4-2

Small-scale expression trials were conducted overnight at 37°C following induction with 0.5 mM IPTG. The cell pellet was disrupted via sonication and the soluble proteins separated from the insoluble material by centrifugation. SDS-PAGE gels were loaded with equivalent quantities (30 μg) of the pellet (P) and soluble (S) fractions.

The Coomassie-stained gel on the left does not show over-expression of the scFv protein at the expected MW of 35 kDa. ScFvs (arrow) are only detectable through immunoblotting (right image). Furthermore, the recombinant scFv was localized exclusively in the insoluble pellet in both of the clones examined.

To ascertain whether the mammalian scFv gene contained amino acid codons not compatible with expression in *E. coli*, its sequence was compared to the codon usage table for *E. coli* using the web-based Graphical Codon Usage Analyzer software (www.gcua.schoedl.de). *In silico* analysis discerned that the scFv 4-2 gene contained seven rare arginine (AGG or AGA) and two leucine codons (CTA) for which complementary tRNAs were not readily available in *E. coli*. Since the codon bias may have limited the expression level of scFv 4-2 in the *E. coli* strains used (DH5α and BL21)

the construct was transformed to the BL21–codon plus (RIL) strain, which expresses plasmid-encoded tRNAs specific for rare codons of arginine and leucine. A small-scale expression trial was conducted to assess whether the correction of codon bias improved solubility of the scFvs. Despite several attempts at optimization, expression of scFvs was neither improved in quantity nor in solubility in this bacterial strain.

Since the murine scFv could not be expressed as a soluble protein, it was necessary to isolate the recombinant protein from the insoluble pellet material following denaturation of the proteins with guanidinium hydrochloride. The solubilized, denatured scFvs were separated from other cellular proteins using a modified IMAC protocol that included urea in all buffers to maintain solubility. Eluates contained few contaminating proteins, however, the scFvs appeared to have been degraded significantly during the purification process evidenced by the presence of a second polypeptide of approximately 20 kDa, which was confirmed by immunoblotting to possess the 6xHis epitope tag. The 20 kDa protein was present in the elution fractions but not in the initial soluble or insoluble fractions of the bacterial cells, suggesting the potential introduction of a protease during dialysis (Figure 16).

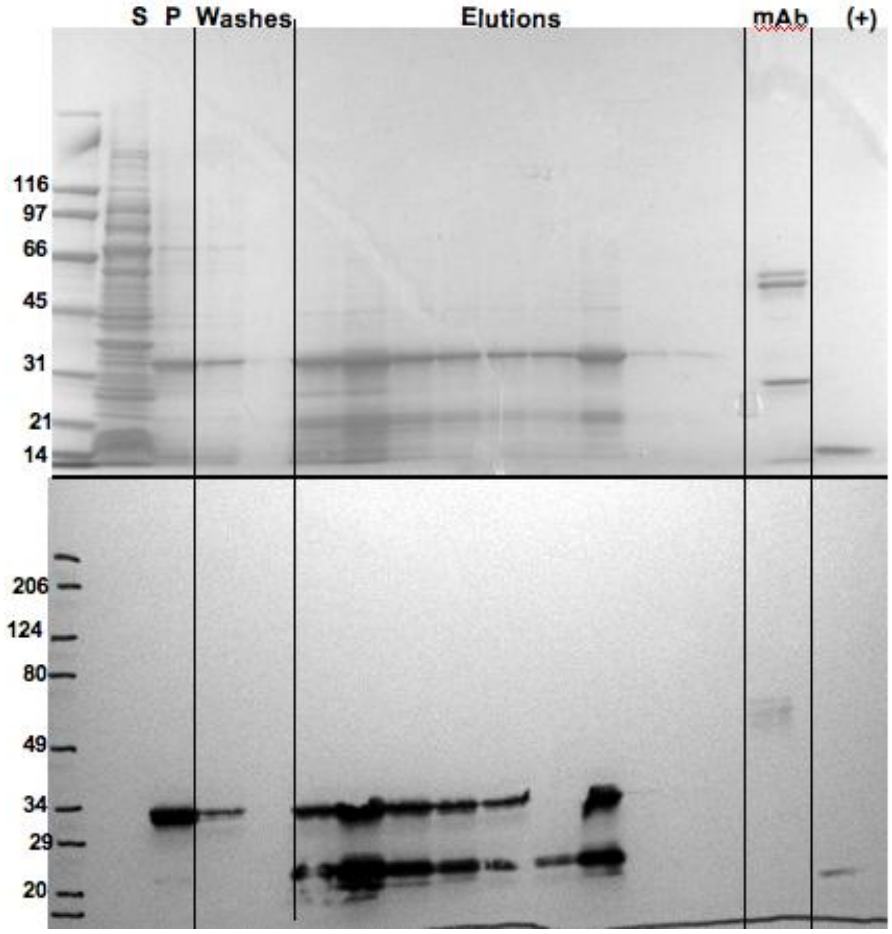


Figure 16. Purification of scFv 4-2 from the Insoluble Fraction

ScFv 4-2 was isolated from the pellet of lysed *E. coli* BL21-codon plus (RIL) using a denaturation IMAC protocol.

The eluates contained few contaminating proteins, however, some degradation of the scFv protein was observed in all of the elution fractions and may have been the result of a protease introduced during dialysis.

The initial soluble fraction (S) obtained from cell lysis and the solubilized pellet material (P) were included as positive controls. The monoclonal antibody Ma1-6 (mAb) was included as MW reference and purified recombinant His tagged *Eut* protein was included as positive control (+) for the anti-6xHis antibody.

The scFv 4-2 purified under denaturing conditions was allowed to refold by removing urea from the scFv-containing fractions through a step-wise dialysis with a 2 – 3 hour incubation in each buffer of decreasing urea concentration [129, 137].

Attempts to demonstrate binding activity of purified scFv 4-2 towards *Pseudomonas* species proved difficult. Initially, the activity of the scFvs was examined using a dot blot protocol identical to the one developed for the monoclonal antibodies (Figure 10). Detection of scFv 4-2 binding was accomplished using antibodies that targeted either the 6xHis or the *c*-myc epitope tag. Unfortunately, both of the secondary antibodies showed substantial non-specific interaction with the target and non-target bacteria by themselves (Figure 17, lane labeled (-)). Densitometric analysis of the signals suggested little binding of scFv 4-2 to the target bacteria, with virtually no interaction seen with live *Pseudomonas* samples. The highest signal intensities were only 20 to 25% above those obtained for the negative control samples (secondary, HRP-conjugated antibody only). Due to non-specific interaction of the secondary antibody, the dot blot assay was deemed unsuitable for the assessment of the binding affinity of scFv 4-2.

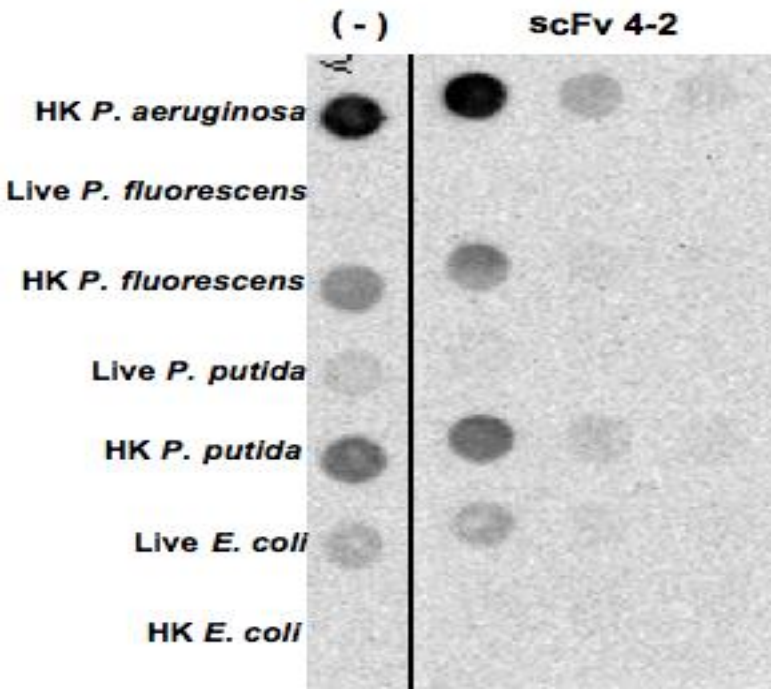


Figure 17. Target Affinity of Purified scFv 4-2 Following Renaturation

Equal numbers of live and heat-killed bacteria were immobilized in decreasing numbers (1:1 dilution) to nitrocellulose by vacuum aspiration. The scFv 4-2 purified by denaturating IMAC was examined for activity toward *Pseudomonas* species using an immunoblot protocol. The secondary antibody used for detection was a goat anti-6xHis HRP-conjugate at 1:1,000 dilution. The negative control (secondary antibody only) is aligned with the scFv-developed blot on the left side (-).

The anti-6xHis HRP-conjugated antibody bound heat-killed samples of all three *Pseudomonas* species. Densitometric analysis of the signal obtained for individual wells minus background suggested that the signal was primarily the product of secondary antibody interaction with bacteria.

To circumvent the non-specific interactions experienced with the dot blot, the outer membrane proteins of *P. aeruginosa* were isolated from lysed bacterial cells that were separated using a sucrose gradient and ultra-centrifugation according to protocols reported in the literature [(119-122, 138)]. Previous dot blot assays visualized antibody binding to the bacterial surface, which was often complicated by non-specific interactions

(Figures 10 and 17). Separation of outer membrane proteins via SDS-PAGE and subsequent immunoblotting would allow for identification of scFv binding to the Ma1-6 target, outer membrane protein H2.

The cell lysate fractionated into three distinct regions (based on turbidity and color) within the gradient that were isolated and resolved by SDS-PAGE. Immunoblots were developed with either the Ma1-6 mAb or with scFv 4-2 in combination with either the anti-6xHis or anti- *c*-myc secondary antibody. The membrane developed with Ma1-6 clearly identified the 20 kDa outer membrane protein H2 in all sucrose gradient samples, however, there was no corresponding signal on either of the membranes developed with scFv 4-2 (Figure 18). Based on these results and those obtained with the dot blot assay, it was concluded that scFv 4-2 did not renature to yield a functional recombinant antibody.

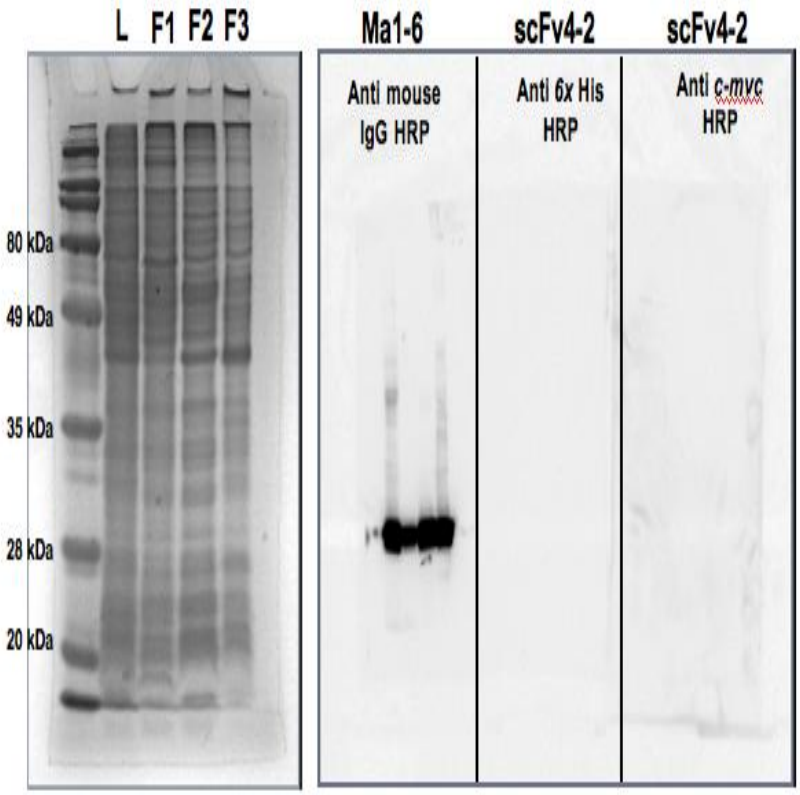


Figure 18. Analysis of ScFv 4-2 Binding Affinity Towards Outer Membrane Proteins of *P. aeruginosa*

Membrane proteins of *P. aeruginosa* were fractionated using a sucrose gradient. The cell lysate (L) and fractions (F1 – F3) containing the membrane protein H2 were separated on a 12% denaturing, polyacrylamide gel.

A Coomassie stain of the membrane fractions is displayed on the left.

Immunoblots were developed with either Ma1-6 or scFv 4-2 as the primary antibody. Secondary antibodies used for visualization are listed on each of the blots above.

The murine scFv 4-2 was generated from a single hybridoma cell line, which did not produce a soluble scFv in bacterial expression trials. The scFv could be recovered from the insoluble material of expression cultures, however, the scFv was unable to bind the target bacteria following renaturation. Construction of the scFv from a hybridoma cell line was a low-throughput method that required a significant investment of time and resources. To expedite obtaining scFvs with the desired characteristics of target

specificity and solubility, a larger diverse antibody repertoire, or library, was constructed. In conjunction with a high-throughput method of selection, the scFv library will provide for a method of isolating scFvs with the desired target-specificity and affinity from a highly diverse pool. This method would eliminate the time delays associated with hybridoma development and selection, circumvent the potential complications encountered with scFv 4-2, and improve the adaptability of the biosensor model by allowing for the selection of scFvs against different bacterial targets.

Construction of an Immunized scFv Library

To facilitate the construction of the scFv library, the spleen, a primary lymph node, from a rabbit immunized with formalin-killed *Bacillus thuringiensis* was obtained. Two rabbits, demonstrating no significant serum antibodies toward the bacterial target, were chosen for the immunization process. Following four rounds of inoculations with formalin-killed bacteria, a final bleed was examined using the dot blot protocol. The antibodies of these final bleeds demonstrated significant antigen recognition capabilities as evidenced in Figure 19. The rabbits were exsanguinated and a spleenectomy was performed.

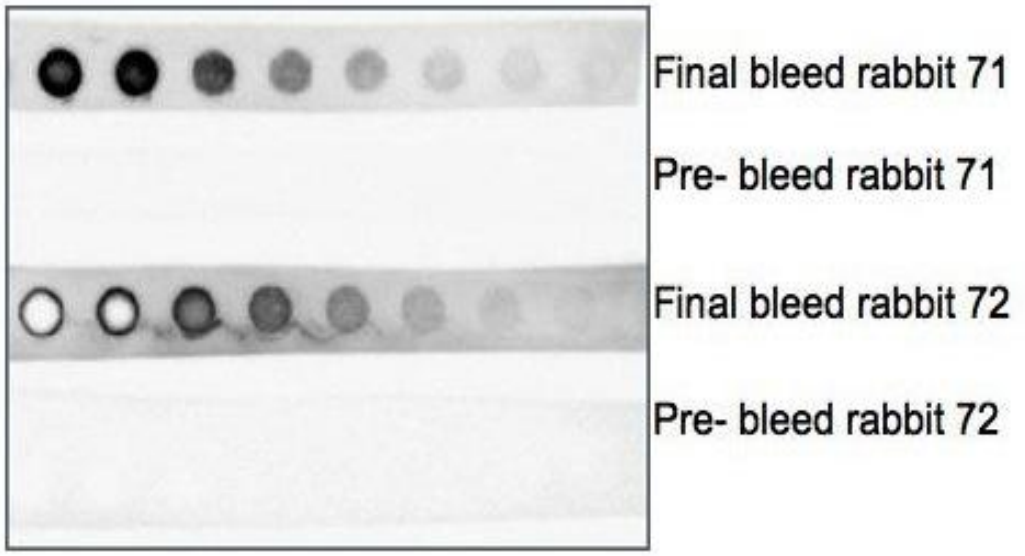


Figure 19. Enrichment of Target-Specific Antibodies through the Immunization of Rabbits with Formalin-Killed *B. thuringiensis*

The serum of non-immunized rabbits was tested to ensure the absence of pre-existing antibodies reactive toward *B. thuringiensis*. Two rabbits were subjected o four rounds of inoculation with formalin-killed *B. thuringiensis* to generate a significant immune response. Final bleeds were compared to the initial pre-bleed samples using a dot blot protocol to demonstrate the increase in antibody recognition of the bacterial target.

Total RNA was extracted from the rabbit spleen and converted to cDNA using the iScript First Strand DNA Synthesis Kit. The product of the cDNA reaction served as template for the subsequent amplification of the variable domain gene sequences. Primer combinations and PCR reaction conditions for amplification of the heavy and light chain genes were obtained from Barbas III *et al.* [(57)]. Despite attempts to optimize reaction condition, not all of the primer combinations produced a PCR product of the expected size as seen in Figure 20. Similar to the construction of the murine scFv, the majority of the light chain primer combinations were successful (lanes 1-8, 10) while many of the heavy chain reactions failed (positives 2-5, 8-11, 15-16). This observation was deemed

inconsequential since rabbits predominantly rearrange a single heavy chain gene, therefore not all primers would be expected to succeed [(64)].

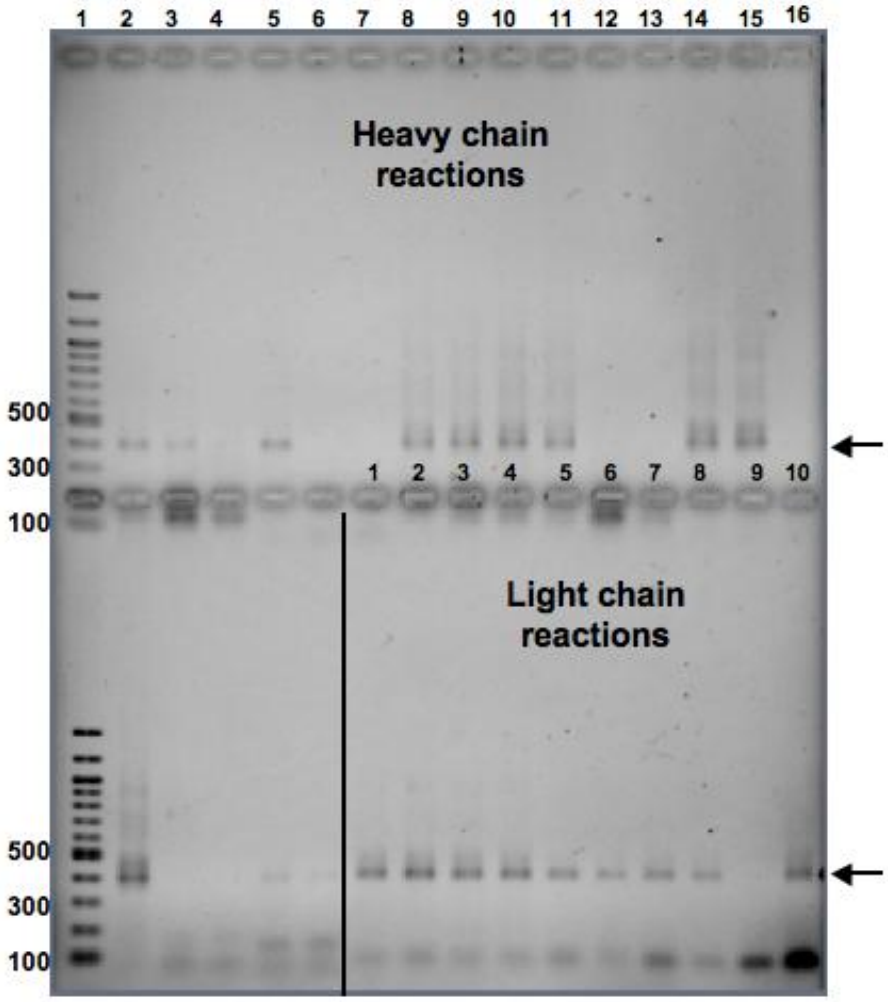


Figure 20. Amplification of Rabbit Heavy and Light Chain Variable Domain Genes

The heavy and light chain variable domain sequences were isolated from a cDNA pool constructed from RNA isolated from a rabbit spleen. PCR products were examined by electrophoresis on a 1% agarose gel. Ethidium bromide staining was used to identify successful reactions, those with a product of approximately 400 bp.

Each lane of the gel represents a different combination of primer sequences. The majority of the light chain reactions were successful (lanes 1-8, 10) while no amplification occurred with several of the heavy chain primer combinations (positives 2-5, 8-11, 15-16).

Although there is no way to accurately calculate the complexity of an scFv library, it can be hypothesized that the diversity cannot exceed the number of transformants obtained following a ligation transformation reaction [57]. Since the diversity of the library directly contributes to the potential of isolating scFvs with the desired target-specificities and affinities, immunized libraries with calculated diversities of $10^5 – 10^7$ are generally sought. Constructing highly diverse libraries often requires numerous ligation transformation reactions, therefore, small-scale (20 μl) control ligation-transformation experiments were conducted to determine the number of large-scale (100 μl) reactions that would be required to obtain a library with sufficient diversity. Based on the number of colonies of the transformation output plates, six large-scale ligation reactions were assembled. Transformation was accomplished through electroporation of XL1-Blue electrocompetent cells. Direct counts of the output plates suggested an estimated diversity of $7x10^6$ clones, however, restriction digest analysis of individual clones determined that only 70% of the clones of the library contained the scFv gene insert for a library size of $4.9x10^6$. Although the diversity of the initial library was smaller than expected, biopanning was conducted against *B. thuringiensis* to isolate a target-specific scFvs and to establish protocols for phage display and the process of scFv selection.

The phage display system was utilized to isolate scFvs specific for target bacteria from both the immunized and naïve scFv libraries. To isolate target-specific scFvs from these libraries, biopanning was conducted against live bacteria immobilized to a 96-well microtiter plate. This method of immobilization ensured that surface epitopes of the bacteria would be in their native conformation within the bacterial cell outer membrane or peptidoglycan layer. Additionally, preliminary antigen purification steps would not be

required since the bacterial surface offered a large number of potential epitopes compared to a single purified antigen. This would expedite the isolation of target-specific scFv for new target bacteria. Unless otherwise stated, four rounds of selection were conducted with increasing stringency of washes against the bacterial antigen.

Individual clones were isolated from biopanning output plates and screened using several different methods throughout the project (Figure 21). In most instances, plasmid DNA was extracted using a colony lysis protocol and digested with *SfiI* restriction enzyme to identify clones containing the scFv gene. As will be demonstrated, non-specific interactions between the M13 filamentous phage and the bacterial antigens required a large number of individual colonies to be examined to identify those containing the scFv gene. High-throughput methods such as the plate lift assay and the microtiter plate assay were utilized in some trials to expedite the identification of clones producing a functional scFv. Once a clone was identified, preliminary trials to assess solubility and specificity of the scFv were conducted. In the initial biopanning trials, solubility was examined first for clones containing the scFv gene using SDS-PAGE and immunoblotting. Soluble scFvs were then purified using affinity chromatography and examined for specificity via ELISA. In later experiments, specificity was considered a priority and examined using scFvs that could rapidly be isolated from the periplasmic space. Although acquired in limited quantities, the scFvs obtained using osmotic shock could directly be tested for specificity and affinity for target bacteria via ELISA. The osmotic shock protocol prevented time delays associated with purifying scFvs that failed to demonstrate desired binding characteristics.

Several different ELISA-based protocols are utilized throughout the biopanning process. In general, phage ELISA protocols utilized equivalent concentrations of phage from each round of biopanning that were diluted to equal OD_{270} measurements in a 1% BSA solution. Specificity ELISAs for individual scFvs were conducted using either affinity purified scFvs or scFvs isolated from the periplasmic space. For both of these preparations, the precise quantity of scFv could not be determined. For affinity purified scFv preparations, elution fractions were diluted to 3 μg/ml in PBS/ 1% BSA for analysis. The osmotic shock solutions were also diluted in PBS/ 1% BSA at a ratio of 1:1 prior to analysis. These dilutions were used in all experiments to maintain some level of consistency. However, as was seen in later trials, there was significant variation in the maximum absorbance values between individual clones. These observations may be indicative of the affinity of the scFv, the product of poor expression or solubility, or some other condition. However, since experimental conditions of expression and purification are identical for each clone, comparison of scFvs between experiments could be made.

