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# An Analytical Survey of a Biotinylated Bacteriophage System for Quantifying Transduction Events in Natural Ecosystems

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# An Analytical Survey of a Biotinylated Bacteriophage T4 System for Quantifying Transduction Events in Natural Ecosystems

A Thesis presented for the

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## Abstract

Bacteriophage transduction events play a contributive role in bacterial gene diffusion in ecosystems comprised of natural microbial populations. The rate at which these phage-mediated genetic transfers occur in wastewater ecosystems is not well understood. This work describes the effectiveness of a genetically engineered biotinylated bacteriophage T4 system as a tool for examining transduction event rates and compositions in environments that pose increased risk of antibiotic resistance proliferation.

Antibiotic use has steadily increased over the past century, giving rise to antibiotic resistance in microorganisms. Agricultural, medical, and industrial use of antibiotics produces waste teeming with residual antibiotic compounds that are collected to form novel microbial ecosystems. These wastewater microbial communities include, among others, bacteria and their viral parasites, bacteriophage. Bacteriophage can transfer genetic material between bacterial hosts through a phenomenon known as transduction, thus creating circumstances that can contribute to the spread of antibiotic resistance genes. Wastewater ecosystems that contain vestigial antibiotic compounds may produce selective breeding grounds for the development of antibiotic resistant microorganisms. This study aimed to elucidate the role of bacteriophage in the spread of antibiotic resistant genes under these environmental conditions using a novel biotin-based tagging and recovery system. This system analyzed the unbiased tagging of bacteriophage particles, infection of host bacteria, and the recovery of biotinylated phage progeny as means to investigate phage-mediated transduction rates.

A recombinant bacteriophage T4 with biotin carboxylase carrier protein (BCCP) fused to the small outer capsid (SOC) acted as the tagged bacteriophage in this work. Numerous techniques for phage propagation and purification were optimized for this application, indicating important information about the feasibility of using this engineered bacteriophage in a natural ecosystem. The genetic instability and susceptibility to selective pressures of the biotinylated bacteriophage, even under optimally controlled conditions, resulted in the deactivation of the phage's biotinylation capabilities. The results of this examination indicate the impracticality of using this genetic-based biotinylation approach as a method for measuring transduction events rates in natural ecosystems.

# Table of Contents

**List of Tables**

**List of Figures**

# Introduction

The most abundant biological entity on earth is the bacteriophage, with global numbers estimated to be in excess of $10^{31}$ (Furhman, 1999). The highly efficient adaptive gene transfer mechanisms employed by bacteriophage exude tremendous influence on microbial ecosystems. Bacterial diversity and population dynamics are linked to bacteriophage effects on a genetic level. Phage mediated gene transfer, or transduction events, in marine microbial systems have been shown to occur at a rate of $2 \times 10^{16}$ events per second (Canchaya, 2003) and among a wide range of bacterial species in freshwater ecosystems (Kenzaka, 2010). Transduction events have been studied in soil (Zeph, 1988), freshwater (Morrison, 1978; Saye, 1987), and marine environments(Jiang, 1998), but the mixture of medical, agricultural, and industrial waste in wastewater treatment facilities is of particular concern with regard to human health. Environments consisting of wastewater and activated sludge contain vast stores of bacteriophage diversity (Ewert, 1980; Hantula, 1991; Muniesa, 1998). Bacteriophage populations actively replicate in these wastewater environments (Khan, 2002) and have been shown to infect hosts across strains, species, and genera (Beumer, 2005; Khan, 2002). Although it has been shown that bacteriophage play a role in the spread of genes that are potentially harmful to humans (Muniesa, 1998; Muniesa, 2004), accurate transduction rates have yet to be determined outside optimized bench scale experiments (Withey, 2005).

The relatively recent increase in phage-based biotechnological applications include bacteriophage therapy for antibiotic-resistant microbial pathogens (Payne, 2000;

Bull, 2002; Matsuzaki, 2005), bacteriophage regulation of wastewater bulking and foaming (Thomas, 2002; Withey, 2005), phage-based biosensors for food and water quality control (Petty, 2007), and phage applications for controlling bacterial contaminants (Clark, 2006). With the expansion of bacteriophage use, environmental discharges are bound to increase. Therefore, identifying and characterizing bacteriophage transduction events in wastewater ecosystems plays a critical role in understanding risks associated with the phage-mediated spread of detrimental human health-associated genes in the environment.

The overall goal of this research is to expand upon established knowledge pertaining to the characteristics, rates, and methods of genetic exchange among genetically engineered microorganisms. A specific objective of this work is to analyze and confirm that a genetically engineered bacteriophage T4 was labeled with a biotin-binding gene, biotin carboxylase carrier protein (BCCP), allowing for isolation of the tagged phage post-infection and subsequent analysis of the accumulated genomic permutations. The aforementioned labeling technique allows for an unbiased approach to identification and isolation of intended phage progeny after a natural infection process followed by bacterial host lysis in a natural ecosystem. This approach technically addresses the proposed hypothesis that genetically engineered bacteriophage can serve as unbiased, indicative vectors of transduction events in an ecosystem. The other expansive hypothesis that is addressed by this work is that bacteriophage have a greater propensity for broad host ranges than is customarily thought, thereby making their role in cross-species dissemination of genetic elements more consequential. This

work is an analysis of experimental designs utilizing a biotinylated bacteriophage T4 as an indicator of host range limitations, a gene transfer agent, and the field application of a genetically modified organism in a wastewater environment.