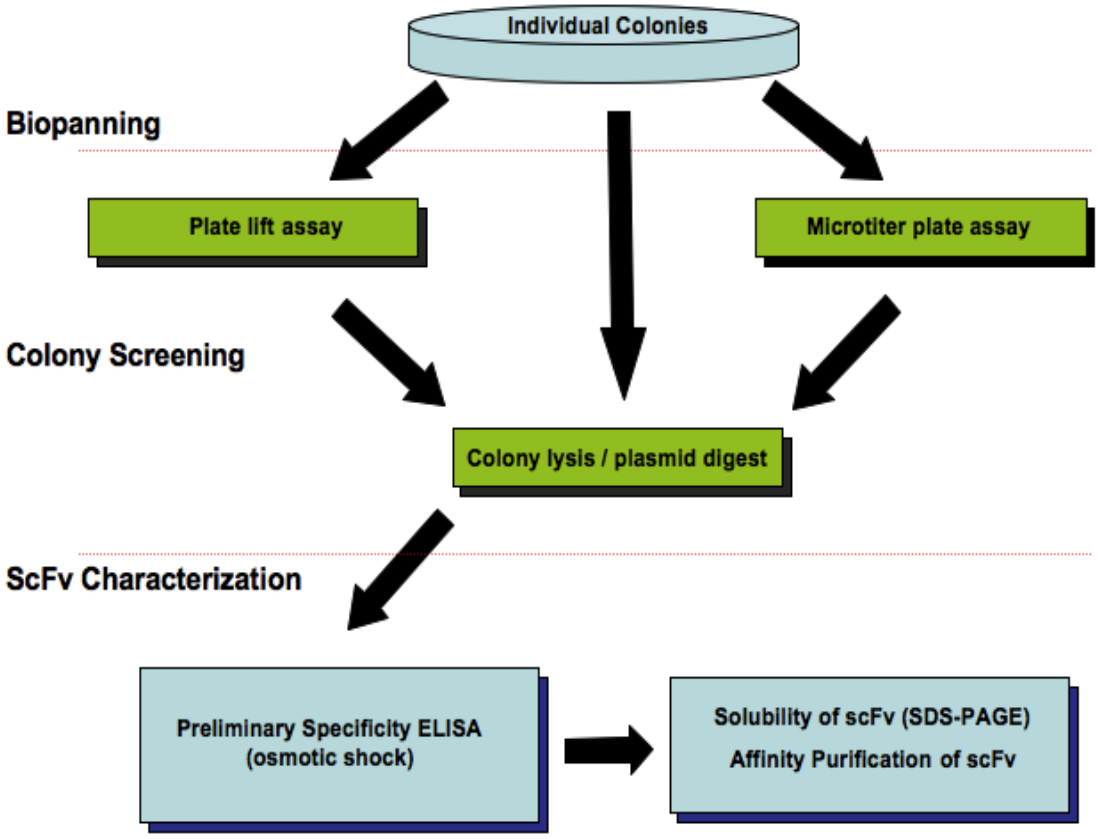


Figure 21. Flow Chart of the Colony Screening Methods

Individual clones from biopanning rounds were screened using one of three methods; colony digest, plate lift, or a microtiter assay. Soluble proteins extracted from the periplasmic space were tested for target specificity prior to more extensive characterizations that required affinity purification of scFvs.

Bacillus thuringiensis Biopanning

A total of 72 clones were screened from the last round of biopanning. A significant number of clones, approximately 90%, contained either no gene insert or a digest product smaller than anticipated (~ 300 bp). Only 10% of the clones examined contained a digest product of 800 bp, the correct size for a complete scFv gene (Figure 22, lane 13).

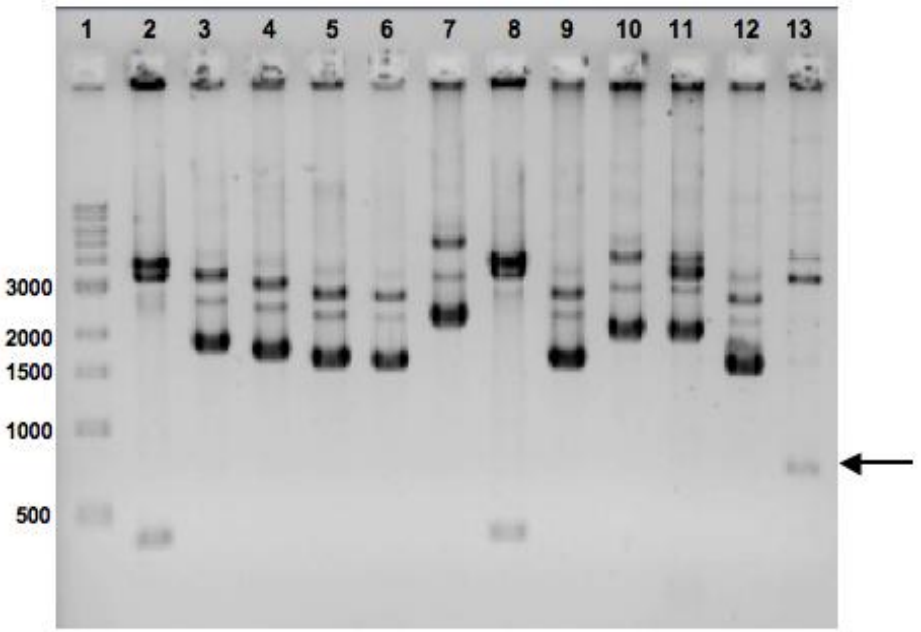


Figure 22. Restriction Analysis of Clones from *B. thuringiensis* Biopanning Round Four

Plasmid DNA from individual colonies of biopanning round 4 output plates were obtained using a direct colony lysis protocol. DNA was digested with the restriction enzyme *SfiI* which cleaves at the 5' and 3' ends of the scFv gene. Digest products were separated on a 1% agarose gel and visualized using ethidium bromide staining.

A minimal number of colonies (lane 13) exhibited a digest product of 800 bp, the anticipated size of an scFv gene (arrow).

Plasmid DNA from clones containing the potential scFv gene was transformed into the suppressor strain DH5α in which the scFv is expressed but translation of the pIII protein is inhibited. Targeting of scFvs to the periplasm was maintained by the *ompA* leader sequence encoded by the phagemid vector. Expression was conducted overnight at

37°C, followed by sonication to lyse cells and centrifugation to separate soluble and insoluble fractions. Solubility of the scFv was assessed via SDS-PAGE and immunoblotting. Poor solubility was observed for the majority of the clones. Of the five clones examined from round four of biopanning, a single clone, k18, exhibited soluble scFvs (Figure 23). The scFvs were isolated from the soluble fraction using nickel affinity chromatography (Ni^{2+}-NTA).

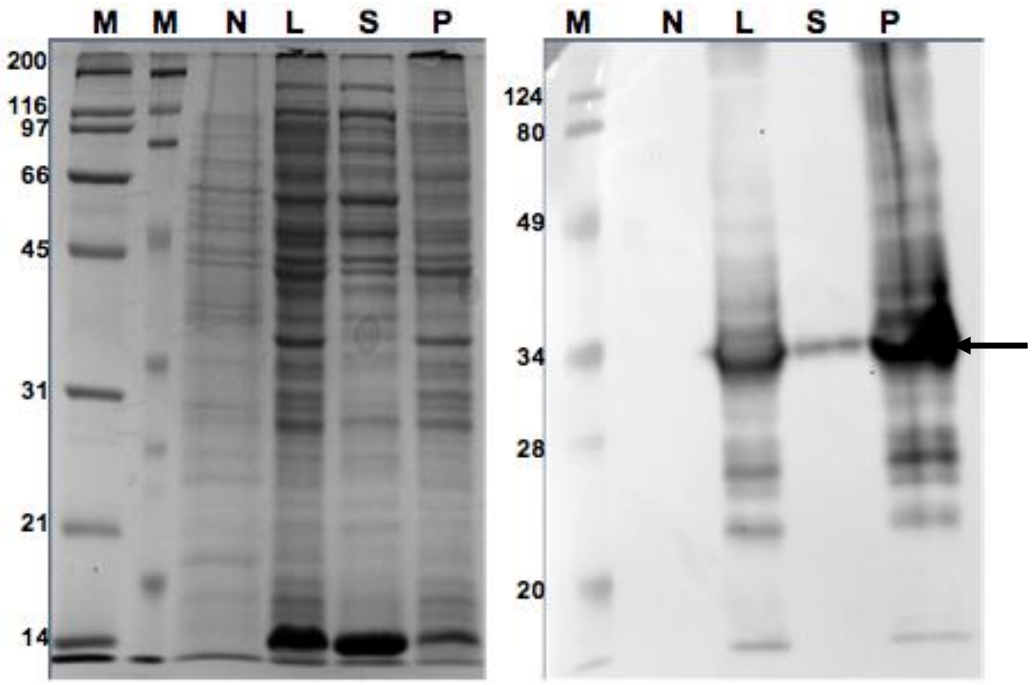


Figure 23. Bacterial Expression of scFv k18

ScFv k18 was expressed in *E. coli* strain DH5α overnight at 37°C following induction with 1 mM IPTG. The cell culture was pelleted, lysed via sonication, and centrifuged to separate soluble and insoluble material. Protein concentration was determined via BCA for the lysate (L), supernatant (S), and pellet (P) to ensure equivalent loads (25 μg) for SDS-PAGE. Non-induced (N) and induced (L) cells were included as negative and positive controls, respectively.

No over-expression of scFv k18 is apparent in any fraction of the expression culture as seen on the Coomassie stained gel (left). Expression of scFv k18 was confirmed through immunoblotting (right with arrow)

Elution fractions from the Ni^{2+}-NTA column contained a considerable number of contaminating proteins (Figure 24). Accurate quantitation of the scFvs was impossible due to contaminating proteins present in higher concentrations of the elution fractions as seen in the Coomassie stained gel. Regardless, sufficient quantities of scFvs were obtained to allow for ELISAs to determine bacterial specificity.

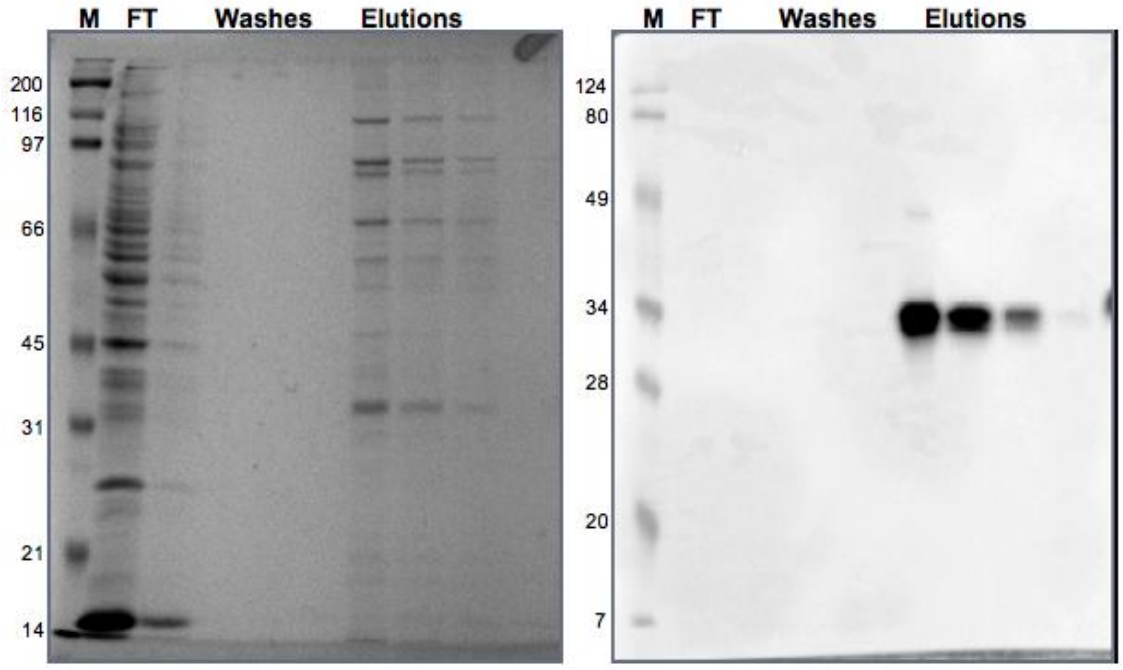


Figure 24. Purification of scFv k18 via Affinity Chromatography

The soluble proteins isolated from an overnight expression culture were subjected to Ni^{2+}-NTAaffinity chromatography to isolate scFv k18. The flow-through (FT), washes, and elutions were resolved on a 12% acrylamide well by SDS-PAGE.

ScFvs were not visible in the elution fractions, which did contain numerous other contaminating proteins (Coomassie stained gel on left). The scFv k18 was visualized in all elution fractions by immunoblotting (right image)

The specificity of scFv k18 was assessed using a panel of bacteria that included the target bacterium *B. thuringiensis* and several non-target bacteria. The scFv k18 exhibited varying affinities for several bacteria of the panel (Figure 25). Although the greatest non-

target interaction occurred with *L. monocytogenes*, a Gram positive bacteria of the panel, substantial affinity was also shown for Gram negative bacterium. The scFv did not consistently recognize a particular bacterial category (enteric, soil, marine, etc.), therefore, affinity for the other non-target bacteria could not readily be explained.

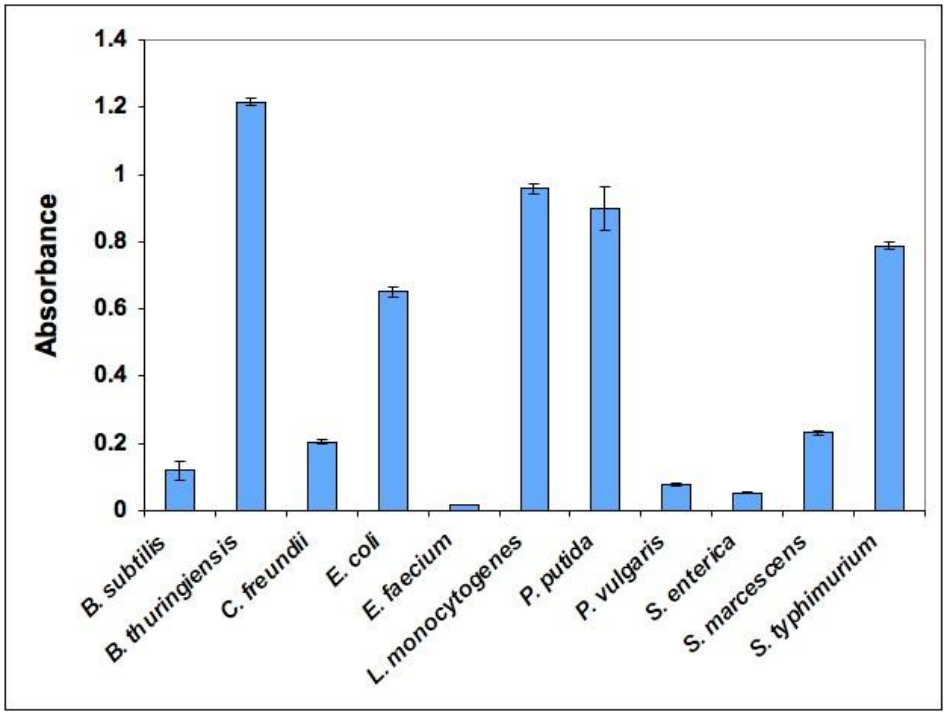


Figure 25. Analysis of the Bacterial Specificity of scFv k18 via ELISA
ScFv k18 was purified from the soluble fraction an overnight expression culture using Ni^{2+}-NTA chromatography. The scFv k18 readily bound the target bacterium *B. thuringiensis* and several other non-target bacteria of the panel.

A multi-specific scFv would result in numerous false positives when incorporated into the biosensor. To improve the specificity of isolated scFvs a negative selection protocol was employed during biopanning. *E. coli* was chosen as the non-target bacterium for several reasons. The scFv k18 demonstrated significant affinity toward *E. coli* in ELISA, which suggested a potentially abundant epitope present on both *B. thuringiensis* and *E.*

coli. Secondly, *E. coli* is a gram-negative bacterium in contrast to the gram-positive classification of *B. thuringiensis*. An initial selection against a gram-negative bacterium may eliminate those scFvs specific for carbohydrate and other epitopes of the lipopolysaccharide layer (LPS) of gram-negatives. Third, *E. coli* is an enteric bacterium while *B. thuringiensis* is found within the soil. The surface structures of the two bacteria would be adapted to survival in their particular habitat; the negative selection would potentially eliminate scFvs targeting conserved epitopes of enteric bacteria.

The initial library was subjected to four rounds of standard biopanning and two rounds of negative selection with *E. coli* as the non-target antigen. To circumvent delays associated with the low-throughput direct method of colony analysis, a plate lift assay was utilized. Phages were amplified from the last round of negative selection and serial dilutions of the culture were plated onto selective media to produce output plates with 20 – 50 cfu. Colonies were replicate plated to nitrocellulose membrane and the activity of their scFvs examined against live *B. thuringiensis* using the protocol of Radosevic *et al.* [(126)]. Seven of the twenty clones present on the output plate produced scFvs that bound the bacterial target. These clones were subjected to small-scale protein expression trials. ScFv isolated from the periplasmic space were tested using a whole-cell ELISA protocol against a limited number of bacteria (*B. thuringiensis, P. putida, S. enterica,* and *E. coli*) to assess specificity (Figure 26). Of the seven examined, four (b1, b3, r1 and r2) demonstrated no activity toward any of the bacterial targets. The scFvs from clone b2 lacked specificity, binding all four bacterial targets with varying affinity similar to scFv K18. Two clones, s24 and s25, expressed scFvs that recognized only *B. thuringiensis*.

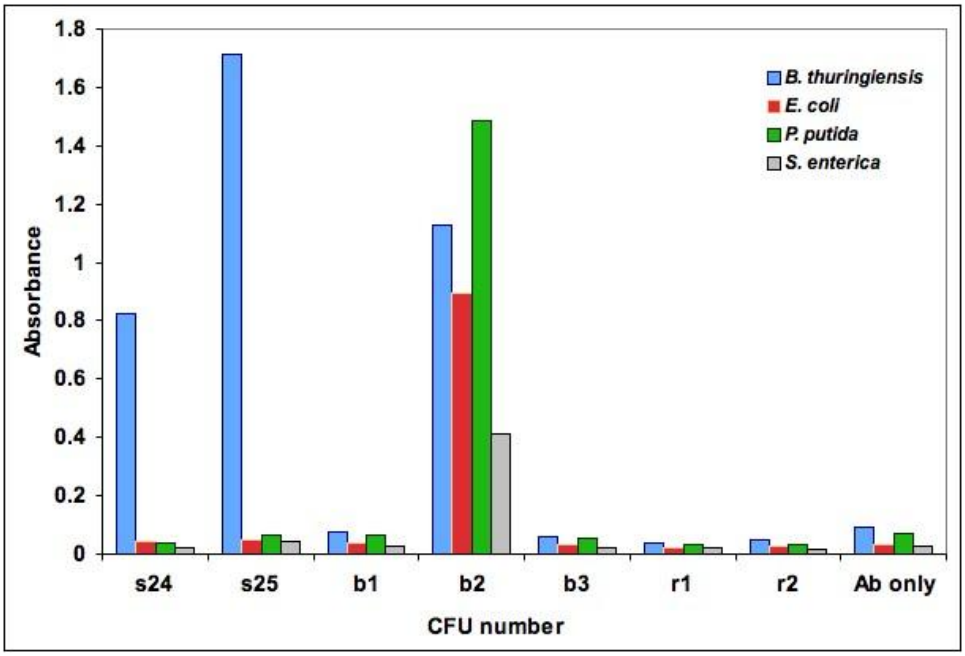


Figure 26. Bacterial Specificity of Clones Isolated through Negative Selection

Soluble scFvs were isolated from the periplasmic space of individual clones identified following negative selection biopanning. The soluble scFv were assayed for affinity toward the target bacterium (*B. thuringiensis*) and several non-target bacteria. The scFv s24 and scFv s25 demonstrated high specificity for the target bacterium.

The scFv s24 proved to be insoluble during small-scale expression trials. Although the scFvs isolated from the periplasmic space of this clone generated a positive signal in the ELISA assay (Figure 26), the quantity of soluble scFvs was insufficient to proceed with purification via affinity chromatography. The scFv s25 was also mostly insoluble, however, there was a sufficient quantity of soluble scFvs to allow for subsequent purification. As was observed with scFv k18, the recovered scFvs were only a minor component in the column elutions and were visible only through immunoblotting. Affinity purified scFv s25 was tested against the complete panel of bacteria via ELISA (Figure 27). The scFv s25 exhibited little to no binding with the negative selection

antigen, *E. coli* or any other bacteria of the panel. An absence of *B. subtilis* and *L. monocytogenes* binding suggests that scFv s25 recognizes an epitope that is not conserved among gram-positive bacteria.

The negative selection technique proved an effective method of enriching for scFvs with high target specificity. Further analysis of specificity against a larger panel of bacteria would be required to confirm monospecificity for *B. thuringiensis*, however, since scFv s25 did not bind all of the other non-target, the scFv was considered an ideal candidate for preliminary trials of the biosensor. In addition to *B. thuringiensis* detection, the biosensor could be tested with the non-target bacterium to establish false positive and false negative statistics for the prototype device.

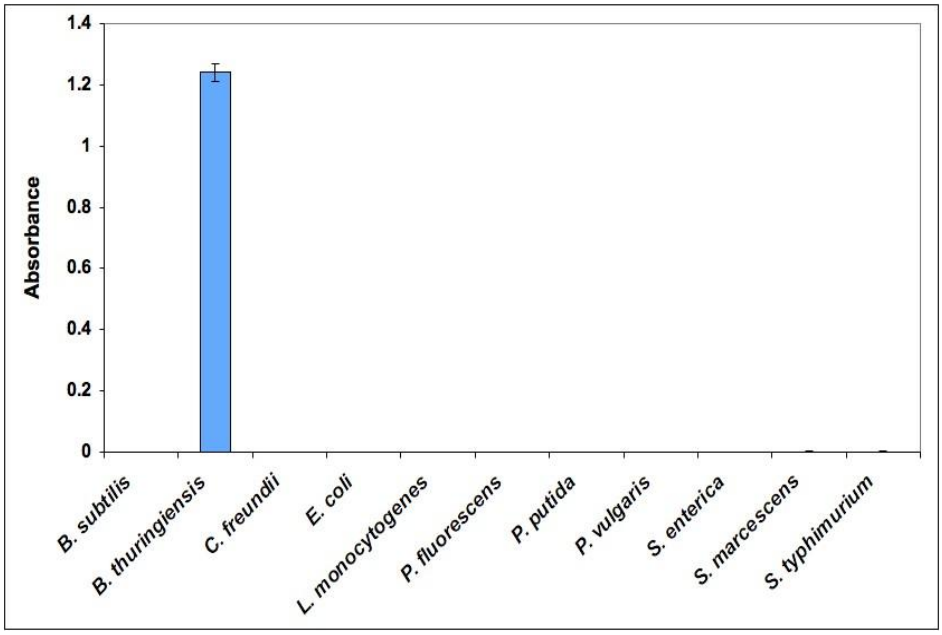


Figure 27. Analysis of the Bacterial Specificity of scFv s25 via ELISA

The scFv s25, isolated via affinity chromatography exhibited significant specificity to the *B. thuringiensis* target. Absorbance measurements for *B. thuringiensis* were ten-fold higher than those contributed by the secondary antibody alone. No significant interaction with non-target bacteria of the panel was detected.

Bacillus anthracis Biopanning

Immunization of the rabbit with *B. thuringiensis* ensured the construction of an scFv library enriched for those capable of binding conserved epitopes of *Bacillus* species. It was postulated that from this library it would be possible to isolate scFvs capable of recognizing other *Bacillus* targets of interest, particularly, *Bacillus anthracis.*

Irradiated vegetative *B. anthracis* cells were obtained from the Biodefense and Emerging Infections Research Resource Repository – Critical Reagents Program, a division of the American Type Culture Collection. Distribution of *B. anthracis,* due to its potential as a bioweapon, is highly controlled. Since only a minimum quantity of the bacteria was received from ATCC (1 ml solution at a concentration of 7.2×10^8 cfu/m), 10^5 bacteria, were immobilized for biopanning compared to the standard 10^7 cells for other experiments. Kristina Clarke conducted five rounds of biopanning against *B. anthracis* following the standard protocol.

Prior to analysis of individual clones, a phage ELISA protocol was employed to assess enrichment of scFvs in each round of biopanning. The results of this assay would determine which biopanning round would be most suitable for screening of individual colonies. Although theoretically target-specific scFvs should be enriched with each successive round, scFv-producing cells grow and divide more slowly than those clones lacking an scFv gene [(18)]. Given the poor growth, individual clones could potentially be lost through excessive rounds of selection. The phage ELISA evidenced enrichment of phage up to the third round of biopanning (Figure 28). The subsequent round showed a marked decrease in absorbance potentially indicating a loss of target-specific molecules. The scFvs capable of target recognition were not significantly enriched in the fifth round

either, indicated by the absorbance value which was still well below the peak seen in round three.

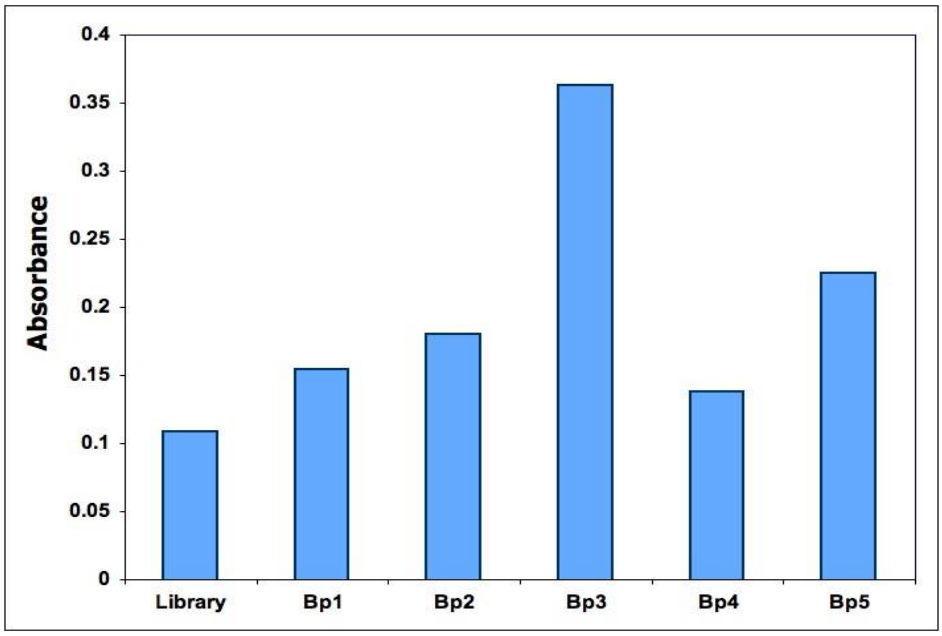


Figure 28. Enrichment of scFvs During Successive Rounds of *B. anthracis* Biopanning
Phage preparations from individual rounds of biopanning were examined via ELISA to visualize enrichment of target-specific scFvs. Biopanning round three demonstrated a greater than three-fold increase in the absorbance value compared to the initial library, suggesting the greatest abundance of scFv capable of binding the target antigen.

Approximately 200 individual clones from the third biopanning round were examined for the presence of the scFv gene. Although the phage ELISA suggested an enrichment of target-specific scFvs in this round, only five clones contained a DNA fragment that could be excised with *SfiI*. Of these, only two clones contained a full-length scFv gene (800 bp).

Small-scale expression studies for the two clones expressing scFv k4 and scFv k5 were conducted in *E. coli* strain BL21. A moderate level of protein expression; i.e. scFv

bands visible on Coomassie stained gels (Figure 29), was seen for both clones. The quantity of scFv present in the soluble fraction was sufficient for subsequent affinity purification. In addition to the scFvs within the soluble fraction, as shown on the immunoblot in Figure 29, both clones exported scFv protein to the culture medium. The culture medium, although not previously examined, was shown by other researchers to contain scFvs released into the medium when expression is conducted at temperatures above 25 °C [(139-141)]. Although scFvs were not purified from the culture medium for these clones, later experiments did examine the potential of obtaining soluble recombinant proteins from this source.

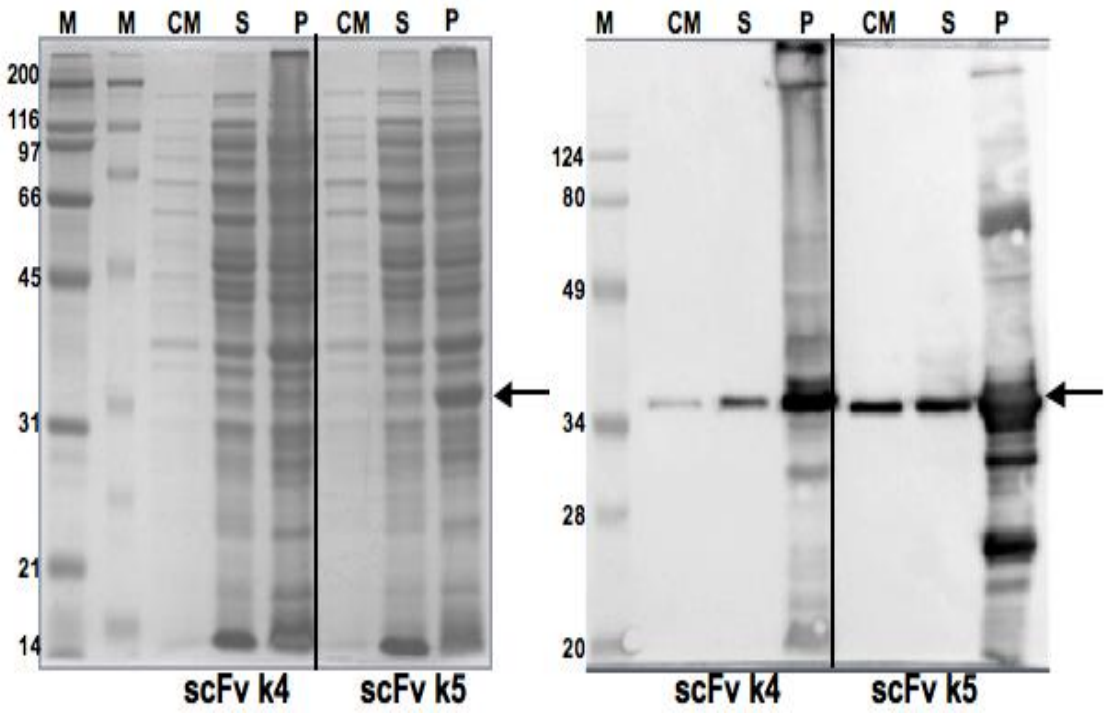


Figure 29. Small-Scale Expression of scFv k4 and scFv k5

Equivalent protein concentrations (25 μg) of the soluble (S) and insoluble (P) fractions of the lysate were resolved using SDS-PAGE. A 20 μl aliquot of the culture medium (CM) was included to determine the extent to which scFvs were secreted from the cells.

Moderate over-expression of the scFv is evident in clone k5 as evidenced by a large protein band at approximately 35 kDa that is well defined on the Coomassie stained image (left image with the arrow). A less visible, though still present, band of the same MW can be observed in the supernatant fraction of scFv k5 as well as the pellet material of scFv k4

A considerable proportion of protein was insoluble for both clones as evidenced by immunoblot analysis (right image). However, both clones produced a sufficient quantity of soluble protein to allow for subsequent IMAC purification and ELISAs.

The scFv k4 and scFv k5 were purified from the soluble fraction of the culture lysate using Ni^{2+}-NTA affinity chromatography. Column elutions were examined with ELISA to determine scFv specificity (Figure 30). Absorbance measurements for scFv k4 were consistently three fold lower than those of scFv k5 for all bacteria of the panel. The scFv k4 did exhibit reduced expression compared to scFv k5 (Figure 29), therefore, it is unlikely that the elutions tested for scFv 4 and scFv k5 contained equivalent concentrations of scFvs. This may have contributed to the lower absorbances for scFv k4. Additionally, amino acid sequence analysis indicated only 76% similarity between the two scFvs. Variations in the amino acid sequences of the CDRs may have contributed to the differences in affinity for the panel bacteria.

Interaction with *B. anthracis* was minimal for both scFvs. Interestingly, the absorbance values for the other gram-positive bacteria (*B. subtilis, B. thuringiensis,* and *L. monocytogenes*) of the panel were three to four times greater than those measured for gram-negative bacteria. Although contradicted by the *B. anthracis* sample, this observation suggested that both scFvs potentially bound an epitope conserved among gram-positive bacteria. To resolve this inconsistency, the *B. anthracis* sample was further examined using microbial techniques to ensure there was no contamination that may explain the results of the specificity ELISA. A Gram stain and light microscopy confirmed that the sample was free of other bacteria or artifacts that may have contributed to the optical density value that was measured. A malachite green stain, a technique to stain bacterial spores, was also performed to further examine the sample. A significant number of spores were present in the sample. A direct count of the cells within the field of view revealed that approximately 60% of the bacteria contained a bacterial

spore and that numerous free spores were present in the sample. The free spores would contribute to the optical density of the sample, therefore cell densities calculated for both biopanning and ELISAs would be overestimates. The high affinity for the other gram-positive bacteria suggested that scFvs k4 and k5 were binding a surface epitope. The lower percentage of intact bacteria in the *B. anthracis* sample combined with the inaccurate calculations of cell density would explain the low absorbance values for both scFv and the *B. anthracis* antigen. A vegetative bacterial sample, that could accurately be quantitated, would be required to assess scFv affinity for the *B. anthracis* target.

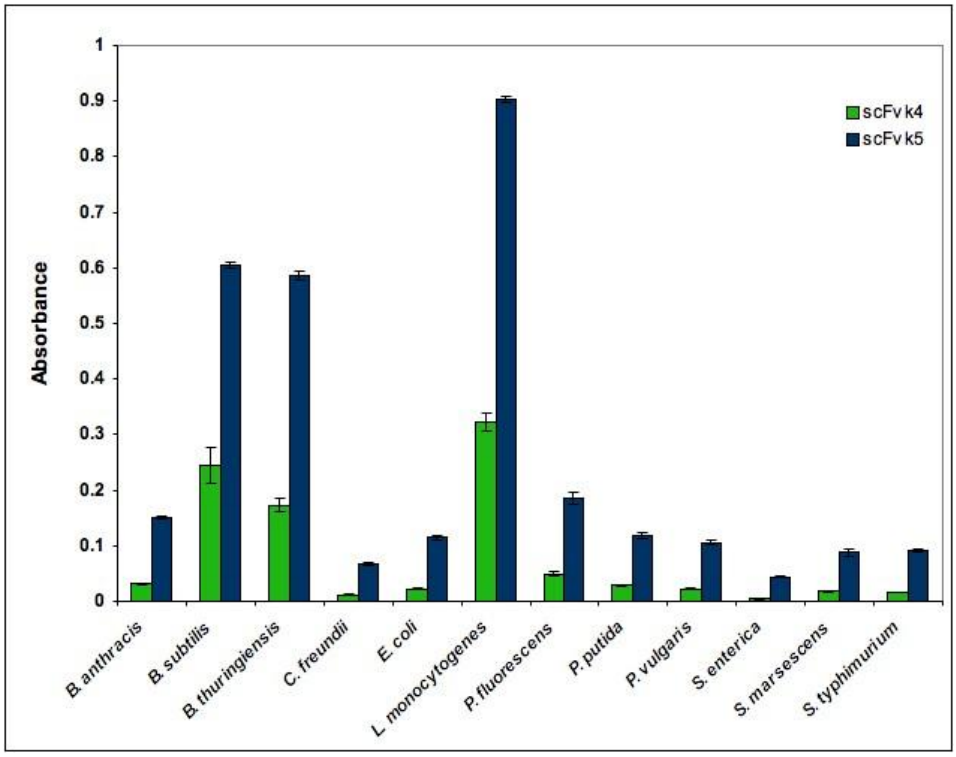


Figure 30. Specificity of scFv k4 and scFv k5 Purified via Affinity Chromatography

Live bacteria were bound in triplicate to a microtiter plate at equivalent cell densities based on optical density. ScFvs k4 and k5 were eluted from a Ni^{2+}-NTA affinity column as a heterogeneous solution

Both scFv k4 and scFv k5 bound primarily gram-positive bacteria of the panel (Abs > 0.1) although scFv k4 exhibited lower affinities compared to scFv k5. ScFv k5 did bind some gram-negative bacteria, however, absorbance values were to 2 to 3 times less than those for the gram-positive bacteria of the panel.

The scFvs highly specific for *B. anthracis* were not readily isolated from the immunized library. While two scFvs that did recognize epitopes of gram-positive bacteria were isolated, no scFv was obtained that possessed the desired monospecificity that would have made them suitable for use in the biosensor. Although the high spore concentration in the *B. anthracis* sample obtained from ATCC provided some insight into the ELISA results for scFv k4 and scFv k5, an scFv should have been isolated that bound the spores themselves. Additionally, since there were still intact cells within the ATCC sample, greater success should have been achieved in isolating scFvs specific for *B. anthracis* surface epitopes.

Since the phage library was constructed from a rabbit immunized with *B. thuringiensis*, a lack of scFv diversity may have contributed to the inability to isolate scFvs to other target antigens. The scFv library was therefore examined against other bacterial targets to assess diversity.

Pseudomonas species Biopanning

The specificity ELISAs of previous biopanning rounds identified scFvs with affinity for *Pseudomonas* species (Figure 25, Figure 26). Biopanning against these targets was conducted to ascertain if *Pseudomonas*-specific scFvs could be isolated from the immunized library. Obtaining scFvs specific for a bacterial target other than the *B. thuringiensis* immunogen would establish the diversity of the library.

Four rounds of biopanning were conducted using the standard biopanning protocol. Analysis of phage preparations from each round of biopanning implied that enrichment of target-recognizing scFvs had not occurred (Figure 31). For both *Pseudomonas* targets, absorbance values measured after biopanning round five were only marginally above

those for the initial library. In contrast, direct counting of colonies on phage input and output plates, performed after each round of selection, continued to produce phage-binding efficiencies (10^{-3} to 10^{-5} % phage bound per round of biopanning) within parameters established by Barbas III *et al.* [57] that are indicative of successful selection. Since phages were recovered from each round of selection it was suspected that scFvs were present, although likely of low affinity since they were not detected in the phage ELISA.

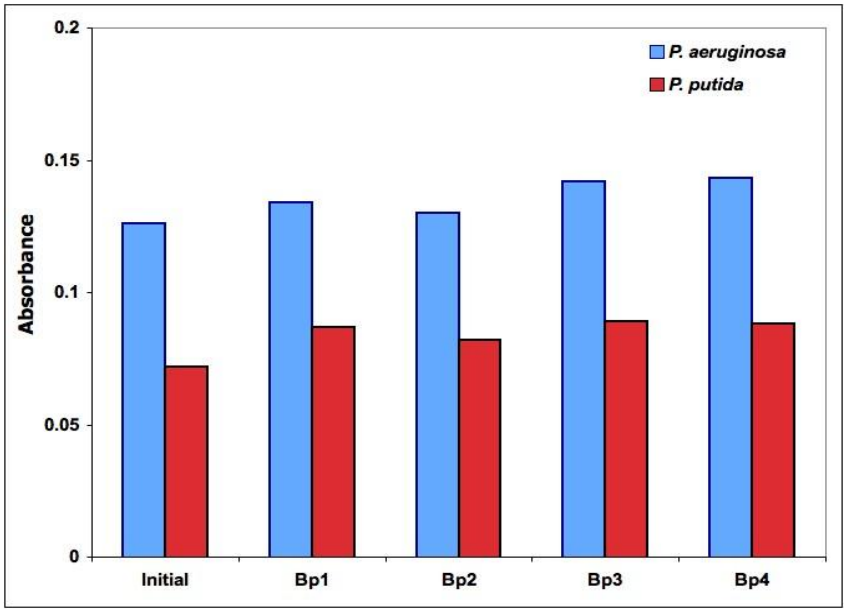


Figure 31. Enrichment of scFvs During *Pseudomonas* Biopanning
Phage preparations from each round of biopanning were examined using ELISA to visualize enrichment of target-specific scFvs. Absorbance values for each round of biopanning did not increase significantly above those for the initial library suggesting that enrichment of scFv capable of binding either *Pseudomonas* species had not been achieved.

Forty clones from the fourth round of biopanning against *P. aeruginosa* were examined to identify those with an scFv gene. None of the clones contained an insert.

Screening of additional colonies from the third and fourth rounds of biopanning rounds also failed to identify clones with an scFv gene insert.

The inability to isolate clones containing an scFv gene and the apparent lack of phage enrichment suggested that the phages recovered during biopanning were the result of non-specific interaction between the phages and the bacterial target. Such interactions have been shown for M13 filamentous phages by other researchers [(142)]. This non-specific binding could occur with an undefined surface structure or perhaps with the capsule of *P. aeruginosa*. Difficulties with *P. aeruginosa* as the target antigen had also occurred in previous experiments. Although it was not determined whether it was the capsule or some other characteristic of the bacterium, inhibition of antibody interaction with target epitopes (Figure 10) and non-specific interactions with secondary antibodies (Figure 17) was previously observed. Continued biopanning and colony screening was therefore not pursued for this bacterium.

In contrast to the results obtained with screens for *P. aeruginosa* scFvs, DNA inserts of either the expected 800 bp or the 300 - 400 bp truncated molecule were identified in sixteen of the forty clones tested from *P. putida* output plates. Small-scale expression in *E. coli* strain DH5α was conducted for the five clones that contained the full-length scFv gene. Two of the clones, p6 and p12, expressed scFvs with sufficient solubility to allow for subsequent affinity purifications and specificity ELISAs (Figure 32). Neither scFv bound the *P. putida* target with high affinity. As with scFv k18 (Figure 25), both scFvs bound a number of unrelated bacteria of the panel.

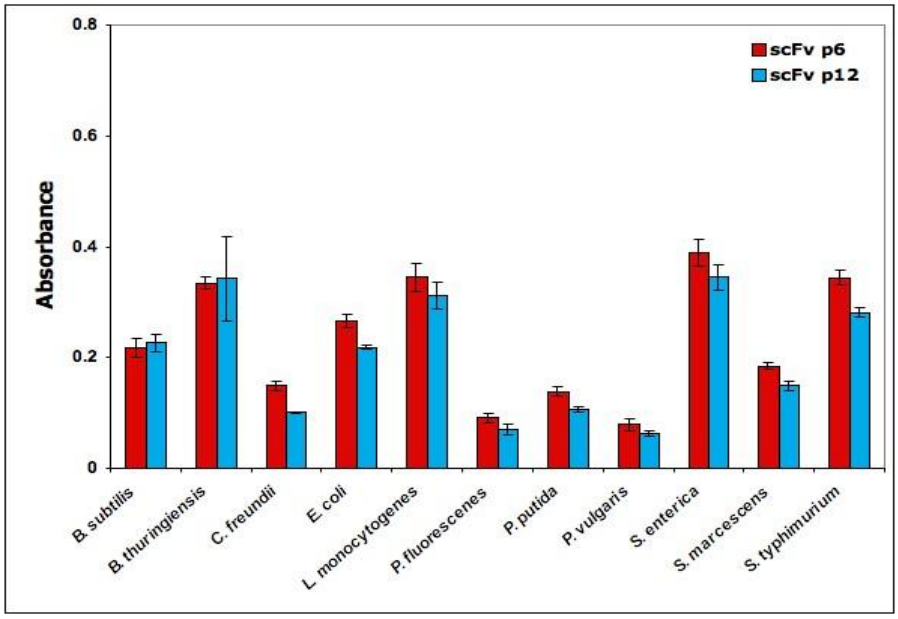


Figure 32. Specificity ELISA of scFv p6 and scFv p12

Bacteria were immobilized in triplicate for specificity assays of scFv p6 and scFv p12 isolated from the soluble fraction of overnight expression cultures using Ni^{2+}-NTA chromatography.

Neither scFv p6 nor scFv p12 exhibited substantial affinity for the *P. putida* target. Both scFvs bound gram-positive and gram-negative bacterial targets with varying affinities.

Two unique clones isolated from the last round of *P. putida* biopanning expressed scFvs that exhibited minimal affinity for the bacterial antigen. While the results of the *P. aeruginosa* biopanning (Figure 31) were likely the product of non-specific interaction from the M13 phage, the lack of scFv enrichment observed for *P. putida* biopanning could be attributed to a low scFv affinity for the target bacterium. Forty percent of the clones examined contained a gene insert that could be excised by *SfiI* digest. Therefore, unlike *P. aeruginosa* biopanning, the percentage of phages bound determined by direct counts of input and output plates was likely the result of phage enrichment and not non-specific interaction with M13. The washes performed during biopanning are not as rigorous as those conducted during standard ELISA and may not have been sufficiently

stringent to dislodge low-affinity scFvs from the immobilized bacteria. In contrast, during the phage and specificity ELISAs, low-affinity scFvs would be removed in the washes leaving no binders to detect. This would explain what appeared to be a lack of enrichment in the phage ELISA as well as the low affinity for the *P. putida* target exhibited in the specificity ELISA (Figure 32). Although additional clones could have been identified, based on the results of the phage ELISA, it is unlikely that any would have shown the desired target specificity or affinity for utilization in the biosensor.

Isolation of scFvs from the immunized library that exhibited high specificity and affinity for *B. thuringiensis* was accomplished through a combination of standard and negative selection biopanning. The immunized library, however, could not be successfully employed to isolate scFvs with similar binding characteristics for other bacterial targets. DNA sequence alignments (ClustalW) of all scFvs of interest (scFvs k18, s24, s25, k4, k5, p6, and p12) did not suggest a lack of diversity of the scFv library. ScFvs were only 80 – 85% similar, with sequence similarity existing primarily within the framework regions of the heavy and light chain genes.

The host rabbit was immunized to enrich for lymphocytes producing antibodies specific for surface epitopes of *Bacillus* species. In such excess, the heavy and light chain genes from these cells may have masked the presence of other non-*Bacillus* antibody genes. To increase diversity, with respect to both library size and antigen-recognition, an scFv library from a non-immunized rabbit was constructed.

Construction of a Naïve scFv Library

Immunized animals are utilized as a source of highly specific antibodies that demonstrate excellent affinities for their target antigens [(21)]. Naïve antibody libraries

circumvent the immunization process by relying on an scFv repertoire that exceeds that of the immunized library and more closely mimics the clonal diversity of the host immune system. Because the diversity is increased, the likelihood of isolating scFvs capable of binding a target antigen should be increased. Naïve libraries have been used with great success by several research groups to obtain scFvs specific for an array of antigens [62, 72, 78]. The naïve library used in this study was constructed to fulfill the adaptability requirements of the biosensor and overcome the limited successes of the immunized library in obtaining scFvs specific for non-*Bacillus* targets.

A naïve library was assembled from the spleen of a non-immunized, 4 - 6 week old rabbit harvested by the Antibody Research Corporation (St. Charles, MO). Primers for the IgG constant domains were used as before, however, since the initial immune response is comprised primarily of IgM molecules, primers specific for the first constant domain of the IgM heavy chain were also designed according to the literature [143-145]. The heavy and light variable domain genes were amplified via PCR from a cDNA pool, gel purified, and combined into a fusion gene using an overlap extension PCR by Kristina Clarke.

To improve the efficiency of transformation, a highly electrocompetent *E. coli* strain, SS320, was generated from MC1061 and XL1-Blue following the protocols of Sidhu *et al.* [124, 146]. Bacterial mating was performed to transfer the F+ genotype of XL1 Blue to the MC1061 strain. This generated *E. coli* strain SS320 that was conducive to phage display and had the added benefit of high transformation efficiency by electroporation. Following bacterial mating, cells were made electrocompetent utilizing the protocol of Barbas III *et al.* [57].

Small-scale ligation–transformation trials were performed as described for the construction of the immunized library to assess both transformation efficiency of the SS320 competent cells and to determine the number of ligations needed to achieve a clonal diversity of 10^9. Based on the results of the initial trials, forty 100 μl ligation reactions were assembled. The library diversity, calculated as described in the construction of the immunized library from the number of transformants following electroporation of SS320 cells, was approximated to 8×10^8.

Salmonella typhimurium Biopanning

The naïve library was subjected to four rounds of standard biopanning against *Salmonella enterica* serovar Typhimurium (referred to as *S. typhimurium*). As with previous experiments, phage preparations from each round of biopanning were examined by ELISA to determine if an enrichment of target-specific scFvs had occurred (Figure 33). The fourth round of biopanning showed a two-fold increase in the absorbance value compared to the initial library. Although the results suggested only a modest increase in the population of scFvs specific for the target antigen, individual colonies were isolated and examined for the presence of the scFv gene.

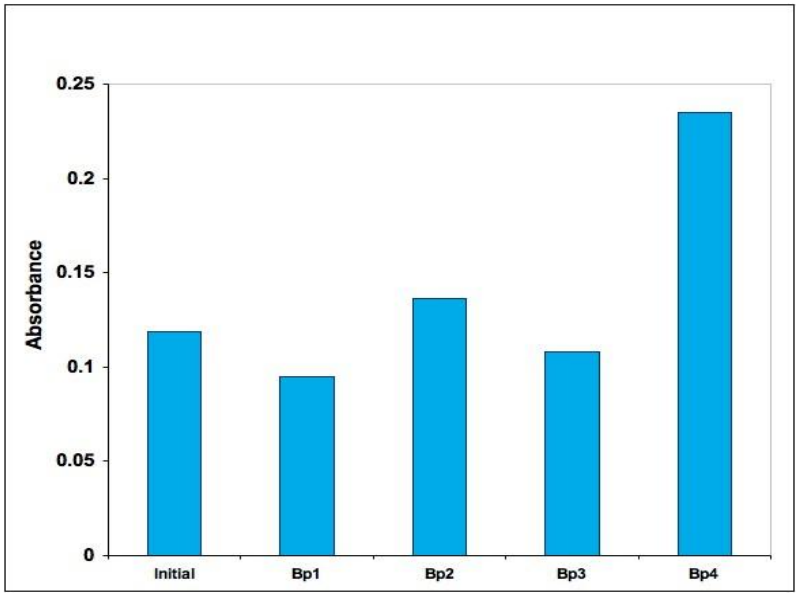


Figure 33. Enrichment of ScFvs During *S. typhimurium* Biopanning

Phage preparations from each round of biopanning were examined for binding affinity toward *S. typhimurium*.

The fourth round of biopanning exhibited a two-fold increase in absorbance compared to the initial library indicative of an enrichment of scFvs recognizing the target bacterium.

More than 200 individual clones were examined from the last round of biopanning (some performed by Kristina Clarke). Unlike previous trials with *P. aeruginosa* and *B. anthracis,* a significant number of the colonies examined (30%) contained an scFv gene. Small-scale expression was conducted in *E. coli* strain BL21 to assess scFv solubility and specificity for bacterial targets. Many of the clones expressed insoluble scFvs that precipitated in the pellet fraction following cell lysis. Nine clones (scFv k20 – k26, scFv s26, scFv s27) expressed recombinant antibodies with sufficient solubility to allow for subsequent purification and specificity ELISAs. Although there was some variation in the affinity for target and non-target bacteria, none of the scFvs demonstrated a substantial specificity for *S. typhimurium*. The specificity ELISA for scFvs from two clones, s26 and

s27, is shown in Figure 34. It was concluded, therefore, that the standard biopanning protocol was not sufficiently stringent to isolate scFvs with improved target specificity.

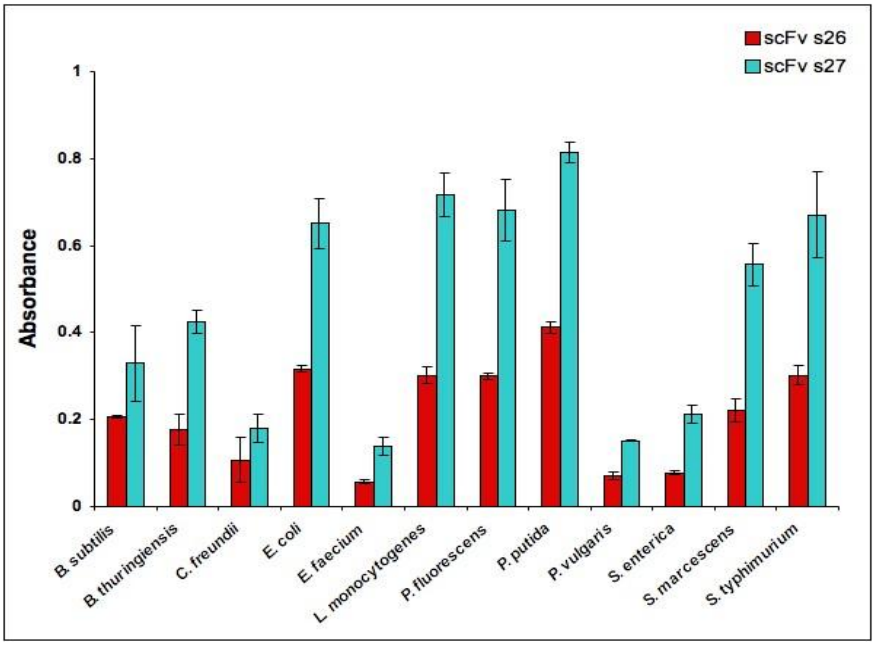


Figure 34. Specificity of scFv s26 and scFv s27 Assessed via ELISA.
The scFv s26 and scFv s27 were purified using a Ni^{2+}-NTA column and analyzed via ELISA to assess target specificity. Values are corrected for background absorbance from the secondary antibody.
Both scFvs lacked specificity for the *S. typhimurium* target and demonstrated affinity for several bacteria of the panel.

To repeat the successes achieved with the immunized library and the isolation of the highly specific scFv s25, a negative selection strategy was employed to enrich for scFvs specific for *S. typhimurium. L. monocytogenes,* a Gram positive bacterium, was chosen as the negative selection antigen because, like *S. typhimurium* these bacteria are flagellated and capable of colonizing the gut of the same host organisms. Removing scFvs that bind highly similar epitopes through negative selection may enrich for those that are specific for unique epitopes of *S. typhimurium.*

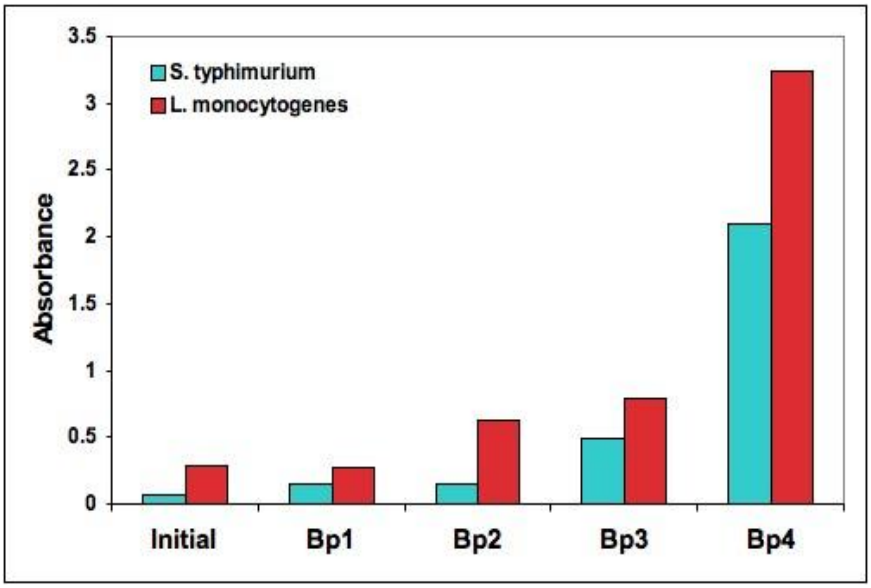


Figure 35. Enrichment of scFvs for *S. enterica* During Negative Selection
Phage preparations from each round of negative selection biopanning were examined via ELISA to assess enrichment of scFvs capable of binding the *S. typhimurium* target.
Affinity for the non-target bacterium was maintained despite the negative selection protocol. An eight fold increase in absorbance was measured for *S. typhimurium* and a thirteen fold for *L. monocytogenes*.

Analysis of the phage preparations by ELISA was performed after two rounds of negative selection. The initial phage ELISA, however, indicated no significant decrease in the interaction of the scFv pool with *L. monocytogenes.* An additional two rounds of negative selection were performed. While the phage preparation of the final round was enriched for scFvs capable of binding the target bacterium, there was also an unexpected increase in binding for the non-target bacterium (Figure 35). The absorbance values for the scFv targeting *S. typhimurium* following four rounds of negative selection increased by nearly a factor of eight compared to the last round of standard biopanning (labeled initial sample - Figure 35). Unfortunately, however, an approximately thirteen-fold

increase in absorbance for *L. monocytogenes* was observed. Analysis of the phage ELISA suggested an enrichment of scFvs capable of binding both *S. typhimurium* and *L. monocytogenes*.

The phage pool from round four of negative selection was amplified and plated on selective medium to isolate individual colonies. A high-throughput microtiter plate method of screening colonies was implemented for analysis of these clones [(59)]. This method permitted several hundred colonies to be examined simultaneously for both scFv expression and affinity for the bacterial target. Two hundred colonies obtained from the output plates were screened in the first application of this method. Of these, 70 colonies (35%) had an absorbance two-fold greater than background (absorbance ≥ 0.1). Positive clones were patched onto solid medium for subsequent plasmid isolation and digestion with *SfiI*. All of the clones examined except one contained an scFv gene smaller than that of a full-length scFv (Figure 36). The microtiter plate assay was repeated using *L. monocytogenes* as the immobilized antigen. A single clone of the 200 examined was identified as a positive. Digest analysis of this clone also produced a smaller than expected scFv gene. The small size of the scFv gene suggests that expressed recombinant antibody is comprised of a single variable domain.

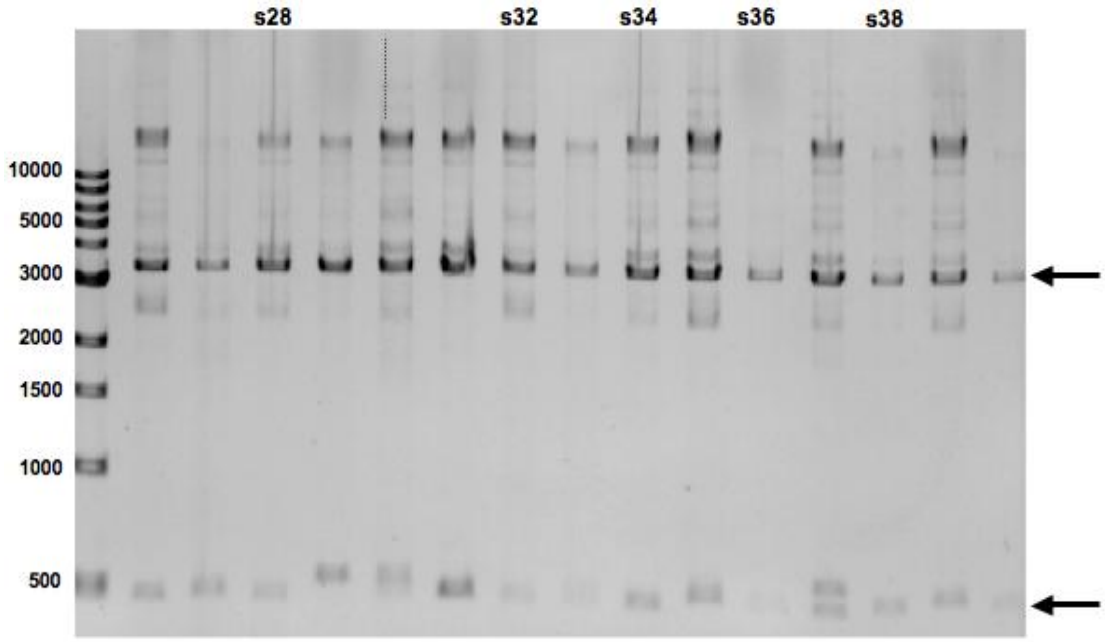


Figure 36. Restriction Digest Analysis Clones Isolated Following Negative Selection Biopanning

Individual colonies identified with the microtiter method of screening were grown on selection medium. Plasmid DNA, obtained through direct colony lysis, was digested with *SfiI* to confirm the presence of the scFv gene.

Clones that will be further examined are identified at the top. The vector backbone and scFv gene are indicated with arrows. All clones isolated from the last round of negative selection contained a truncated scFv gene of 500 bp or less.

The truncated scFv genes were amplified from individual clones via PCR and sent to Northwoods DNA, Inc. for DNA sequencing. Analysis of the nucleotide sequence would identify which of the variable domain genes was persisting through numerous rounds of selection by forming a molecule capable of antigen binding. Sequence analysis showed that the majority of the sequenced genes lacked a full-length light chain variable domain gene (Figure 37). The linker sequence that facilitated heavy and light chain gene fusion through oePCR, although not complete in all of the clones examined, could readily be identified. Protein BLAST analysis was performed with the putative heavy chain gene,

the amino acid sequences downstream of the linker. For all six scFv genes sequenced the heavy chain variable domain gene of *Oryctolagus cuniculus* (rabbit) exhibited the greatest similarity with an E value $\leq e^{-47}$.

scFv s28
ELVMTQTEGTKGWSNLWWRWLGGGGGGSSRSSQSLEESGGRLVTPGTPLTLTCTASGFSLSSYNMGWFRQAPGKGL
EWIGYIYAGSGSTWYASWAKGRFTISKTSTTVDLKITSPTTEDTATYFCARGGGFYSGFNLWGPGTLVTVSSGQPKAPS
VTSGQAGQ

scFv s32
ELVLTQSPSGGGGSGGGGGGSSRWDPADRHRLLWWRWLGRWWGWSSRSSQSLEESGGRLVTPGTPLTLTCTVSGFS
LSSYDIYWVRQAPGKGLEYIGYISYGGSTYYASWAKGQFTISKTSTTVDLKISSPTTEDTATYFCARKPADYSDMFNMW
GPGTLVTVSSGQPKAPSVTSGQAGQ

scFv s34
ELVLTQSPSGGGGSGGGGGGSSRSSGTQLTVTGSSGGGGSGGGGGGSSRSSQSLEESGGRLVTPGTPLTLTCTASGFSL
SSYYMTWVRQAPGKGLEWIGIVSNFGDTYYASWAKGRFTISRTSSTTMDLKITSPTTEDTATYFCARDYLYAGIFIFGL
WGPGTLVTVSSGQPKAPSVTSGQAG

scFv s36
ELVMTQTPGPRDRAGDPILWWRGSGGGGGGSSRSSQSVKESEGGLFKPTDTLTLTCTASGFSISSYRMGWVRQAPGKG
LEWIGFINSYGRTYYASWAKSRSTITRNTNENTVTLKMTSLTAADTATYFCAREPGYSASGSGGSIWGPGTLVTVSSGQP
KAPSVTSGQAGQ

scFv s38
ELVLTQTPSPVSAAVGGTVSISCQSSQSVNSNNWLSWFQQKPGQPPKLLIYGASTLASGVPSRFKGSGSGTQFTLTISDVQ
CDDAATYYCLGEFSCSSTDCNAFGGGTEVVVKSSGGGGSGGGGGGSSRSSQSVEESRGRLVTPGTPLTLTCTISGFSLSN
YAMGWVRQAPGKGLEWIGAIDSSATTYYATWAKGRFTISKTSTTVDLKITSPTTEDTATYFCARYWDDYGDYAFDPWG
PGTLVTVSSGQPKAPSVTSGQAGQAPPPPPQGRIPAHWRPLLVDPSSVPSLMHSLSIL

scFv k18
ELVMTQTPSPVSAAVGGTVTISCQSSESVYNNNELSWYQQKIGQPPKLLIYRASKLASGVSSRFSGSGSGTQFTLTISGVQ
CDDAATYYCLGGASSRDYKAFGGGTELEILSSGGGGSGGGGGGSSRSSQSVEESGGGLVTPDEALTLTCTVSGIDLSTYT
MSWVRQAPGEGLEWIGAVNRDGITYYASWAKGRFTISKTSTTVDLKMTSPTTEDTATYFCVRSVTGGATGLNTWGPGT
LVTVSWGQPKAPSV

Figure 37. Sequence Analysis of Truncated scFv Genes
Sequence analysis of individual clones isolated from negative selection biopanning of the naïve library. The conserved amino acid sequence contributed by the forward primer used in library construction is highlighted in purple and the scFv linker sequence is highlighted in green. The scFv k18 gene is included to illustrate the characteristic portions of the typical full-length scFv. With the exception of scFv s38, all the scFvs isolated from the last round of biopanning lack a complete light chain gene.

The negative selection process apparently eliminated the full-length scFvs that were present during the initial biopanning rounds and enriched for truncated molecules that were only a minor product initially. Other researchers have utilized recombinant antibodies comprised of only the heavy chain variable domain of unusual immunoglobulins of sharks and camels for the detection of a number of antigens *(68, 69, 147,*

[148]. In light of these successes, the single domain, truncated scFv clones isolated from the negative selection were further examined for protein expression and specificity against the bacterial panel. The cell pellets that remained in the microtiter expression plates were lysed and the soluble proteins resolved via SDS-PAGE. The molecular weight of the recombinant proteins, visualized with an immunoblot (Figure 38), varied from 15 kDa (scFv s28) to 29 kDa (scFv s36).

The low molecular weight of the scFvs visualized on the immunoblot was expected for all of the molecules except scFv s38. The scFv s38 gene was a full-length gene (Figure 37) that, based on *in silico* translation analysis using the Transeq program, contained an open reading frame. The gene sequence was further examined using the Graphical Codon Usage Analyzer software, which identified a sequence of seven consecutive codons whose tRNA complements are in very low abundance (<10%) in *E. coli*. This nucleotide sequence may have prematurely terminated translation of the scFv by causing the ribosome to stall. A second potential translation start site was identified within the light chain using GeneHacker software. In combination, these discoveries explain the low molecular weight (20 kDa) scFv (Figure 38) produced by the full-length scFv gene.

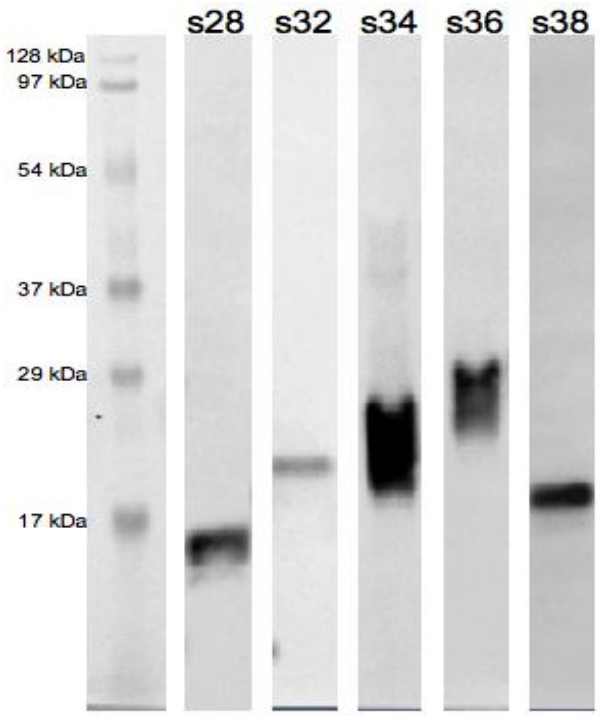


Figure 38. SDS-PAGE Analysis of Truncated scFv Expressions

Bacterial pellets were recovered from microtiter plates and lysed at 100°C in loading buffer. Proteins were resolved on a 12% acrylamide gel and transferred to nitrocellulose for immunoblotting.

The scFvs varied significantly in molecular weight. Given the small size of the cell pellet, equal cell densities may not have been examined. Expression of individual scFvs could not be accurately compared between clones.

Proteins were isolated from the periplasm of small-scale expression cultures to assess specificity of the truncated scFv molecules (Figure 39a). Unexpectedly, none of the scFvs showed affinity for either of the *Salmonella* species examined. Additionally, the majority of scFvs tested continued to bind *L. monocytogenes* despite multiple rounds of negative selection biopanning. Based on the results obtained with the ELISA, the single domain scFvs appear to form functional antigen-binding domains capable of binding bacterial epitopes, although as with the full-length scFvs isolated prior to negative selection (Figure 34), there was not significant specificity for *S. typhimurium*. In contrast, the scFv

s32, bound all bacteria of the panel with high affinity, displaying absorbance values ten to twenty- fold higher than those of the other scFvs tested (Figure 39b). While other scFvs such as scFv k18 (Figure 25) have been identified that bind several bacteria of the panel, scFv s32 interacts strongly with all bacteria currently used. Further testing of this scFv would be required to determine if the results of the specificity ELISA were the product of

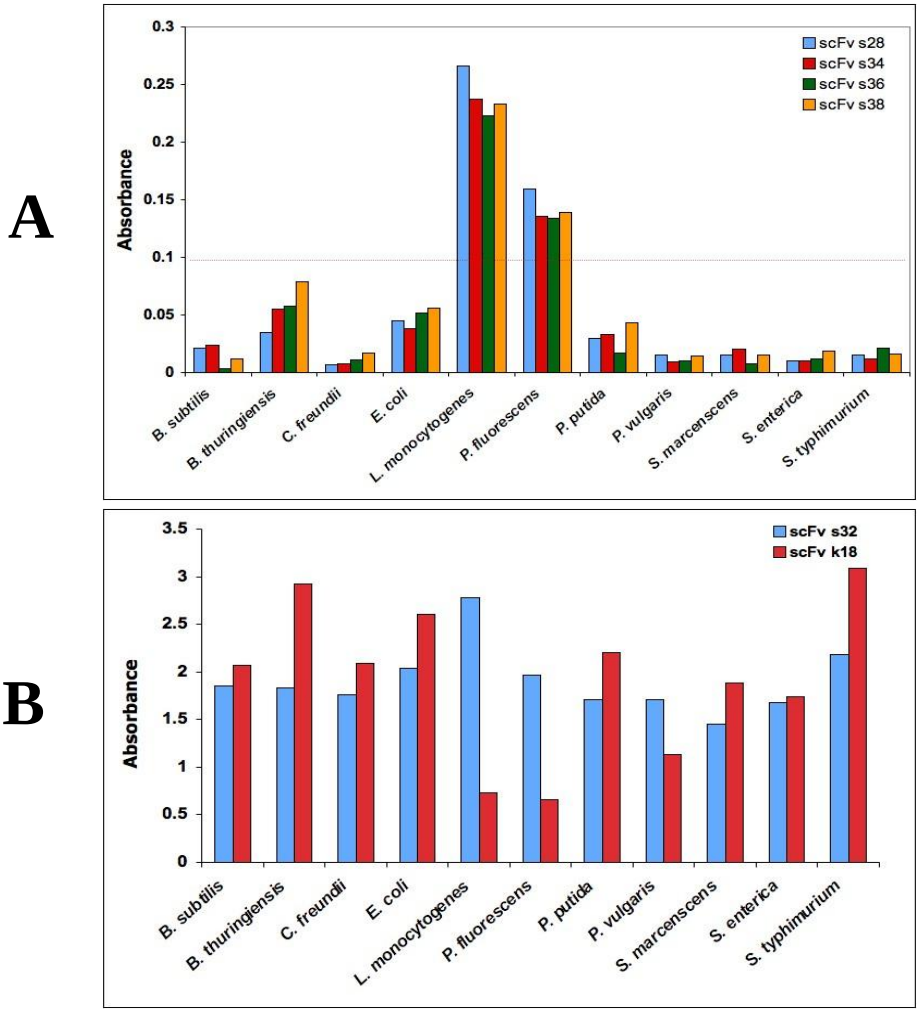


Figure 39. Specificity of Truncated scFvs from Unique Clones Identified Following Negative Selection Biopanning

Soluble scFvs s28, s32, s34, s36, s38 isolated via osmotic shock were tested for bacterial specificity with ELISA.

The scFvs s28, s34, s36, and s38 bound only *P. fluorescens* and the negative selection antigen *L. monocytogenes* with significant affinity (Abs > 0.1). Interestingly, none of these scFvs had affinity for the *S. typhimurium* target. In contrast, scFv s32 bound all bacteria (panel B) with high affinity, similar to the full-length scFv k18.

true antigen-antibody interactions.

With the exception of scFv s25, all of the scFvs obtained through biopanning lacked substantial specificity for target bacteria. While initially deemed advantageous, the complexity of the bacterial cell surface and the numerous potential epitopes may have been inhibitory to the isolation of scFvs specific for unique epitopes of target bacteria. To examine this possibility, biopanning was conducted by Kristina Clarke against purified O-antigen isolated from *Salmonella typhimurium*. The O-antigen is a complex of oligosaccharides attached to proteins of the outer cell surface. These complexes, which are often associated with virulence, can be used to identify particular strains of *S. enterica* [(149, 150)]. Utilization of the O-antigen during biopanning would enrich for scFvs with improved specificity toward *S. typhimurium* by limiting the number of epitopes available during selection.

The naïve library was subjected to five rounds of biopanning against the O-antigen, at which point a phage ELISA was performed to assess enrichment of antigen-specific scFvs (Figure 40). The microtiter plate assay was used initially to screen 60 individual colonies from the last round of biopanning. Eighteen of clones examined (30%) bound the immobilized O-antigen (Abs > 0.1). Positive clones were transferred to antibiotic containing solid and liquid medium to facilitate further analysis via *SfiI* restriction enzyme digestion. A single clone, k2, contained a full-length scFv gene insert. Plasmid DNA from three of the other clones contained a gene product of approximately 200 bp all others were empty vectors.

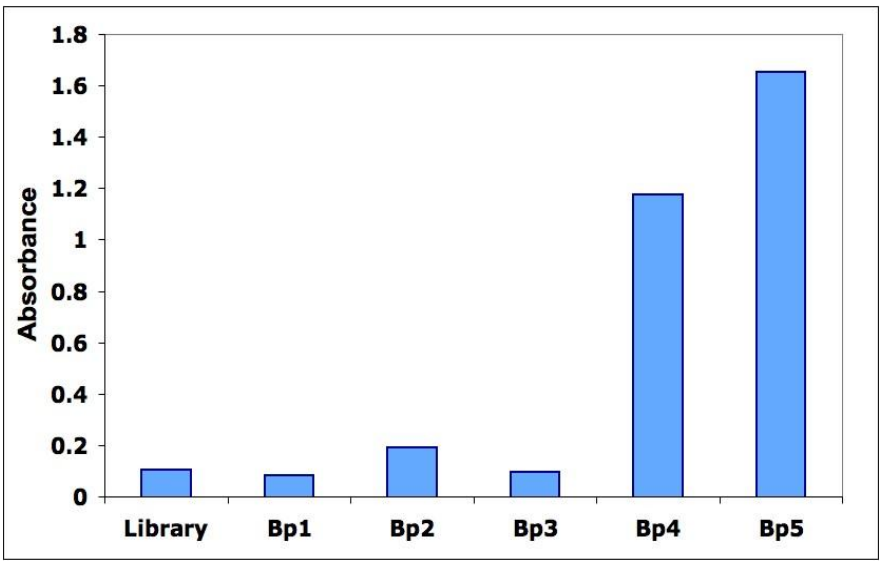


Figure 40. Enrichment of scFvs Specific for *S. typhimurium* O-antigen
Phage preparations from each round of biopanning were assessed using ELISA to ascertain if enrichment of scFvs with affinity for the O-antigen of *S. typhimurium* had occurred. A dramatic increase in scFvs binding the target antigen was evident in the fourth and fifth rounds of selection. A fifteen-fold increase in the absorbance measurement between the initial and last round of biopanning was observed.

Small-scale expression and specificity studies of scFv k2 were conducted as previously described. Clone k2, as with other clones, did not produce an over-abundance of recombinant protein during expression trials, scFv k2 could only be identified through immunoblotting. Although the majority of the protein was insoluble (Figure 41), sufficient quantities of soluble scFvs were isolated via Ni^{2+} -NTA affinity chromatography to allow specificity to be examined via ELISA. The scFv k2 did demonstrate affinity for the O-antigen itself and both bacteria of the *Salmonella* species. However, as with other scFvs (scFv k18 – Figure 25 and scFv b2 – Figure 26), scFv k2 readily bound several bacteria of the panel (Figure 42).

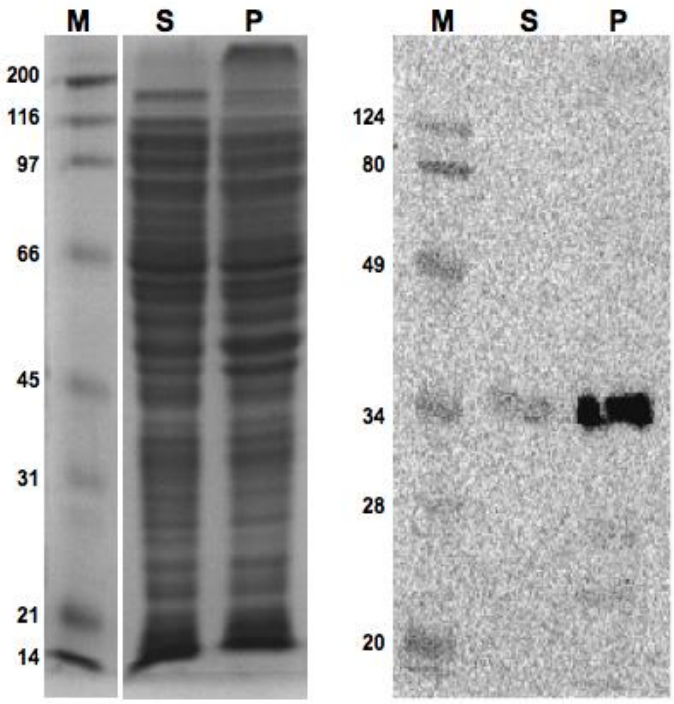


Figure 41. Small-Scale Expression of scFv k2

The soluble and insoluble fractions obtained following cell lysis were separated via SDS-PAGE.

The scFv was not evident on the Coomassie stained gel (left image) but was identified using a standard immunoblotting protocol (right image).

The majority of the scFvs are found in the insoluble pellet fraction.

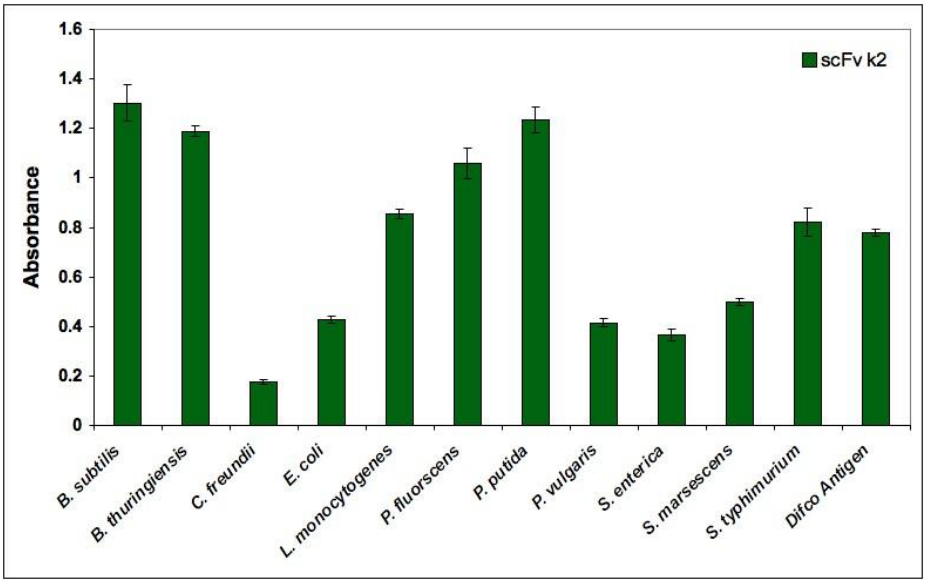


Figure 42. Analysis of Bacterial Specificity of scFv k2

The scFv k2 was purified using IMAC from the soluble fraction of an overnight expression culture.

ScFv k2 readily bound the O-antigen and both *Salmonella* species in addition to all of the non-target bacteria of the panel. Greater affinity for non-target gram-positive bacteria compared to the O-antigen used in biopanning was observed.

Additional colonies were screened from the fourth and fifth rounds of biopanning, however, no other full-length scFv genes were identified. Although single domain antibodies isolated in previous biopanning attempts displayed binding capabilities (Figure 36), clones with a truncated scFv gene from these experiments were not examined. The scFv gene was less than 200 bp in these clones, smaller than a typical variable domain (300-330 bp).

Although antibodies specific for carbohydrates of the LPS have been reported in the literature [(151, 152)], attempts to isolate O-antigen specific scFvs from the naïve library were unsuccessful. It could not be determined based on these rounds of selection whether the

inability to isolate a highly specific scFv was the product of the poor choice of antigen or the diversity of the naïve library.

The naïve library was also selected for scFvs against *L. monocytogenes* by Kristina Clarke. Phage preparations from each round of biopanning were assessed via ELISA, however, no significant enrichment of scFvs was observed similar to the results of the *Pseudomonas* biopanning (Figure 31). Several hundred colonies were screened using the direct colony lysis protocol, however, a clone containing the scFv was not identified. Biopanning of the initial library was repeated for the *L. monocytogenes* antigen to ensure failures were not the product of unrecognized experimental complications. After four rounds of selection using the standard protocol, two hundred additional colonies were screened using the microtiter plate assay. None of the clones exhibited an absorbance measurement above the background (Abs > 0.1). The failures of the biopanning against *L. monocytogenes* were not anticipated due to the high affinity shown by other scFvs for this antigen (Figures 25, 30, and 32). Additional biopanning was not conducted since at the conclusion of this project a suitable explanation for the observed complications had not been established.

Although several bacteria and a purified surface antigen were employed as targets, no scFvs with improved target specificity were isolated from the naïve library. Library construction, through the random combination of heavy and light chain genes, is thought to produce a highly diverse scFv repertoire. While the true diversity of the scFv pool cannot easily be determined, the DNA sequences of scFvs isolated during biopanning were compared to assess variability within the CDR sequences. The heavy chain gene sequences of all clones of interest were aligned using ClustalW. Nucleotide similarity

varied from 65 – 83 % identity for the heavy chain variable domain gene. ScFv genes isolated from the naïve library displayed greater sequence variability compared to those of the immunized library (80 – 85%), however, since the library did not yield scFvs with the desired binding characteristics, the overall library diversity may still be inadequate.

Expression and Purification of scFv

Expression of scFv

ScFvs were initially expressed in either *E. coli* strain DH5α or BL21, suppressor strains that inhibit the translation of the pIII phage capsid protein, to assess solubility and provide sufficient quantities of scFvs to allow for subsequent specificity assays. For these trials, the scFv gene was maintained in the phage display vector pComb3 XSS, which provided for periplasmic localization of scFvs. Expression was generally conducted at 37°C with 1 mM IPTG added to induce expression. Cultures were maintained for 12 - 16 h prior to periplasmic extraction of soluble proteins via osmotic shock or lysis of the cell pellet and Ni-NTA purification of scFvs from the soluble fraction. Under these conditions, all of the scFvs demonstrated low expression and poor solubility (Figures 23, 29, and 41). Several different induction conditions (temperature, IPTG concentrations, duration of expression), expression vectors, and bacterial strains were examined in an attempt to improve both expression and solubility of scFvs. The scFv k18 was used for these studies since it exhibited slightly improved expression levels compared to other scFvs. A summary of plasmid expression vectors and the *E. coli* strains examined can be seen in Table 2.

Table 2. Plasmid Vectors Examined to Optimize scFv k18 Expression

Vector	Bacterial Strain	Expression	Solubility
pComb3 XSS	DH5α / BL21 / BL21 Codon Plus	+	++
pProEx Htc	BL21 Codon Plus	+	+
pET21 c	BL21 Codon Plus	+	+
pET41 c	BL21 Codon Plus	++++	+

Note.

Expression of scFv k18 was conducted in a number of bacterial strains and plasmid vectors in an attempt to optimize expression. The pComb3 XSS vector targeted scFvs to the periplasm which improved solubility. The pProEX and pET vectors utilized cytoplasmic expression which decrease solubility slightly. Expression of scFv k18 as a GST-fusion (pET41 c) dramatically improved the expression.

Solubility and expression are rated on a scale of one (lowest) to four (highest) indicated by the "+" sign.

Expression conditions such as temperature, IPTG concentration, and duration of induction were examined for scFv k18 in the pComb3 XSS vector. None of the examined conditions succeeded in increasing expression of the recombinant proteins. As in the preliminary trials, scFvs were detectable only via immunoblotting. A slight improvement in solubility was observed for lower temperature induction (20 - 25°C) trials. Adjustments to the IPTG concentration and the duration of induction had little affect.

Codon bias analysis was conducted using the web-based Graphical Codon Usage Analyzer (http://gcua.schoedl.de/). *In silico* analysis of the scFv k18 gene identified nine amino acid codons whose corresponding tRNA is present in low abundance in *E. coli.* To compensate, expression studies were conducted in BL21 – Codon Plus – (RIL) *E. coli,* a

strain that provides plasmid-encoded tRNAs for six of the nine rare codons. Expression in this bacterial strain did not, however, improve scFv expression or solubility.

Although the pComb3 XSS vector provided the advantage of periplasmic localization, other research groups utilize commercial vectors to improve expression and/or solubility of scFvs [52, 54]. The pProEx Htc, pET21c, and pET41c were chosen for cytoplasmic expression of scFvs. Similar to pComb3 XSS, all vectors incorporated a terminal 6x His tag onto the recombinant protein to allow for subsequent IMAC chromatography. The pProEx and pET41 vectors incorporate the 6xHis affinity tag at the C-terminus while the pET21 incorporates the sequence at the N-terminus of recombinant proteins. Expression with the pET21 vector was investigated to determine if the position of the 6xHis epitope affected expression or solubility The pET41 vector differed from the other two vectors in encoding a portion of the glutathione-S-transferase (GST) protein upstream of the multiple cloning site (MCS). The GST-fusion has been demonstrated to improve expression and solubility of recombinant proteins [153]. Since all vectors utilized a similar MCS, a single set of primers were designed to facilitate the addition of *BamHI* and *HindIII* restriction enzyme sites at the 5' and 3' ends of the scFv gene respectively. As previously described, PCR products were purified using agarose gel electrophoresis and cloned into the pCR Blunt II TOPO vector for confirmation of the DNA sequence. The scFv gene was cloned into the aforementioned expression vectors using standard restriction digestion, ligation, and transformation protocols.

Cytoplasmic expression of scFv k18 in pProEx or pET21 vectors did not result in significant improvements in quantity or solubility of the scFv (Table 2). This observation was consistent regardless of the expression conditions. In contrast, expression in the

pET41 vector did visibly improve the quantity of scFv k18 expressed. A defined protein band of approximately 60 kDa (scFv 35 kDa , 25 kDa GST) was visible following SDS-PAGE and Coomassie staining (Figure 43). The immunoblot, however, suggested that translation was still slightly inhibited. A number of secondary products, which may be the product of premature termination of translation, were identified on the immunoblot. Expression conducted at lower temperatures (20 - 25°C) and IPTG concentrations had little to no effect of the accumulation of these proteins. Expression at 16°C was not examined, however, and may have decreased the accumulation of these molecules.

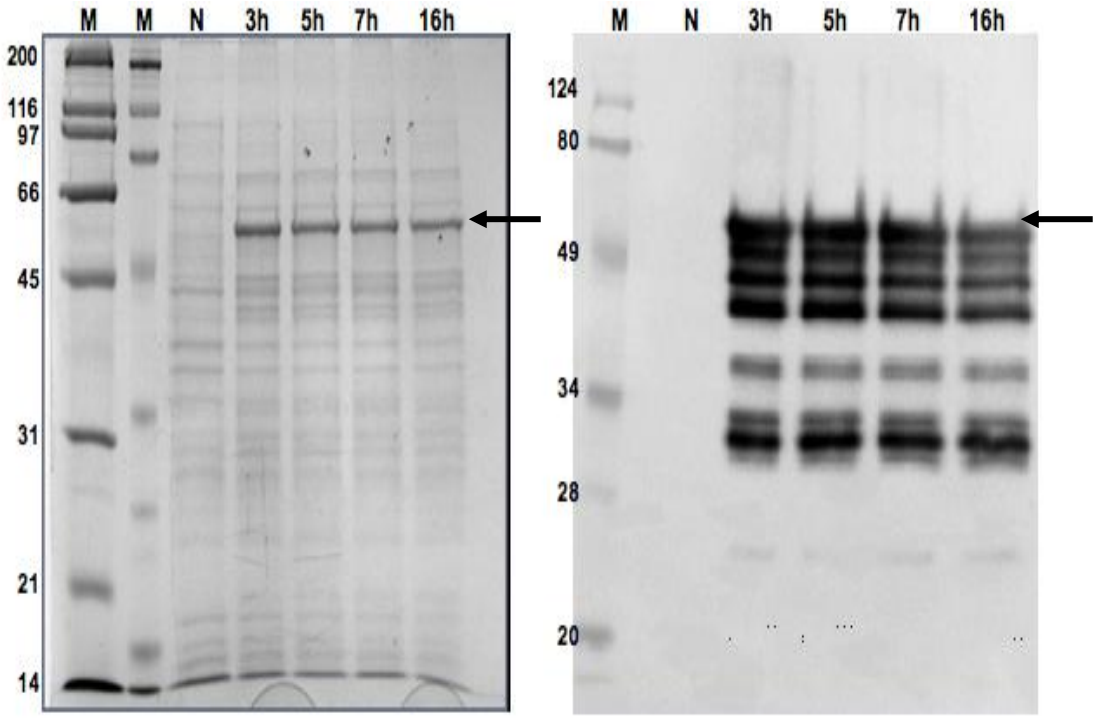


Figure 43. Small-Scale Expression Trial of scFv k18 in the pET41 Vector

Expression of scFv k18 in pET41c vector was examined for various durations. Individual 3 ml reactions were removed at the time points identified.

The GST-scFv fusion is visible on the Coomassie stained gel (left image) as an over-expressed protein of approximately 60 kDa (arrow). The non-induced sample (N) does not contain a corresponding band serving as negative control.

The immunoblot (right image) identifies several products that may represent premature termination products. The uppermost band (arrow) is approximately 60 kDa and is likely the GST-scFv k18 fusion protein

Expression of the GST-scFv fusion protein was conducted in larger volumes to better assess the solubility of scFv k18 and allow for subsequent purification and confirmation of scFv k18 activity. The fusion protein was largely insoluble (Figure 44) when expressed at either 25 °C or 37°C, aggregates of scFvs were evident in the pellet samples when visualized via immunoblotting.

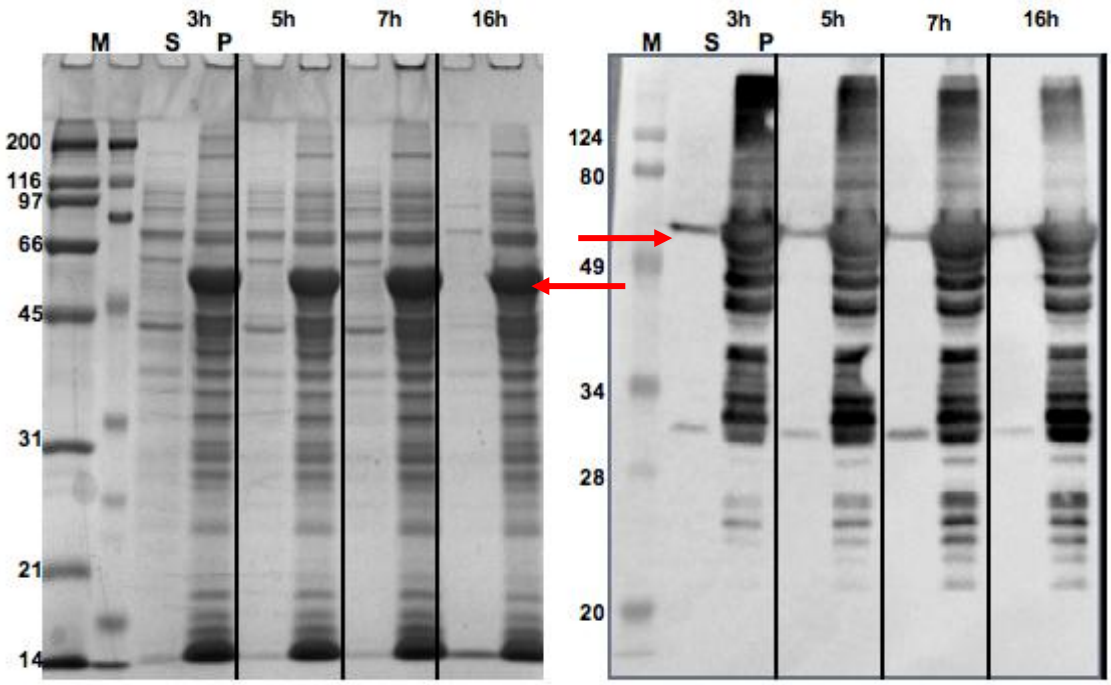


Figure 44. Assessing Solubility of pET41 Encoded scFv k18

Bacterial pellets were lysed using lysozyme, B-PER II, and sonication. Sample loads consisted of a defined quantity of protein (25 μg) for the pellet sample (P) gel and an equivalent volume of the supernatant (S).

The GST-scFv fusion is clearly discernable (arrows) in the insoluble material on both the Coomassie stained gel (left image) and the immunoblot (right image). Aggregates and incomplete translation products of the GST-scFv fusion are evident on the immunoblot.

Despite poor solubility, sufficient quantities of soluble GST-scFvs were available for purification via Ni-NTA affinity chromatography. As with previous purifications (Figure 24), elutions contained numerous contaminating proteins in addition to the fusion proteins. Although, the GST-tag could be used enzymatically removed, the fusion protein was left intact to determine if the GST portion affected the affinity or specificity of the scFv. If the GST portion did not affect scFv-binding properties it could potentially be utilized for immobilization of scFvs for QCM and SPR studies improving the probability

that scFvs will be immobilized in the proper orientating to ensure activity of the antigen-binding site.

The GST-scFv fusion proteins obtained via affinity chromatography were tested for target affinity using the standard ELISA protocol. The scFv k18 fusion protein displayed high affinity for all bacteria of the panel (data not shown). Given the initial, low target-specificity of scFv k18 (Figure 25), the initial ELISA results for the GST-scFv k18 could not be easily interpreted. To better characterize the effects of the GST-fusion, scFv s25, an scFv with excellent target specificity, was cloned into the pET41c vector. Following affinity purification, the binding characteristics of the two fusion proteins were examined via ELISA. The two fusion proteins displayed proportional binding to all bacteria of the panel (Figure 45). This suggested that binding of the bacterial targets occurred either with the GST portion of the fusion protein itself or was the result of GST addition to the scFv molecule leading to a disruption of the scFv structure. Further studies would be needed to better characterize these fusion proteins and establish the potential usage of the pET41c vector for expression of other scFvs.

Expression of scFvs was consistently low, regardless of bacterial strain, for all expression vectors except pET41c. To be an effective alternative to monoclonal antibody production, a system of producing large quantities of scFv would have to be developed. These experiments indicated that this would not be accomplished through the use of commercial vectors or bacterial strains. Although not investigated, yeast expression and bacterial fermentors offer additional avenues of increasing scFv production.

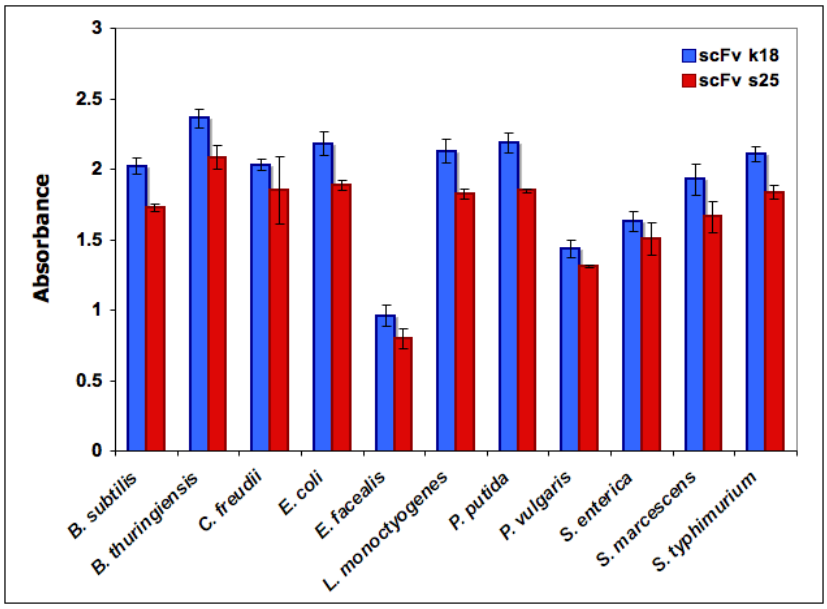


Figure 45. Bacterial Specificity of GST-scFv k18 and GST-scFv s25

The scFv k18 and scFv s25 genes were cloned into pET41c. The pET41 vector encodes a portion of the GST protein in frame with the scFv gene resulting in a fusion protein. The fusion proteins were isolated from the soluble fraction of expression cultures and purified via Ni^{2+}-NTA affinity chromatography.

Both GST-scFv preparations demonstrated proportional binding to all bacteria of the panel. The GST-scFv s25 fusion exhibited a complete loss of the specificity for *B. thuringiensis* previously shown for scFv s25. The similar affinities demonstrated for the two very different scFvs suggests GST interaction with bacteria of the panel.

Affintiy Purification of scFv

Because variation in recombinant protein expression and solubility existed between individual clones, experimental conditions were maintained to allow for comparison of results. Expression was induced in 500 ml cultures (OD_{600} = 0.6 – 0.8) with 0.5 mM IPTG, and then conducted overnight (12-16 hours) at 25°C. Cell pellets were lysed with lysozyme, B-PER II bacterial extraction reagent, and sonication as previously described. The scFvs were recovered from the soluble fraction using Ni^{2+}-NTA column with a 3 ml bed volume. Proteins were eluted from the column with 250 mM imidazole and collected

in three fractions, the volume of the first two fractions was equivalent to the bed volume, while the third fraction was twice the volume. Elution and wash fractions from the column were always examined at a maximum volume (25 μl) on subsequent SDS-PAGE gels. Unless otherwise stated, optimization of the purification protocols was accomplished with clone k18.

ScFvs were routinely purified from the soluble material of expression cultures using a Ni^{2+}-NTA agarose resin. For all purification attempts, regardless of clone, a considerable number of contaminating proteins were also retained by the column matrix and present in the elution fractions. Initially, it was believed that due to the low concentration of the scFvs within the soluble fraction, excess Ni^{2+} ions of the column matrix were binding other cellular proteins. Significant reductions in the volume of the column bed were made but with little benefit to the scFv purity. Additional modifications to the manufacturers protocol included:

1) Salt concentrations of the wash buffers increased to 300 mM NaCl in buffers A/C and 1.5 M in buffer B
2) Imidazole (25 mM) added to all column buffers
3) pH of the wash buffers decreased to pH 6.0 for buffers A/C and to pH 4.0 for buffer B.

While none of these changes were definitive improvements to the protocol, utilization of all three modifications did seem to diminish the number of contaminating proteins in the elution fractions (Figure 46).

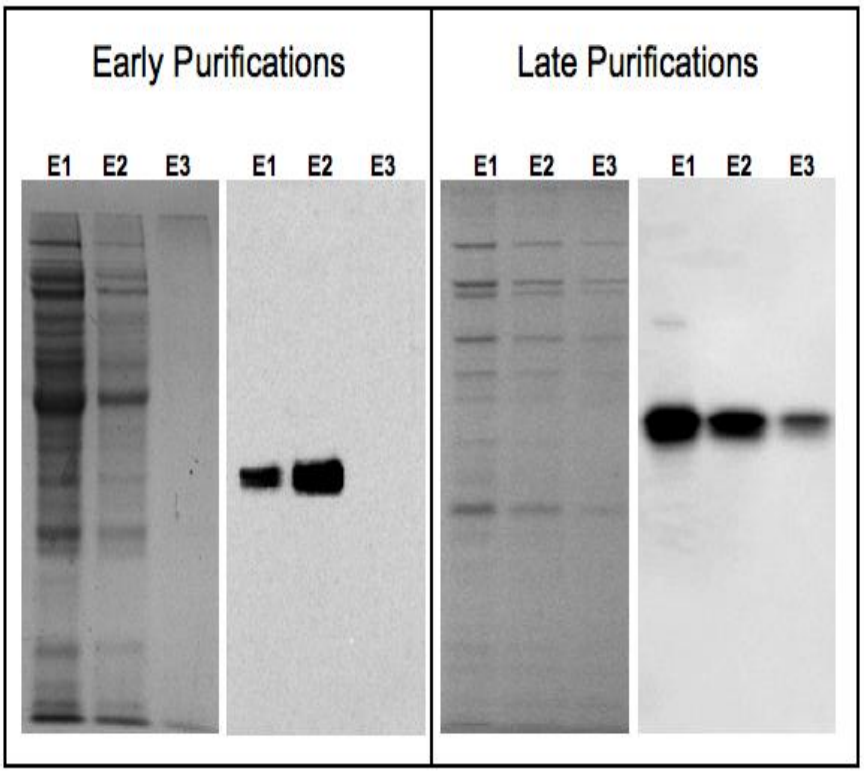


Figure 46. Comparison of the Abundance of Non-scFv Contaminants in Eluates Obtained from Ni^{2+}-NTA Chromatography

The elution fractions obtained from purifications conducted one year apart were compared to visualize improvements in purification methods. The scFv clone, expression conditions, and volume of elutions examined were consistent between the two experiments.

Elutions from early purification attempts (left box) contained a high concentration of non-scFv proteins as evidenced by the Coomassie stained gel (left image). The concentration of contaminating proteins was reduced in later purifications (right box) through adjustments to the salt concentration and pH of the wash buffers.

Accurate quantitation of scFvs in elutions fractions was impossible due to contaminating proteins that were present in greater abundance. To circumvent this complication, a second purification strategy was employed. A small affinity column was assembled using EZview red anti-HA affinity gel (Sigma) as the column matrix. Although this purification step allowed for the removal of the majority of the contaminating proteins from the elution fractions (Figure 47), the anti-HA matrix was

only available as an immunoprecipitation reagent (500 μl volume) and therefore not feasible as a primary method of purification.

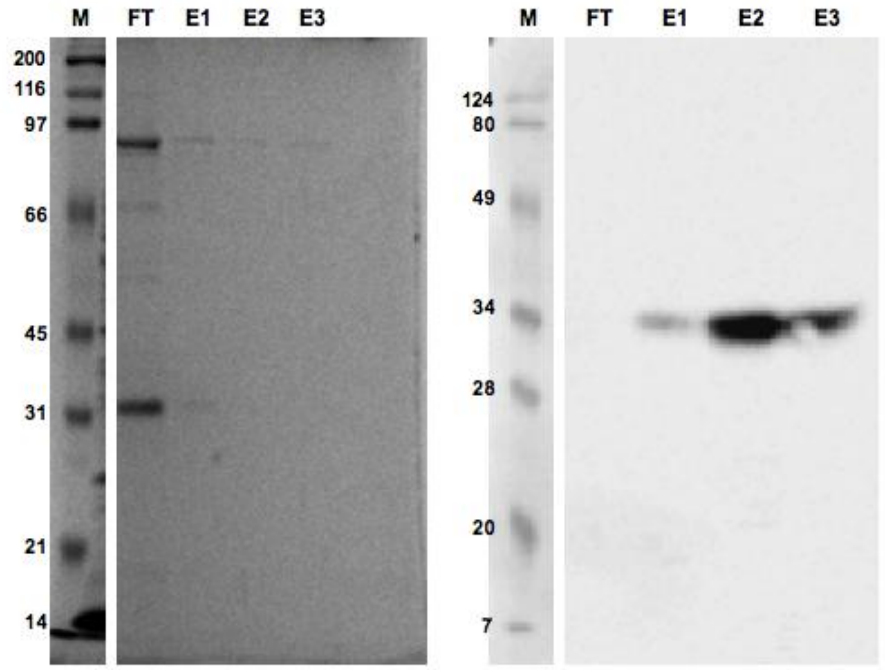


Figure 47. Purification of scFvs Using Anti-HA Affinity Chromatography

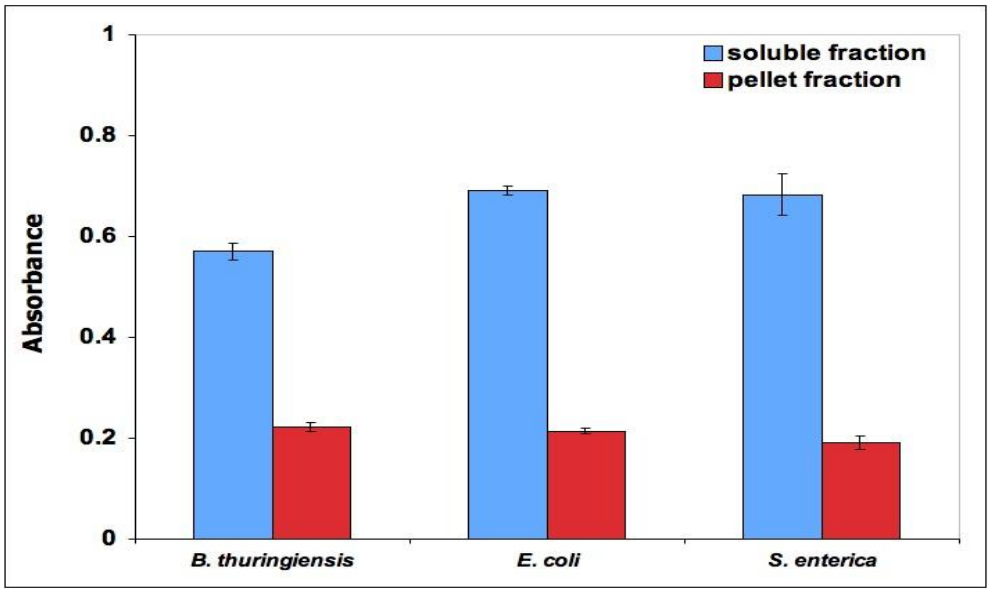
ScFv-containing elutions from Ni-NTA affinity chromatography were combined and subjected to a second column purification using an anti-HA affinity matrix. The flow-through (FT), and all three elutions (E1-E3) were loaded at equivalent volumes (20 μl) to a 12% denaturing, polyacrylamide gel. The scFvs were not visible on the Coomassie stained gel (left image) due to low concentration. The scFvs, however, could be detected in all three elution fractions via immunoblotting (right image).

Although some contaminating proteins are still faintly visible in the elution fractions, the majority passes through in the initial column loading.

The scFvs eluted from the anti-HA column were concentrated with PEG-8000, dialyzed, and quantitated via BCA. The purified scFvs were then included on immunoblots to establish standards of scFv concentration. These standards provided a method of calculating the concentration of scFvs in elutions from Ni^{2+}-NTA columns using densitometric analysis of immunoblots.

Given that most of the desired protein was separated into the insoluble fraction, a denaturating Ni^{2+}-NTA purification protocol was attempted as previously described with the murine scFv 4-2. The scFvs k18 and s25 were refolded using an on-column renaturation method prior to elution. For comparison of activity following purification, scFvs were purified in tangent from the soluble and insoluble fractions of overnight expression cultures and then assessed using a standard ELISA protocol (Figure 48). The denaturing process directly affected the activity of both scFv k18 and scFv s25. The scFv k18 preparations maintained affinity for target bacteria, however, as evidenced by the low absorbances for each antigen, some portion of the scFvs had lost activity. The measured absorbances for the denatured/renatured scFvs was three-fold less than those of scFvs purified from the soluble fraction. The loss of activity was more dramatic for scFv s25 samples, a near complete inactivation of the scFv was observed. Since both scFvs displayed a significant loss of binding activity, the denaturation/renaturation purification method was deemed unsuitable for recovery of the scFvs from the insoluble pellet.

A



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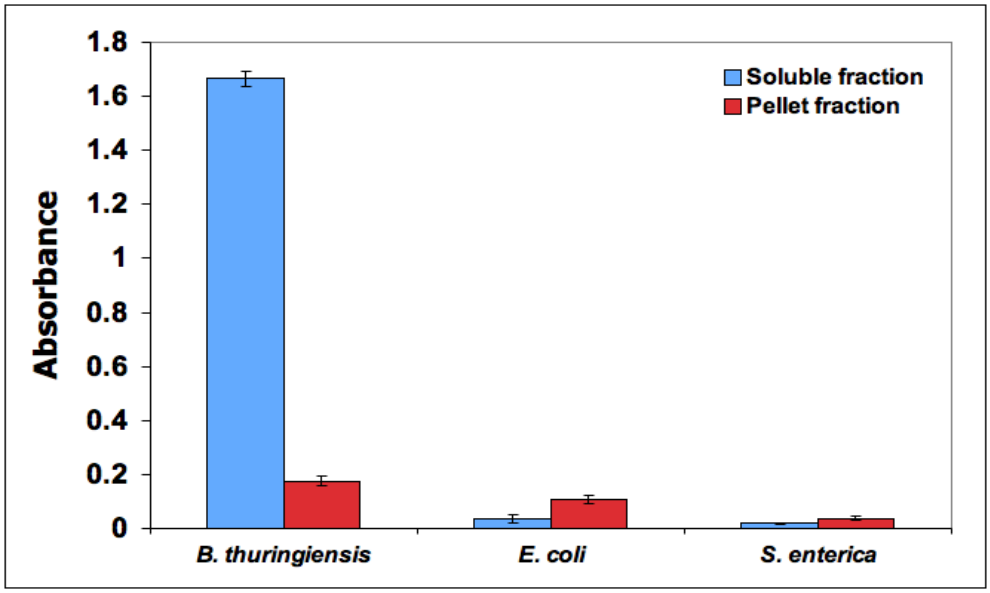

Figure 48. Comparison of Target Affinity for scFvs Purified from the Soluble and Insoluble Fractions of Expression Culture Cell Lysates

The scFv k18 (panel A) and scFv s25 (panel B) were purified from both soluble and pellet fractions of an overnight expression culture.

Equal concentrations of total protein (eluates) were tested for target affinity via ELISA. The scFv s25 subjected to denaturation/renaturation exhibited little to no binding activity. In contrast, denatured/renatured scFv k18 continued to bind target bacteria, however, absorbance values were decreased three-fold suggesting some decrease in the number of functional recombinant antibodies.

Several clones isolated during biopanning experiments exported scFvs to the culture medium during expression trials (Figure 29). Although this phenomenon was not observed with clone k18, clone s25 did export scFvs to the surrounding medium when expression was conducted at 37°C (Figure 49). The scFvs were purified from the culture medium using a standard IMAC protocol and tested for activity via ELISA (Figure 50). Unfortunately, scFvs obtained from the culture medium exhibited different binding interactions with the panel bacteria. In contrast to the highly specific activity of scFv s25 purified from the supernatant, culture medium purified scFv s25 bound several non-target bacteria. Comparison of elution fractions from the two purification methods via SDS-PAGE did not indicate degradation of the scFvs, therefore, the differences in activity may have been the result of scFv tertiary structure.

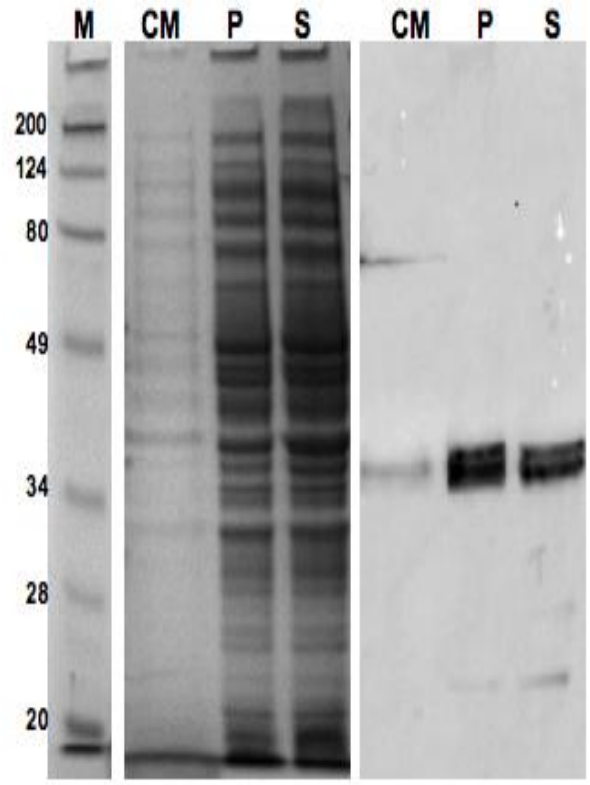

Figure 49. Small-Scale Expression of scFv s25

Expression was conducted at 37°C to encourage scFv export to the culture medium. Equivalent concentrations of pellet (P) and supernatant (S) fractions were examined in parallel to an aliquot (25 μl) of the culture medium (CM) on a 12% acrylamide gel.

Although the immunoblot suggests a minimal scFv concentration in the culture medium, the volume examined is only a fraction of the available volume (1/20,000) compared to the volume of the other two fractions (1/6,000).

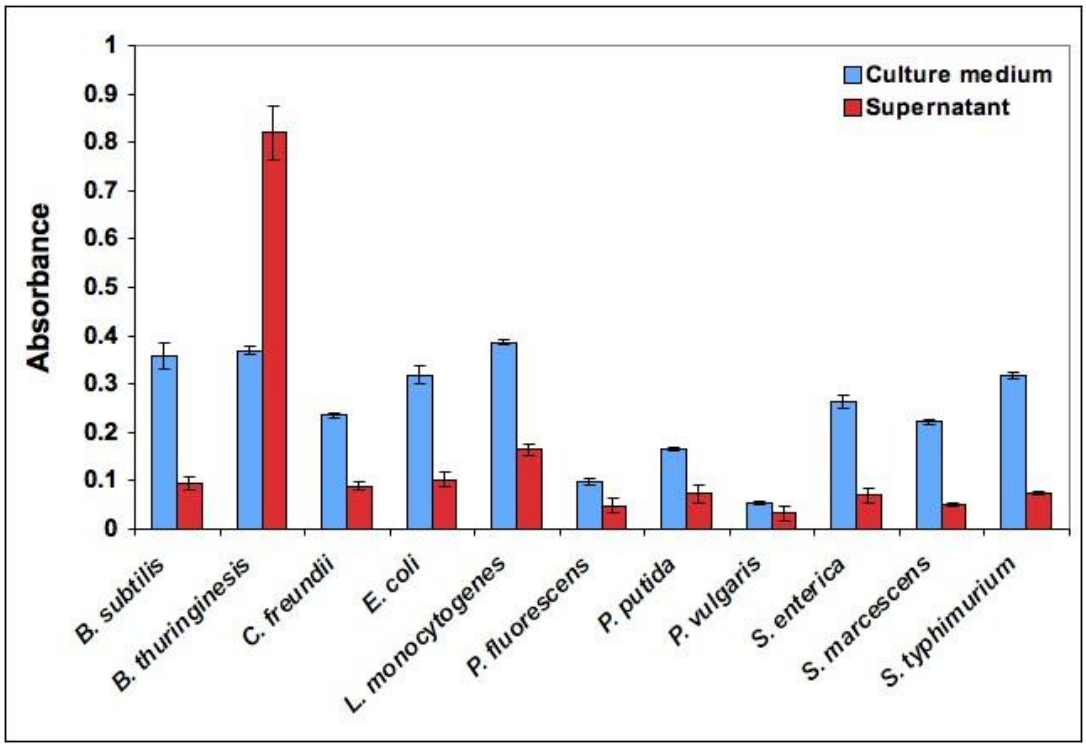

Figure 50. Comparison of Target Specificity of scFv s25 Purified from the Culture Medium and the Soluble Fraction of Expression Culture Cell Lysates

The scFv s25 was purified using a Ni^{2+}-NTA affinity column from either the culture medium or the soluble fraction of the lysed cell pellet. Equivalent total protein concentrations were examined via ELISA to assess specificity of the purified scFvs.

The scFv s25 purified from the soluble fraction exhibit high affinity for the *B. thuringiensis* target, in contrast to scFvs purified from the culture medium. The scFvs obtained from the culture medium demonstrated not only a reduced activity (lower absorbance values) but also a loss of target specificity.

Absorbance values above background (which was subtracted) were observed for scFv from the soluble fraction. The duration of the colorimetric reaction was longer than in previous trials, which may have contributed to this observation.

The oxidizing environment of the periplasm allows for the formation of disulfide bonds that are often critical for scFv activity. These bonds may not form in scFvs exported to the culture medium or may be later oxidized while in the culture medium itself. Although purification from the culture medium was not applicable to scFv s25, the method may prove advantageous to purification of recombinant proteins from other clones.

Several modifications to the manufacturer protocol for IMAC were employed to improve the purity of scFvs obtained from expression cultures. These improvements did eliminate some of the contaminating proteins present in the elutions of early purifications, however, a second affinity purification method was required for complete removal of these molecules. Purification of scFvs from both the insoluble pellet material and the culture medium was also investigated. Although scFvs could be purified from both fractions, the specificities and affinities of these scFvs were unlike those of scFvs from the soluble fraction. The scFvs used in later ELISA and QCM studies were therefore only obtained from the soluble portion of expression cultures.

Integration of Antibodies into the Quartz Crystal Microbalance (QCM) Detector

The prototype biosensor was modeled on a QCM platform for detection of bacterial targets. The gold electrode of the QCM crystal is amenable to numerous attachment chemistries making it well suited for the immobilization of target-specific scFvs. Formation of the SAM generated a surface of free carboxylic acid groups to which proteins could be immobilized through the formation of an amide bond facilitated by an EDC/NHS reaction. Monoclonal antibody Ma1-6 and scFvs were attached directly to the SAM layer through the EDC/NHS conjugation reaction. An additional immobilization

protocol indirectly attached to the surface by way of an IgG molecule that was specific for the C-terminal HA-epitope tag was also employed (Figure 51).

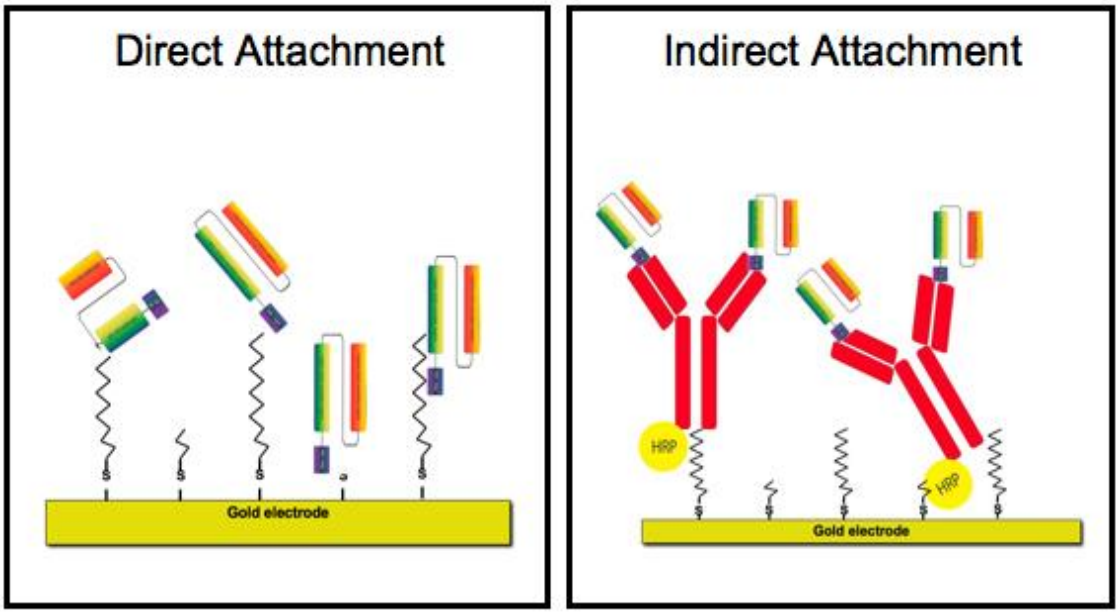

Figure 51. Diagrams of scFv Immobilization Strategies
The direct method of attachment (left panel) involved the formation of amide bonds between the activated acid group of the SAM and a free-amine group of the scFv. Neither orientation nor activity of the scFv was ensured.
The indirect method of attachment (right panel) utilized the rat anti-HA HRP to immobilize the scFv to the surface. The scFvs were attached through a C-terminal HA epitope that ensures both orientation of the scFv antigen-recognition domain and activity.

Monoclonal antibody Ma1-6, purified via Protein G chromatography, was immobilized to the surface at saturating concentrations based on the conclusions of Prusak-Sochaczewski *et al.* [(33)]. Bacterial detection was assessed using live *P. putida* suspended in degassed PBS buffer. Bacterial concentrations of 10^2 cfu/ml and 10^3 cfu/ml were repeatedly examined to establish a limit of detection for the sensor with a surface comprised of immobilized mAbs. A consistent decrease in frequency ranging from -25

Hz to -40 Hz was measured for bacterial concentrations of 10^3 cfu/ml. Analysis of bacterial concentrations of 10^2 cfu/ml, however, demonstrated inconsistent measurements that ranged from +5 Hz to -15 Hz. The limit of detection (LOD) for immobilized IgG molecules was therefore established at 10^3 cfu/ml.

Initially, scFvs obtained from IMAC purifications were directly immobilized to the surface through formation of the amide bond. A total of 3 μg of total protein (8.7 pmoles of scFvs based on densitometry) were used in the initial immobilization and detection trials. *S. typhimurium* samples were tested at a concentration of 10^6 cfu/ml using the standard detection protocols. A significant decrease in frequency was not attained suggesting little to no interaction between bacteria and the surface. The scFv concentration was increased to 7 μg of protein (20.3 pmoles of scFv) in an attempt to saturate the crystal surface. Again trials were conducted with cell densities of 10^6 cfu/ml. A decrease in oscillation frequency was observed in the majority of experiments; however, the frequency change was not consistent from between trials (Table 3).

Table 3. Detection of S. typhimurium Using QCM and Direct Immobilization scFv k18

Date	**Concentration**	**Frequency change (Δf)**	**Resistance change (ΔΩ)**
2009-04-15	10^6	+3 Hz	-15 Ω
2009-04-16	10^6	-59 Hz	+38 Ω
2009-04-16	10^6	-16.7 Hz	+2 Ω
2009-05-14	10^6	-7.4 Hz	+2.5 Ω
2009-05-14	10^6	+1.4 Hz	+1 Ω

Note.

A protein solution containing 20 pmole scFv k18 was directly immobilized to the SAM using an EDC/NHS reaction to facilitate amide bond formation. *S. typhimurium* cells were diluted in degassed PBS (pH 7.4) to OD_{600} = 1.0. A 10 μl aliquot (10^6 cfu/ml) was added to 500 μl PBS on the surface of the QCM crystal.

The frequency change (Δf) was calculated from the subtracting the final frequency measurement following three washes of the surface after sample incubation from the initial baseline frequency.

Direct attachment of scFv to the surface lacked reproducibility in individual trials.

The direct attachment of scFvs proved an ineffective method of generating a QCM crystal capable of consistent target detection. An immobilization scheme was developed that utilized the C-terminal HA–epitope tag to ensure proper orientation of the scFv antigen-recognition domain and therefore activity of the scFvs. The rat anti-HA HRP antibody used for immunoblot and ELISA protocols was readily attached to the surface by way of the standard carbodiimide reaction. This immobilization strategy had several

benefits compared to the direct method of attachment. The IgG molecule is substantially larger than the scFv and conjugation to the surface is less likely to occur at a position that is critical for antigen recognition ensuring activity of the molecule. Each IgG molecule tethered to the surface can bind two scFvs increasing target binding through avidity. Additionally, the IgG molecule was commercially available and could be attached to the surface at defined concentrations. Once saturation of the available IgG binding sites with scFvs was determined, the indirect immobilization of scFv allowed for a greater reproducibility of sample analysis (frequency versus concentration) compared to the direct method of attachment.

For indirect attachment of scFvs, crystal surfaces were prepared by first immobilizing 37.5 units of the rat anti-HA HRP antibody using the previously described protocol, followed by the addition of 5 μg of total protein from the eluates of the Ni^{2+}-NTA chromatography columns (15 pmoles of scFv k18). Increasing the molar concentration of the scFvs added to the anti-HA surface did not improve the frequency change for bacterial samples. It was therefore concluded that saturation of the anti-HA surface had been attained. A minimum molar concentration of scFvs required for saturation was not determined. A standard curve of frequency change in relation to bacterial concentration was constructed using the SRS system and scFv k18 (Figure 52). The data collected would serve to both validate the biosensor during later trials and determine a limit of detection (LOD) for the device.

The standard curve was constructed for bacterial concentrations ranging from 10^1 – 10^6 cfu/ml. Individual data points were the average of 15 – 20 trials, the majority of which were obtained from single crystals measurements. In later trials it was determined

that the crystal surface could be regenerated with a 0.2 M glycine-HCl (pH 2.5) solution incubated on the crystal surface for 60 s followed by a second scFv incubation step. This reduced some of the variability observed when using several different crystals. A linear relationship was observed between the decrease in frequency and bacterial concentrations between $10^1 – 10^4$ cfu/ml. Cell densities greater than 10^4 cfu/ml, however, did not conform to this linearity. It was suspected that the surface was saturated at these elevated

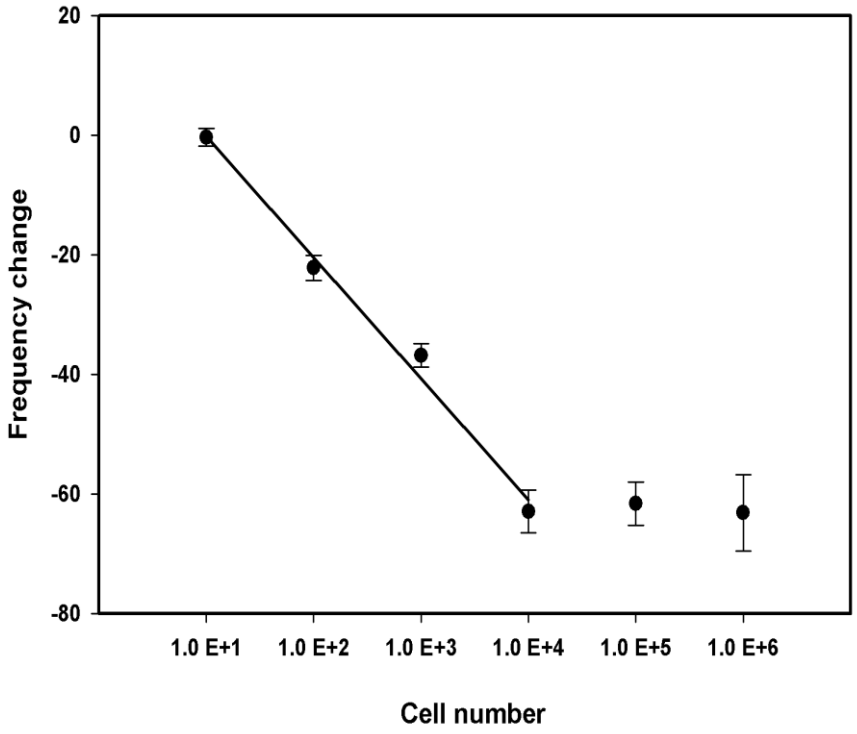

Figure 52. Standardization of *S. enterica* Detection Using a QCM Platform and scFv k18

ScFv k18 was immobilized to the gold electrode using an indirect attachment protocol. Baseline and final frequency measurements were recorded from a 500 μl volume of PBS (pH 7.4) solution.

A linear relationship between Δf and cell number was observed from $10^1 – 10^4$ bacteria/ml. Cell densities above 10^4 did not conform to the linear relationship suggesting saturation of the surface with bacteria

Error bars represent the standard error of the mean rather than standard deviation since there was significant variation in the Δf value between individual crystals

bacterial concentrations.

The standard curve was developed with scFv k18 due to the poor expression and solubility of scFv s25, which limited the quantity of scFvs available. The lack of specificity, however, made scFv k18 unsuitable for accurate validation of the prototype biosensor since negative (non-target) controls were not available. In contrast, scFv s25 allowed for analysis of several non-target bacteria to demonstrate accuracy (low false positive) of the device. The SRS system was initially used to confirm that scFv s25 produced frequency changes that correlated to the established standard curve. Real-time frequency measurements could also be obtained using the SRS system permitting a visible comparison of target and non-target bacterial interaction with the surface (Figure 53). Negative controls were repeated several times with both *B. subtilis* and *S. typhimurium*.

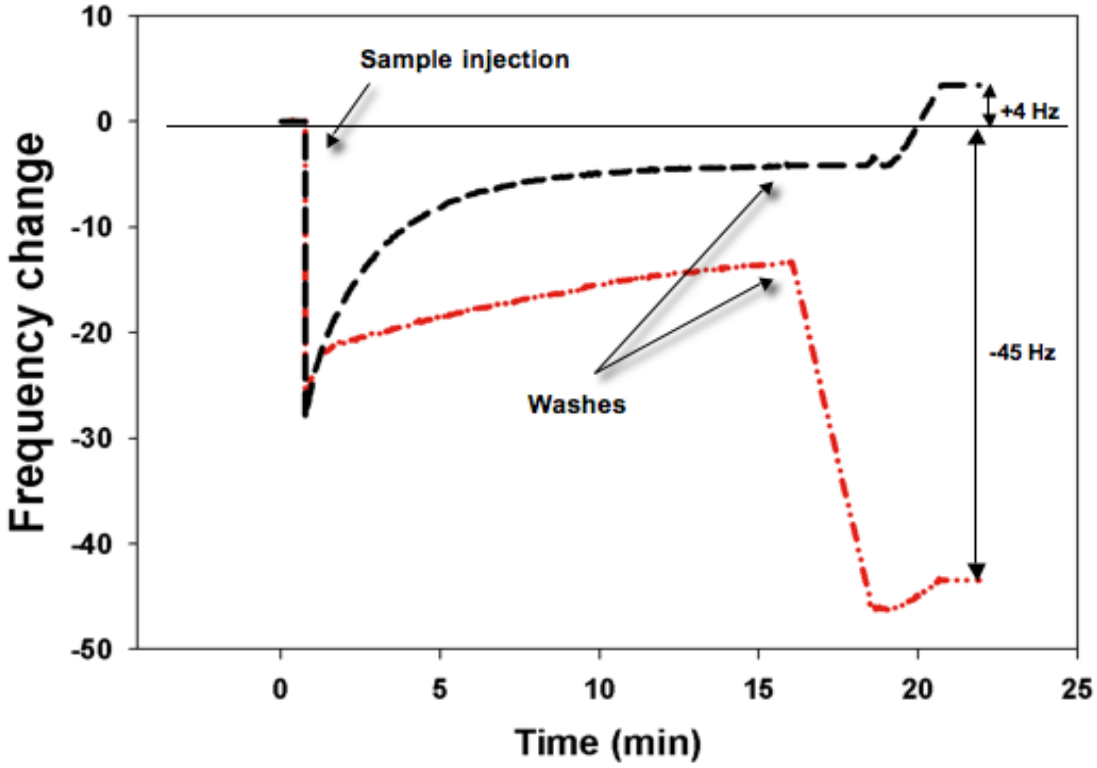

Figure 53. Real-time Analysis of Target and Non-Target Bacteria Using the QCM Detector

The SRS system and scFv s25 were used to monitor the interaction of target (*B. thuringiensis* -..-..) and non-target (*B. subtilis* ----) bacteria with scFv s25 immobilized to the surface. Bacteria were examined at a concentration of 10^3 cells/ml following a standard detection protocol. The frequency for both samples demonstrated an increasing slope, however, the non-target bacteria plateaued closer to the initial baseline following the wash steps. The target bacteria produced a significant decrease in frequency (positive) while the non-target returned to within error of the baseline (negative)

The biosensor was tested using the previously described protocol with both target and non-target bacteria. Fifteen measurements of varying bacterial concentrations for the *B. thuringiensis* target were obtained, of these trials, a single measurement failed to produce a positive signal. The device was also tested with the aforementioned negative control

bacteria with concentrations ranging from 10^3 - 10^5 cfu/ml. For all such trials the instrument relayed the expected negative result. These experiments validated the utilization of a recombinant single chain Fv antibody, immobilized indirectly through the C-terminal HA-epitope, and the quartz crystal microbalance platform for the detection of target bacteria from an aqueous sample.

CHAPTER V

DISCUSSION

Systems for the production of full-length antibodies, from either polyclonal or monoclonal sources, are established and routinely used by industry for the acquisition of monospecific antibodies for commercial uses. However, obtaining antibodies from animals and mammalian cell cultures is quite expensive and not feasible for application in a mass-produced biosensor. To satisfy the low cost and adaptability requirements of the biosensor, alternative systems that mass-produce recombinant antibody molecules were explored. Two single-chain Fv antibody (scFv) libraries were constructed and a high-throughput phage display system employed to select recombinant antibodies that bound specific bacterial targets. This system was compared to full-length antibody production to assess which method would best satisfy the requirements of a commercial biosensor.

Monoclonal Antibodies from Bioreactors

Hybridoma cell cultures have been utilized for production of antibodies with defined specificity since their initial discovery by Köhler and Milstein in 1975 [(15)]. As demand for antibodies increased, several commercially available systems of maintaining dense hybridoma cell cultures were developed to facilitate the large-scale production of monoclonal antibodies (mAbs). These bioreactors utilize a hollow fiber network of permeable membranes that allow the passage of nutrients into and removal of waste products from the growth chamber in which cells are maintained (Figure 7). Great success has been demonstrated with these systems and yields of up to 10 g/L of mAb from harvested media have been reported [(131, 132)].

The Fibercell System (Frederick, MD) was utilized for the maintenance of the murine hybridoma cell line Ma1-6 (ATCC CRL-1783). Although the growth conditions of hybridoma cultures are similar in most regards, subtle differences in antibody production and nutrient requirements are commonly observed between individual cell lines [154, 155]. To optimize antibody production for the Ma1-6 cell line, the quantity of antibody purified from the ECS media was examined in tandem with the rate of glucose consumption and the pH of the circulating media on a daily basis. Antibody production was optimal when cells consumed half of the available glucose of the circulating media every two days. Increased consumption was the result of excessively high cell densities, which did not exhibit an equivalent increase in antibody production. On average, a single cartridge of the Fibercell system could produce 3 – 4 g of mAb per month at a cost of $200 per bioreactor for media and supplies. The cost of the bioreactors themselves, in addition to that of the media and fetal bovine serum supplement, made mAb production quite expensive.

Purification of mAbs was accomplished with either Protein G or DEAE Affi-gel blue chromatography. Both purification strategies produced a mAb free of contaminating serum proteins that maintained antigen recognition when tested via immunoblotting. Although purification using the DEAE Affi-gel Blue column required an initial ammonium sulfate precipitation and concentration of column elutions, the method was considerably more cost effective than purification using Protein G- based affinity columns. It is feasible that the protocol developed for DEAE Affi-gel Blue purification could be adapted for commercial production of monoclonal antibodies, although the true

cost would have to account for delays associated with the initial precipitation and subsequent concentration steps.

Production of mAbs from hybridoma cell cultures is not without its disadvantages. Aside from the cost and difficulties of maintaining these bioreactors for long periods of time, the process of isolating hybridoma cultures that produce a target-specific antibody is also quite laborious. Generation of hybridoma cell lines requires immunization of animals, typically mice, over a span of 8 – 12 weeks. This is followed by cell fusions to form the hybridoma cultures and limiting dilutions to isolate clonal cultures, a process which often requires several weeks [156, 157]. Selection of cell lines that produce a mAb with the desired characteristics of affinity and specificity requires an additional 3 – 9 months. This process would have to be repeated for each new target antigen. Additionally, once the hybridoma cell line is established the scaling up of the culture from T-flasks to the bioreactor can take 3 – 4 weeks. In total, six months to a year are required to establish a hybridoma cell line that produces antibody specific for a new target antigen on a commercial scale.

Although large-scale hybridoma production of mAb was successful, this system of obtaining target-specific antibodies is likely too costly to be integrated into a commercial biosensor that is capable of identifying multiple bacterial pathogens. Additionally, the time constraints inherent in the development of new cell lines limit the adaptability of the system to emerging threats. In contrast, recombinant antibodies constructed from only the antigen-binding domain of full-length antibodies can be produced in bacteria in a considerably shorter time frame, thereby significantly reducing the expense associated

with antibody manufacture. Large-scale growth in fermentors would allow for bacterial production of antibodies significantly decreasing the cost of biosensor commercialization.

A Recombinant Antibody Was Constructed from the Genes Encoding the Fv Region of the Ma1-6 Monoclonal Antibody

Construction of scFvs from the mRNA of hybridoma cells is well documented in the literature [18, 23, 63]. Primer sequences for amplification of the heavy and light chain variable domain sequences from mouse had previously been described by Zhou *et al*. [67]. These primers were designed to bind conserved sequences of the 5' and 3' ends of both heavy and light chains. Although there is substantial sequence conservation in these regions, the primer pool was still large, consisting of ten reverse and four forward primers for the heavy chain and nine reverse and six forward primers for the light chain. Zhou *et al.* performed extensive *in silico* sequence analysis to ensure that this primer pool was sufficiently degenerate to amplify the vast majority of murine IgG variable domain genes. The determination of effective primer sequences by Zhou *et al*. expedited construction of the murine scFv.

Krebber *et al*. suggested that not all scFvs derived from hybridoma cells are functional molecules capable of binding the target antigen [18]. Often this is the result of non-functional mRNAs, contributed by the endogenous IgG genes of the myeloma cells, which may be amplified and incorporated into the scFv constructs [18, 55, 158]. Such events can significantly complicate the interpretation of results from *in silico* analysis since genes originating from the myeloma cells contain sufficient homology to be identified as an Fv gene. Plasmid DNA from twelve clones derived from the heavy chain PCR reactions of scFv 4-2 was sent for DNA sequence analysis to verify that they contained

the mouse immunoglobulin gene. Of the twelve, six were identical to one another and contained an open reading frame (ORF). A BlastN analysis of the nucleotide sequence suggested a high similarity to other reported mouse immunoglobulin gene sequences (E value = $5e^{-141}$). Four of the remaining clones were nearly identical to the preceding six sequences (98% similar), but contained a stop codon close to the 3' end and two silent mutations. The final two clones exhibited regions of similarity to the preceding clones (74% similarity) and were identified as potential murine immunoglobulin genes (E value = $1e^{-84}$), however, these sequences did not contain an ORF. While the genes of four of the clones may have been the result of mutations incurred during PCR, the final two clones were dissimilar enough from the other clones to have originated from a different DNA sequence, potentially an endogenous myeloma gene. Although a putative heavy chain gene was identified, the Ma1-6 variable gene sequences have not been reported in the literature and therefore validation of their identity was not possible.

As was seen with scFv 4-2, scFvs often aggregate during protein expression *(159-161)*. Although recovery of the insoluble scFvs from the pellet fraction was possible, the recombinant antibodies exhibited no target binding ability. Purification of denatured recombinant proteins is often used for the recovery of scFvs from the insoluble pellet of bacterial expression cultures *(54, 159, 162)*, however, the conditions of purification, as potentially seen with scFv 4-2, may prove too harsh for the recovery of active scFvs from some clones.

The constant domains of a full-length antibody, which are absent in scFvs, contribute to the stability of the protein *(34)*. The absence of these stabilizing regions may have contributed to the lack of binding affinity exhibited by scFv 4-2, which may have been

unable to refold properly following denaturation. The addition of constant domain sequences to form a recombinant Fab has been shown by some research groups to recover or improve the antigen-binding activity of poorly functioning scFvs [(163-167)]. The DNA sequence of scFv 4-2 suggested that the gene did encode the IgG heavy and light chain variable domains, and the protein therefore should have folded into an active recombinant antibody. Since no target affinity was observed for the renatured scFv 4-2, it is possible that there were inadequate interactions between the heavy and light chain scFv subunits to stabilize the antigen-binding domain.

Although other research groups have successfully employed hybridomas as the source of the genetic material for construction of scFvs [(18, 23, 63)], the Ma1-6 cell line did not yield a functional scFv. Therefore, continuation of recombinant antibody research focused on the construction of a larger, more diverse pool of immunoglobulin heavy and light chain genes that would improve the probability of isolating scFvs that can detect a variety of bacteria. Additionally, an antibody library lends itself to high throughput selection of scFvs based on their binding affinity and specificity, circumventing the difficulties observed with the potential lack of stability shown by an individual scFv. To expedite the identification of scFvs specific for a defined target (*B. thuringiensis*), an immunized scFv library was therefore constructed.

Target-Specific scFvs Can be Isolated from an Immunized Library

An scFv library with a clonal diversity of $5x10^6$ was constructed from the spleen of a rabbit immunized with formalin-killed *Bacillus thuringiensis*. The clonal diversity of the scFv library was initially calculated at $7x10^6$ determined from the number of transformants obtained following electroporation. However, analysis of individual clones

following biopanning indicated that many lacked the scFv gene insert. The pComb3 XSS vector was designed with *SfiI* restriction sites that are dissimilar in their nucleotide sequence and therefore facilitate proper orientation of the scFv gene in the vector during ligation. Restriction digest analysis, however, confirmed that ligation of the vector backbone without an scFv insert did occur with appreciable frequency. A random sampling of 50 clones from the initial library indicated that 70% contained the scFv gene. The calculated clonal diversity was adjusted to $5x10^6$ based on this observation.

The rabbit was chosen as the host largely due to its ability to generate antibodies to antigens that are often not immunogenic in other rodents [(168-170)]. Additionally, rabbits tend to use a single heavy chain gene that undergoes somatic hypermutation during diversification. This characteristic requires a smaller primer pool for library construction compared to other organisms [(56, 64, 171)].

The initial rounds of selection demonstrated some complications inherent in the biopanning process and the pComb3 XSS phagemid vector. Individual colonies were examined with a colony lysis/restriction digest protocol to detect the scFv gene following four rounds of biopanning. The majority (89%) of the colonies examined lacked an scFv gene insert, a significantly higher percentage than observed with the initial library. As shown in Figure 22, a careful inspection of the digestion products for samples lacking the scFv gene revealed a DNA species of approximately 3000 bp at a position similar to that of the linearized vector backbone. Since the *SfiI* restriction sites were likely corrupted during ligation, uncut plasmid DNA from these aberrant clones probably migrated during electrophoresis as a compact circular molecule with an apparent size of 1500 bp. Although similar results are not described in the literature, the high abundance of the

empty vector compared to scFv gene-containing clones was explained by Krebber *et al.*, who suggested that *E. coli* containing empty plasmid vectors would possess a growth advantage over those having to replicate larger plasmids with inserts [18, 172, 173]. Given that the frequency of empty vectors was greater following four rounds of biopanning, the results of restriction analysis of individual clones from biopanning output plates agree with Krebber's explanations.

Recombinant antibodies were isolated from clones that contained a confirmed scFv gene insert and their specificity and affinity assessed via ELISA. Although, like scFv 4-2, many of the scFvs were insoluble (six of the initial seven tested), scFv k18 was sufficiently soluble to allow for subsequent purification. Specificity assays, however, established a potential complication associated with biopanning against whole bacterium, since scFv k18 readily bound the majority of the panel bacteria. Because of the complexity of the bacterial surface, some cross-reactivity was expected due to conserved bacterial epitopes such as flagella, common receptors, etc. To diminish the scFv population that recognized these conserved bacterial epitopes, a negative selection protocol was adopted. The method was quite successful according to the initial trial, resulting in the isolation two scFvs (scFv s24 and scFv s25) that were highly specific for the target bacterium *B. thuringiensis* (Figures 26 and 27). Although scFv s24 was insoluble and could not be tested against the entire bacterial panel, ELISA results suggested that scFv s25 exhibited a high degree of specificity. This scFv could not be declared monospecific based on tests with the limited bacterial panel; the high specificity indicated that the negative selection protocol was a powerful tool for limiting scFv cross reactivity. Based on these results, particularly the lack of binding interaction observed

with other Gram-positive bacteria such as *B. subtilis*, it was assumed that the epitope recognized by scFv s25 was unique to *B. thuringiensis.*

The initial scFv library was constructed from the spleen of a rabbit immunized with a specific antigen to ensure enrichment of scFvs capable of binding a particular target bacterium. However, the spleen, a primary lymph node, should also contain mature lymphocytes that produced antibodies specific capable of targeting other antigens. Through the random combination of these and other heavy and light chain variable domain genes the library should display a highly diverse population of recombinant antibodies that could potentially bind other antigens. To assess the potential of using the immunized library for selection of scFvs specific for other bacteria, several alternate bacterial targets were used as antigens in biopanning experiments.

Biopanning of the Immunized Library against Non-*Bacillus*Targets Produced scFvs with Affinity for Multiple Bacteria

Because *B. anthracis, B. thuringiensis, and B. cereus* belong to the same family of highly similar bacteria [(174-176)], *B. thuringiensis* is often used as a simulant for the more pathogenic *Bacillus* species. The scFv s25, isolated through biopanning against *B. thuringiensis*, exhibit only minimal interaction with *B. anthracis* when tested via ELISA. It was therefore, assumed that scFv s25 bound an epitope unique to the *B. thuringiensis* species and not other closely related bacilli. Since the immunized library was generated against a highly similar, simulant bacterium; the immunized library was screened for scFvs that bind epitopes conserved among *Bacillus* species.

Five rounds of biopanning were conducted against the *B. anthracis* bacterium from which the recombinant antibodies scFv k4 and scFv k5 were isolated. These two scFvs

bound *B. subtilis* and *B. thuringiensis* with high affinity, but did not demonstrate similar affinity for the antigen used during selection, *B. anthracis* (Figure 30). The low affinity for *B. anthracis* compared to other gram-positive bacteria can be attributed to the high spore content and low vegetative cell content in the ATCC sample, which led to a miscalculation of cell number for biopanning rounds and ELISAs. This observation, however, does not explain the lack of specificity for *B. anthracis*, nor does it explain why scFvs were not isolated against the spore of this bacterium. Although these scFvs lacked the specificity needed for incorporation into the biosensor, their further characterization did yield important information for to future work to improve scFv affinity and bacterial expression.

Interestingly, the absorbance measurements for target and non-target bacteria from specificity ELISAs were proportional for scFv k4 and scFv k5 (Figure 30), with those for scFv k4 consistently three-fold less than those of scFv k5. This similar binding pattern suggests that the two scFvs may target the same surface epitope. The concentration of scFvs in eluted fractions could not be determined and the differences observed in ELISA may be attributed to this. While the concentrations of the partially purified scFvs may have contributed to the ELISA results, the two scFvs also differed significantly in the amino acid sequences of their respective complementarity determining regions (CDRs), which form the antigen-binding domain. Alignment of the amino acid sequences for the entire protein (ClustalW) indicated that the two scFvs were 76.2% similar. As expected, the amino acid compositions of framework regions of both heavy and light chains were very similar. The greatest variation in sequence was observed within the CDR3 loop of the heavy chain gene. The scFv k4 contains two more arginine residues than scFv k5,

which possesses an additional aspartic acid residue not present in scFv k4. The charge differences in this region, since CDR3 is reputed to be most involved in antigen recognition [177], likely contributes to the observed differences in epitope affinity. These observations suggest that the CDR3 domain of the heavy chain could serve as an initial target for mutagenesis to improve scFv affinity. In support, several research groups have targeted this region of the heavy chain to improve the characteristics of scFvs [178, 179].

Both scFvs exhibited improved solubility and expression levels compared to other scFvs isolated from the immunized library (scFv k18, scFv s24, scFv s25). Kipriyanov *et al*. demonstrated that specific amino acid residues could be changed to improve solubility of scFvs and encourage their export into the culture medium [180]. A comparison of their primary structures with those of other scFvs with similar characteristics (export – scFv s25; improved solubility – scFv k18) may be able to identify specific amino acid residues or motifs that are linked to these properties and may represent good targets for substitution. This analysis was not performed at this time, however, since the number of scFvs available for comparison was too low to yield reliable prediction.

In addition to biopanning against *B. anthracis*, the immunized library was screened against the gram-negative bacteria *P. aeruginosa* and *P. putida*, whose outer surface was expected to differ significantly from that of the initial gram-positive immunogen. Biopanning rounds with *P. aeruginosa* and *P. putida* as antigens failed to generate any significant enrichment of scFvs, as evidenced by the phage ELISA results (Figure 31). Further analysis of individual clones from biopanning output plates, however, suggested different reasons for this lack of success.

The inability to select *P. aeruginosa*-specific scFvs by biopanning was probably the result of non-specific interaction between the bacteria and the M13 filamentous phage, which is known for non-specific binding to a number of different substances [(142)]. Non-specific interactions with *P. aeruginosa* samples (attributed to the bacterial capsule) and secondary antibodies (Figure 17) were repeatedly observed in experiments. Previous displacement assays with latex beads showed similar interaction and binding of *P. aeruginosa* to gold surfaces through capsule and formation of a biolayer has been documented in the literature [(181, 182)]. In combination, these binding events would ensure that phages would be recovered from each round of biopanning regardless of the presence or absence of target-specific scFvs. Phage clones that do not express an scFv fusion protein are considerably more abundant than those displaying recombinant scFv protein, since the empty phagemids have a significant growth advantage, as previously discussed. Although the biopanning protocol could have been adapted to include washes of increased stringency that eliminate non-specific interactions, attempts to isolate scFvs specific for this antigen were not continued in favor of trials with antigens that pose fewer potential complications.

Unexpectedly biopanning against the *P. putida* antigen also met with little success. Similar to *P. aeruginosa*, *P. putida* possesses a capsule at the mid-log stage of growth, however it was likely absent from the overnight stationary-phase cultures used for biopanning [(183)]. The phage clones that were recovered following each round of selection were likely not the product of non-specific interactions as was observed for *P. aeruginosa*, since several clones contained an scFv gene insert. Two unique clones, p6 and p12, isolated from the final round of *P. putida* biopanning, produced sufficient

quantities of soluble scFvs for subsequent ELISA analysis. Although these scFvs did bind a number of non-target bacteria of the panel, they exhibited little affinity for *P. putida* itself (Figure 32). In combination with the phage ELISA results (Figure 31), which suggested no significant enrichment of scFvs that recognized the bacterial antigen, the results of the specificity ELISA suggested that the scFvs expressed from clones selected through biopanning exhibited poor antigen affinity. The low affinity scFv-phage from each round of selection would have readily been displaced during the phage and specificity ELISA since theses protocols involve more stringent wash conditions than those employed in typical biopanning.

Although two low-affinity scFvs were isolated from the immunized library, based on affinities demonstrated by other scFvs (scFv 18 – Figure 25, scFv k5 – Figure 30) for *Pseudomonas* species, greater success was expected. The scFv k18 readily bound *P. putida* with only slightly less affinity than that demonstrated for its target antigen of *B. thuringiensis*. Similarly, although the affinity was three-fold less than for target bacteria, both scFv k4 and scFv k5, isolated through *B. anthracis* selection, bound *P. fluorescens* and *P. putida*. Although none of these scFvs bound *Pseudomonas* species with high specificity, they did indicate that scFvs with higher affinities for *Pseudomonas* surface epitopes were present in the initial scFv library. Although repeating the biopanning process against *P. putida* may have yielded scFvs with improved affinities, additional selection against the *P. putida* antigen was not conducted. The *P. putida* bacterium, in contrast to *P aeruginosa*, was not deemed an antigen of sufficient concern to any of the target fields (agricultural, medical, biodefense) to justify additional selection experiments.

Based on the results from biopanning against bacterial targets other than *B. thuringiensis,* it was assumed that the immunized library lacked the clonal diversity necessary to isolate scFvs with high specificities for other bacterial antigens. The project, therefore, shifted to the construction and selection of scFvs from a naïve scFv library. Unlike the immunized library, which was constructed from tissues containing an abundance of matured lymphocytes in response to immunization, a naïve library is expected to contain a gene reservoir of improved diversity. Since there is not an excess of mature immunoglobulin genes that produce antibodies for a specific target, variable domain genes present in low copy in the immunized tissues may be more likely to be included in the scFv repertoire of the naïve library [(57)].

A Naïve Library Was Constructed to Increase scFv Diversity against Bacterial Targets

The naïve library was constructed to determine if scFvs with improved target specificity could be isolated from an antibody reservoir of increased diversity and assembled from genes obtained from lymphocytes not matured toward a specific antigen. Since all of the scFvs isolated from the immunized library exhibited some affinity for *B. thuringiensis* regardless of the antigen used during biopanning, the naïve library was constructed to determine if this was the result of the limited diversity of immunoglobulin genes of the lymphocyte pool obtained following immunizations.

While the successful acquisition of target-specific scFvs from the immunized library relies on a large scFv population with affinity for the target antigen, the naïve library depends on a high clonal diversity ($10^9 – 10^{10}$ clones) and high-throughput selection to isolate scFvs with the desired specificities and affinities against a larger, more diverse

range of antigens [20, 184, 185]. To increase clonal diversity, primers for IgG and IgM constant domains were used during library construction. Antibodies of the IgM isotype are produced during the primary immune response. Generally exhibiting low affinities for the target antigen, the immunoglobulin genes encoding IgM antibodies have undergone little to no gene recombination and are therefore most similar to initial germline genes [35, 36, 186]. Combining variable domain sequences for both IgG (matured) and IgM (naïve) genes would increase the diversity of the scFv library. Finally, since clonal diversity is limited by the efficiency of bacterial transformation, a highly electrocompetent *E. coli* strain was generated. Despite these efforts, the calculated clonal diversity of the naïve library was $8x10^8$. Although an order of magnitude lower than desired, it was considered acceptable for proof-of-concept trials.

Salmonella typhimurium was chosen as the initial bacterial target with which to test the naïve library against gram-negative bacteria. As shown with the *Pseudomonas* species, the immunized library was only minimally successful with these antigens. Additionally, this bacterial target promoted the applicability of the device to prominent agricultural outbreaks that were occurring at that time. The standard biopanning protocol produced several clones that expressed scFvs with varying levels of affinity for target and non-target bacteria (Figure 34). Although a greater affinity for gram-negative bacteria was shown by scFv s26 and scFv s27, substantial binding of the gram-positive bacterium *L. monocytogenes* also occurred. Given the successes of the previous negative selection biopanning experiment, in which highly specific scFv s24 and scFv s25 were selected (Figure 26), *L. monocytogenes* was employed as the non-target antigen to remove scFvs that may have been targeting epitopes conserved among enteric bacteria. However, rather

than improving specificity for *S. typhimurium*, all scFvs from the final round of selection had no affinity for the original antigen (Figure 39a). These experiments, while unsuccessful in isolating a suitable scFv, led to several insights about the recombinant antibody system.

The scFvs isolated from the last round of negative selection were comprised of only the heavy chain variable domain, as evidenced by DNA sequence analysis (Figure 37). In contrast to full-length scFvs, these truncated molecules would rely on the three CDRs of the heavy chain to form an antigen-binding domain. It is well documented that the heavy chain variable domain can, by itself, bind target antigens independent of contributions from light chain CDRs [(187-189)]. Although not seen with the clones isolated in this study, single domain antibodies can show significant specificity for their target antigens [(190, 191)]. The absence of the light chain CDRs in the antigen-binding domain was therefore most likely not the primary contributor to the observed lack of scFv specificity. As with non-specific interactions observed with other scFvs, this was liley a consequence of whole bacteria as the antigen and the presence of numerous conserved surface epitopes that must exist between bacterial species. Although an scFv specific for *S. typhimurium* was not isolated, these experiments did show that clones with truncated scFv genes, which were not characterized in previous biopanning rounds, could prove suitable for bacterial detection in later studies.

The naïve library was generated to improve the potential acquisition of scFvs with high target specificity. However, clones isolated from the naïve library continued to exhibit specificity for multiple bacterial targets similar to those scFvs obtained from the immunized library. This observation suggested that the inability to isolate scFvs for non-

Bacillus bacteria from the immunized library was probably not attributable to the low library diversity but likely the result of either biopanning conditions or the complexity of the bacterial surface as a selection antigen. The bacterial surface, particularly that of gram-positive bacteria, contains countless membrane proteins and structural features that could potentially serve as epitopes to which scFvs can bind [(192)]. Invariably, some epitopes are conserved or very similar among bacteria and contribute to the potential for cross-reactivity of scFvs with non-target bacteria. Epitopes unique to a particular bacterium may not be abundant on the bacterial surface and therefore scFvs specific for these structures may easily be lost through iterative rounds of biopanning.

The vast majority of biopanning experiments reported in the literature are directed against specific, purified antigens (proteins, carbohydrates, or small molecules). Utilizing a defined epitope allows for enrichment of a limited pool of scFvs during biopanning. In contrast, biopanning against the complex bacterial surface leads to an enrichment of scFvs that may bind any one of countless epitopes that may or may not be unique to the particular bacterium. To improve the probability of isolating *Salmonella*-specific scFvs, the naïve library was subjected to five rounds of selection against a purified oligosaccharide, the O-antigen, of *S. typhimurium*. Restriction analysis of phagemid DNA from individual clones again showed a high frequency of vectors that lacked the scFv gene, further validating the explanation offered by Krebber that clones containing an empty phagemid vector have a significant growth advantage over those that harbor the scFv gene [(18)]. A single clone, k2, was obtained from the last round of biopanning. The scFv expressed by this clone bound the O-antigen, whole *S. typhimurium cells*, and all other bacteria of the test panel with varying degrees of affinity (Figure 42). Cross-

reactivity with other gram-negative bacteria was anticipated, since these have similar carbohydrate structures on the outer lipopolysaccharide (LPS) layer. The scFv k2, however, also readily bound gram-positive *L. monocytogenes* and *Bacillus* species with very high affinity. Although scFv k2 was selected with a carbohydrate antigen isolated from a specific bacterium, this scFv continued to display affinity for all bacteria of the panel. The LPS does contain unique epitopes since antibodies against bacteria-specific LPS have been previously isolated [150, 151, 193]. The lack of specificity of scFv k2 and the absence of other clones available for characterization suggest a complication with current biopanning protocols, perhaps with the stringency of washes or the inability to limit amplification of the clones containing the empty phagemid vectors.

The poor specificity exhibited by scFv k2, and many of the other clones isolated in previous trials, illustrated a difficulty that was encountered throughout the various selection experiments. The bacterial surface contains countless potential epitopes formed from both unique and conserved bacterial structures. Epitopes that are highly abundant on the bacterial surface will likely be the target of scFvs that are enriched through iterative rounds of selection. Those scFvs that target low-copy epitopes will be less abundant during phage amplification and, since clones expressing full-length scFv already have a growth disadvantage, may gradually be lost through iterative rounds of selection. Therefore, using the current protocols, it would be nearly impossible to isolate scFvs with affinity for low-copy unique bacterial epitopes. In contrast, unique bacterial epitopes that are abundant on the bacterial surface would allow for the selection of scFvs from the library. Although other researchers have been able to isolate scFvs that recognize their bacterial targets, in these studies only limited screening was performed to assess

specificity for non-target bacteria and significant cross-reactivity was observed between closely related species [88, 194]. The high target specificity of scFv s25 may be the exception rather than the norm when panning against whole bacteria.

Although only limited success was achieved in isolating scFvs with high target specificity from the rabbit scFv libraries constructed in this project (scFv s24 and scFv s25), the successful selection of scFvs from a library with desired binding characteristics for a specific antigen is well documented in literature [60, 92, 195, 196]. What is evident in both the literature and these experiments is that individual antigens will likely require unique selection strategies to improve specificity. This includes not only the stringency of washes but also the platforms used for antigen immobilization [58, 69, 89, 197, 198].

The biopanning protocol could almost certainly be manipulated by increasing the stringency of washes beyond that of the standard protocol. In combination with phage ELISA assays, changes in selection conditions could be assessed to determine if this leads to an enrichment or loss of scFv with affinity for the target antigen. Similarly, the negative selection protocol, which was successful in initial trials, may also prove suitable for later selection rounds. However, rather than using a single bacterium as the negative agent, selection against several non-target bacteria could be conducted to assess which is most beneficial for the elimination of non-specific scFvs. Finally, the greater the diversity of the scFv library, the more likely an scFv with particular characteristics can be recovered. The naïve and immunized library were of sufficient calculated clonal diversity compared to libraries used by other researchers, however, improving diversity should lead to greater success in isolating a target-specific scFv. Although these methods were

not explored, there are many methods of improving clonal diversity during library construction.

The naïve library was constructed from cDNA synthesized from the mRNA of spleen cells using protocols described in the literature [18, 56, 57]. In rabbits, however, the appendix is also a location of B cell maturation and may serve as secondary source of lymphocytes that can be used for library construction [171, 199, 200]. The appendix, or gut-associated lymphoid tissue (GALT), develops lymphoid follicles shortly after birth and maintains a high population of mature B cells. Knight suggests that greater than 50% of the B-cells within the GALT are actively dividing at the early stages of development [171]. Given the age of the donor rabbit (4-6 weeks), the GALT would have also been an excellent source of diverse variable domain genes. Including the GALT tissues as a source of RNA during library construction would have improved library diversity.

Naïve libraries assembled from the immunoglobulin genes of multiple donors are frequently reported in the literature [21, 62, 78]. Although such libraries are usually generated from human peripheral blood lymphocytes, the concept of utilizing several donors could be explored using the protocols already developed for the construction of the rabbit scFv library. Particularly with older rabbits, matured heavy and light chain genes would be present in varying ratios in the spleen tissue depending on antigen exposure of the animals. Within the spleen of a single rabbit, certain variable domain sequences may exist in such low abundance that they could potentially be lost during library construction, particularly when the library is of limited complexity. Utilizing the spleens of several rabbits would increase the miscellany of variable domain sequences available for library assembly.

As an alternative to the aforementioned methods, the CDRs of the heavy and light chains can be randomized directly utilizing a variety of PCR techniques during library construction. Methods such as CDR shuffling [(60, 201)] and direct randomization of CDRs [(69)] have been used as effective methods of substantially improving the diversity of the scFv library. With CDR shuffling, forward and reverse primers are designed for conserved nucleotide sequences of framework region 2 and framework region 3. Following the initial PCR to isolate the heavy and light chain genes, a second PCR amplification using the original primers and the primers of the framework regions segments the variable domain genes. These segments are then recombined randomly through regions of complementarity in a third oePCR reaction. Goldman *et al.* utilized CDR shuffling to increase the diversity of a llama single-domain antibody library from 10^6 to 10^9 [(60)]. Liu *et al* randomized the nucleotide sequence and length of CDR3 in an scFv library derived from spiny dogfish sharks [(69)]. The randomized CDR3 genes were then cloned back into the library, thereby substantially improving the diversity of the existing library. Application of these methods to the naïve and immunized rabbit libraries constructed in this study may increase the scFv diversity and eliminate the need to generate additional libraries from other rabbits.

A final alternative that deviates from the strategy of using a naïve library to isolate target-specific scFvs is based on the work of Li *et al.* who immunized rabbits with multiple haptens [(73)]. It should be possible to use several bacteria as immunogens to simultaneously enrich for lymphocytes producing multiple target-specific antibodies. The spleen could then be extracted for construction of a library that is already enriched in matured heavy and light chain genes that are specific for all of the immunogens. Given

the success with the initial *B. thuringiensis* immunized library, this method should improve the potential for isolating target-specific scFvs against bacterial antigens. Additionally, since numerous immunogens can be used, a single library could potentially yield several scFvs that are specific for different targets. Selection of the bacterial immunogens would be critical to the success of this method, and it would need to be determined experimentally whether rabbits should be immunized with bacteria that are similar or diverse in nature with respect to habitat, Gram classification, possession of flagella, etc..

Only Limited Quantities of scFvs are Obtained from Small-Scale Bacterial Expression Cultures

Poor solubility is an inherent complication associated with bacterial expression of scFvs [(195, 202)]. In addition, poor expression levels, with yields ranging from 0.05 – 1 mg scFv / L, are also not uncommon [(203-205)]. Expression trials of scFvs were conducted in a number of bacterial strains, and with different expression vectors. However, only limited improvements in the solubility or expression levels were achieved. Cytoplasmic expression conducted in the pProEx and pET21 vectors did not yield increased amounts of recombinant protein. In contrast, cytoplasmic expression using the pET41c vector, which generates an N-terminal GST fusion, did improve expression but not solubility. The GST-scFv fusions, however, no longer exhibited bacterial specificity in ELISAs (Figure 45). This lack of specificity may have been the result of the GST portion of the molecule interacting with the bacterial surface and would explain why GST-scFvs s25 exhibited affinities that were similar to GST-scFv k18. The pET41 vector encodes a cleavage site downstream from the GST position of the recombinant protein that can be

used to remove the non-scFv portion of the fusion. Removal of GST was not explored in this study because the necessary protease can only be obtained commercially and at significant expense. Should this additional step be required for the purification of scFvs, the cost of biosensor production would rise considerably.

Poor solubility of scFvs was an inherent condition of all clones during expression trials (Figures 23, 28, and 49). Although solubility of all scFvs could be improved slightly by lowering the induction temperature during expression and decreasing the IPTG concentration, scFv yields from the soluble material were low (45 – 60 μg / L). To aid in the recovery of scFvs, denaturing chromatography and renaturation through step-wise removal of the denaturant by dialysis were used for the purification of scFv k18 and scFv s25 from the insoluble pellet. This method of purification was marginally successful for scFv k18 but completely ineffective for scFv s25. The renatured scFv k18 did maintain some bacterial affinity, however, when tested via ELISA, eluates obtained from standard and denaturing chromatography (Figure 48a) showed significant differences in affinity for bacterial targets.

Eluates from both purification methods were resolved using SDS-PAGE. Densitometric analysis of immunoblots indicated only 25% less recombinant antibody in the eluates of the denaturing column, an insufficient difference to account for the three-fold lower affinity observed in ELISA. These observations suggested that some portion of the scFvs recovered from the pellet fraction did not refold into the native, functional conformation. The renatured scFv s25 eluates exhibited only minimal activity that was eight-fold lower than that of scFv s25 eluates recovered with the standard purification (Figure 48b). Success in obtaining a functional molecule following denaturating affinity purification

appear to vary with individual scFvs and must be determined experimentally for each clone since other research groups have been able to successfully isolate scFvs using the denaturation/renaturation purification method [206, 207].

High-level accumulation of recombinant proteins within in the periplasm can often lead to secretion of these proteins into the culture medium when expression is conducted at temperatures between 25°C and 37°C [128, 140]. This phenomenon was observed for several of the scFvs isolated in these studies (scFv s25, scFv k4, and scFv k5). Since scFvs exported to the culture medium were soluble, a standard affinity purification protocol could be utilized. The scFv s25 was purified from the culture medium and tested for target specificity via ELISA. Unfortunately, the extracellular scFvs did not exhibit the high specificity for *B. thuringiensis* characteristic of scFv s25 purified from a soluble cellular protein extract (Figure 50). The *ompA* leader sequence of the pComb3 XSS vector targets scFvs to the periplasmic space, which provides an oxidizing environment to facilitate disulfide bond formation during expression. Georgiou *et al.* suggested that high level expression of recombinant proteins can lead to an inhibition of the synthesis of native outer membrane proteins, which compromises the integrity of the outer membrane leading to semi-specific secretion of periplasmic recombinant proteins to the culture medium [140]. In conjunction with early studies by Bowden *et al.* which showed that high-level expression and targeting of recombinant proteins to the periplasm often overwhelms the folding machinery [208], it is possible that scFvs within the culture medium are not folded into a native conformation, perhaps lacking the disulfide bonds formed within the periplasmic space. Reduced stability of the antigen-binding domain would explain why scFv s25 was still capable of binding bacterial targets, but no longer demonstrated the

high specificity characteristic of a well-defined antigen-binding domain. While scFv s25 purified from the culture medium did not exhibit typical target affinity, the method per se may hold potential for the purification of other scFvs since the success with this method has been documented in the literature [209, 210].

The expression and purification of scFvs using bacterial shake flasks (50 ml – 2 L volumes) was a limiting parameter in scaling up the recombinant antibody system, because of the low yields of recombinant antibodies obtained in these studies (45 – 60 μg scFv/L of culture) and documented in the literature [52, 195, 211]. Recovery of scFvs from expression cultures would need to achieve higher yields equal to those obtained with hybridoma bioreactors to be a successful substitute. Alternatives, although not explored, are available for the large-scale production of recombinant proteins and have been demonstrated an as effective method of mass-producing functional scFvs.

The research groups of Feldhaus *et al.* and Liu *et al.* have demonstrated that expression in *Saccharomyces cerevisiae* and *Pichia pastoris* yeast cultures significantly increases that quantity of scFv that can be recovered, with yields ranging from 0.4 -15 mg/L [54, 212, 213]. Yeast expression cultures are, however, not without their own problems. Liu *et al.* showed that further genetic manipulation of the scFv gene was necessary to correct for codons bias of *P. pastoris* [212], and Feldhaus *et al.* demonstrated that expression in *S. cerevisiae* often leads to hyperglycosylation of the recombinant protein, which was shown to interfere with binding affinity [54].

Expression of scFvs in *E. coli* quiescent cells maintained in bacterial fermentors has been shown to produce significant levels of recombinant protein [141, 159, 214]. Quiescent *E. coli* cells are non-growing, yet metabolically active cells generated through the over-

expression of the *Rcd* transcript [215]. The *Rcd* protein represses chromosomal genes through nucleoid condensation [141]. Because quiescent cells are no longer dividing, cell resources are focused on the production of plasmid-encoded foreign gene products [216]. Summers *et al.* utilized quiescent cells to express scFvs with yields of 37 mg/L in traditional baffled flasks, a nearly three orders of magnitude higher yield than that obtained in this study for scFv k18 expression cultures. The group typically obtained 150 mg/L of recombinant scFv from fermentors [141]. Yields from fermentors vary between individual clones, however, recoveries greater than 3 g of scFv/L culture are routinely demonstrated in the literature [159, 203]. Utilization of either of these methods for scFv manufacture would ensure low-cost production of sufficient quantities of target-specific scFvs for integration into a commercial biosensor.

Monoclonal and Recombinant Antibodies Suitable for Biosensor Applications

Full-length mAb and recombinant scFvs were tested with the QCM platform to ensure that either type of antibody could be used in the biosensor. Full-length immunoglobulin molecules are routinely employed in QCM sensors for the detection of bacterial targets [26, 28, 30, 109, 112]. The Ma1-6 mAb was tested to examine the efficiency of attachment to the mixed acid self-assembled monolayer and to determine the limit of detection. Consistent measurements sufficient to generate a positive signal were obtained for concentrations of 10^3 cfu/ml, consistent with LOD values reported by other research groups [30, 109, 110]. Although lower LOD values have been reported for QCM detectors that utilize mAbs, significant differences in variables such as the crystal oscillation frequency and antibody immobilization strategy in these systems prevented a meaningful comparison of the performance to the prototype biosensor [31, 32]. To lower the overall cost of biosensor

production, the recombinant antibodies isolated from the scFv libraries were examined to determine if they would be sufficient substitutes for the mAb, capable of LODs equal to that of Ma1-6.

Two different methods of scFv immobilization were employed to activate the QCM crystal for bacterial detection. Initial experiments were conducted using a strategy identical to the one employed for immobilization of monoclonal antibodies. While successful for the Ma1-6 antibody, the direct method of immobilization was unsuccessful for scFvs. In trials with immobilized scFv k18, QCM analysis of bacterial samples produced highly variable frequency measurements for each of the concentrations examined (Table 3). This was observed regardless of the molar concentration of scFvs immobilized to the surface and can most likely be attributed to the structure of the scFv protein, which is primarily comprised of amino acids that are either part of the antigen-binding (CDRs) or structural stability (framework). The scFv k18 contains seven lysine and eight arginine residues, the primary targets of the carbodiimide reaction. Three of the lysines and three of the arginines are located within the CDRs themselves, which form loop structures that project away from the ß-sheets formed by the framework regions. The lysines and arginines of the CDRs are therefore excellent targets for the formation of amide bonds with the SAM of the crystal. In the event that attachment to the surface occurred through one of these residues, the antigen binding capabilities of the scFv could be lost due to steric hindrance as the binding domain would be oriented toward the crystal surface. Similarly, a reaction that targets amino acids of the framework regions may also lead to loss of scFv activity by disrupting the tertiary structure of the molecule. Although amino acids of the framework regions and CDRs can be identified using the variable

domain numbering system established by Kabat [123], which amino acid are utilized for attachment to the surface cannot be determined *a priori*. Although some success in generating a functional crystal surface has been reported when free cysteine and histidine and residues were used for surface immobilization of the scFv [217-219], these methods were not attempted in this study.

The indirect method of scFv immobilization to the QCM slide, in which the C-terminal HA eptiope tag of the scFv binds to a monoclonal anti-HA antibody that is immobilized on the surface, proved significantly more successful in demonstrating a linear relationship between bacterial concentration and frequency change. This method of coupling ensured orientation of the scFv antigen-binding domain and therefore the capability to bind target bacteria. Purified monoclonal anti-HA IgG antibody is commercially available and therefore could be immobilized in well-defined quantities. Utilizing an excess of the scFv containing fractions obtained from Ni^{2+}-NTA chromatography ensured saturation of the available HA-binding sites of the IgG antibodies. As shown in the standard curve (Figure 52), scFvs immobilized using this method were repeatedly able to detect 10^2 cfu/ml, a improvement in sensitivity by an order of magnitude.

The scFv s25 was first tested with the Stanford Research System QCM apparatus to ensure that the target specificity observed with ELISA would be maintained following immobilization to the gold electrode. These initial trials allowed for the acquisition of real-time frequency measurements that visualized target and non-target bacterial interactions with the crystal surface (Figure 53). Once the specificity of scFv s25 was confirmed, the prototype biosensor was tested with bacterial samples of *B. thuringiensis,*

B. subtilis, and *S. typhimurium* to establish the LOD for the detection of target bacteria and to assess the accuracy of identifying target bacteria compared to non-target bacteria (false positive / false negative). The LOD measured with the biosensor correlated with the results obtained with the SRS system and scFv k18 exhibiting a LOD of 10^2 cfu/ml of *B. thuringiensis* in a PBS solution. Samples containing non-target bacteria did not generate a frequency change sufficient to produce a positive result.

This project concluded that recombinant antibodies could be isolated from an scFv library using high-throughput techniques. The scFvs selected in that manner could readily be incorporated into a QCM-based biosensor for the detection of target bacteria. Although additional studies are required to optimize scFv production, bacterial expression of scFvs offers a low cost alternative to hybridoma-derived mAbs. While several complications with the phage display system were encountered, future endeavors with more diverse libraries may overcome these difficulties. The scFvs isolated through biopanning were expressed in bacterial hosts and utilized for detection using both a commercially available QCM apparatus and the prototype biosensor constructed by collaborators. A method of immobilizing scFv to the gold electrode was developed that ensured both directionality of the antigen-binding domain and activity of the scFv. The prototype biosensor was successfully employed in the detection of target bacteria.

CHAPTER VI

CONCLUSIONS AND FUTURE WORK

Methods of producing bacteria-specific IgG antibodies and recombinant scFvs were explored for the development of a QCM-based biosensor. Both types of antibodies were amenable to integration into the QCM-platform and demonstrated reproducible detection of target bacteria to concentrations of 10^3 and 10^2 CFU/ml, respectively. The recombinant antibody system, however, offered several advantages over monoclonal antibody production and suggests that scFv libraries are a superior system for application into an adaptable biosensor.

Selection of unique clones, whether from hybridomas or scFv-expressing *E. coli* cultures, requires a considerable initial investment of time as immunizations of a host organism with the target antigen are conducted. The immunization process, excluding complications such as antigen purification, requires several months before necessary materials can be obtained for both hybridoma (lymphocytes) and library (spleen or similar material) generation. While both full-length and recombinant antibody production both require these initial immunizations, a single scFv library could yield recombinant antibodies for several target antigens. In contrast, development of a hybridoma cell line would require immunizations for each target antigen. Although highly specific scFvs were not isolated from the naïve library, such a source of recombinant antibodies has been utilized by other research groups with great success [63, 72, 184]. Through a combination of DNA techniques (mutagensis, CDR shuffling, *etc*) to improve diversity and adjustments to the biopanning protocols, the scFv libraries generated in this study could be further utilized to screen for scFvs with the desired binding characteristics.

The diversity of the scFv library and power of phage selection provide a high-throughput method of isolating scFv with desired target-specificity and stability. Future endeavors with the phage display system would need to employ selection strategies that ensure scFv binding specificity and affinity under harsher conditions than utilized in the preliminary trials of this study. Since the biosensor will likely be used to analyze complex aqueous samples or unknown solids, inclusion of detergents in the sample buffers will likely be required to limit non-specific interactions. The biopanning process is ideally suited for the manipulation of binding conditions with respect to temperature or chemical composition to isolate scFvs that are tailored to particular applications [(60, 88)].

In addition to the benefits of high-throughput selection, the recombinant antibody system allows the antigen-binding domain to be reshaped through direct manipulation of the heavy and light chain genes themselves. It has been extensively demonstrated that scFv affinity for a specific target can be improved through an affinity maturation process [(50, 220-222)]. The scFv gene can be subjected to mutagenesis, followed by high-throughput screening of the biopanning process to identify clones with improved affinity. A suitable protocol for assessing affinity of the new scFvs would need to be developed based on surface plasmon resonance or flow cytometry.

Of primary concern in the development of any commercial product is the cost of production. Production and purification of monoclonal antibodies are considerably expensive, a single Fibercell bioreactor cartridge maintaining the Ma1-6 cell line produced an average of 3 – 4 g of antibody per month at a cost of approximately $200 in media and reagents. Although several of these cartridges could be maintained concurrently, the expense of fetal bovine serum and DMEM media made antibody

production prohibitively expensive. In contrast, scFvs could be produced in high yields in bacterial fermentors. Medium for *E. coli* growth can be prepared at a fraction of the expense for hybridoma maintenance and reported yields for scFvs produced in fermentors are comparable to those for hybridoma-derived monoclonal antibodies. Large-scale production of scFvs in quiescent cells of fermentors should be explored to ensure that commercial production of the biosensor is feasible.

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