## Background

The established description of the lytic bacteriophage T4 infection cycle includes the phage attachment to specific receptor sites on the host cell's surface. Numerous molecules can act as phage receptor sites and proteins that are exploited by phage generally serve important roles for the effective functioning of the bacterial cell. After phage adsorption to the host cell, the phage injects its DNA through the bacterial cell wall. This process requires energy and initiates conformational changes in the phage structure. These structural changes result in the contraction of the phage tail sheath, thereby forcing the hollow tail into the host cell. Small fibers located on the end of T4 phage tails serve as anchors for the baseplate attachment to the cell surface receptors. As the phage capsid is drawn to the cell surface, the baseplate transforms from a hexagonal shape into a star shape, tail diameter increases as it is shortened, and an internal pin-like apparatus contacts the outer membrane of the host cell. It is during this time that the viral genetic material is injected into the host cell through the tail. This injection process is powered by a proton motor force, compliments of the bacterial cell. After the phage DNA is injected into the host cell, early viral genes responsible for phage genome replication and modification of host cell machinery for maximum phage reproduction are expressed. Bacteriophage T4 synthesizes concatemers, or long continuous chains of genomic DNA, which are later enzymatically cut to produce

discrete phage genomes. Later phage genes that are expressed produce protein subunits for capsid construction. Progeny phage capsids are packed with phage genomes and enzymes are produced to lyse the host cell. These lysis-inducing enzymes include holin, which disrupts the cell's cytoplasmic membrane, and lysozyme, which degrades peptidoglycan in the cell wall. This disruption of the cell wall and membrane causes the host cell to burst, allowing for the release of the progeny bacteriophage (Abedon, 2005).

Transduction events contributing to lateral gene transfer in microbial environments were considered negligible until the ubiquity of viruses in nature was better understood. Bacteriophage mediated transduction is now thought to play a significant role in microbial evolution (Winebauer, 2004). During the infection cycle, the packaging of foreign DNA into replicating phage capsids follows two trends: specialized and generalized. During specialized transduction, distinct portions of the host genome are incorporated into the phage capsid, thereby becoming part of the progeny phage genome. By contrast, generalized transduction consists of the replicating phage taking up any DNA fragments residing in the microenvironment of a host bacterial cell. A bacteriophage that has the ability to undergo generalized transduction can incorporate a host genome in its entirety, limited only by the holding capacity of its capsid. This can occur in theory, however, it is more likely that small, random fragments will be mixed with the replicating phage genome due to the phage capsid holding capacity typically being several orders of magnitude smaller than the host genome. Once these abundant segments of DNA are packaged into the capsids of phage progeny, they can be

delivered to other bacterial hosts, allowing for this foreign DNA to be incorporated into the bacterial host genome. DNA fragments that are taken up by transducing bacteriophage can include exotoxin genes (Casas, 2006), genetic mobile elements (Breitbart, 2003), and antibiotic resistance genes (Muniesa, 2004). Bacterial genome analyses have shown that up to 20% of bacterial genomes are made up of these phage related genetic elements (Casjens, 2003). Specifically, it has been found that 15% of the *E. coli* genome consists of genes that have been acquired through horizontal gene transfer (Lawrence, 1998).

The constant interactions between bacteria and phage produces vast opportunity for repeated transfer of genetic material not only between hosts, but between phage populations. A conceptual paradigm lies in the T-even bacteriophage with regard to horizontal gene transfer mechanisms. These phage have been shown to share a core genome that constitutes approximately 12-15% of their coding potential and some of the genes that make up this genome have spread to the genomes of a diversity of bacterial genera (Petrov, 2010). Several distinct transduction-related processes that are utilized by T-even bacteriophage include evidence for highly variable DNA modification systems that can produce homologous recombination events with co-infecting phage within the host (Mosig, 2006). The genome of bacteriophage T4 shows evidence of a particularly active enzyme system for homologous recombination that has evolved to mediate non-homologous exchanges between slightly similar or disparate genetic sequences in all DNA-based biological systems (Petrov, 2010). These exchanges can occur as genetic replacements resulting from traditional reciprocal exchanges with foreign genetic

entities, or as genetic additions produced from the initiation of DNA replication through the invasion of intracellular phage DNA pools by free 3' ends of foreign DNA (Mosig, 2006). Intron and intronless homing endonucleases are coded and deployed by T-even bacteriophage, both of which utilize homologous recombination between phages co-infecting a bacterial host to transfer of genetic information from the endonuclease-encoding genome to a endonuclease-free recipient (Edgell, 2010; Belfort, 2009; Belle, 2004; Liu, 2003). These homing endonucleases can contribute to lateral gene transfer by mobilizing introns, flanking DNA sequences, and distant genes or gene clusters in phage. Additionally, the head size plasticity of phage T4 plays a critical role in the ability to take up extra, foreign DNA during infection (Mosig, 2006). The T4 DNA packaging system can encase approximately 170 kb of foreign DNA into a capsid with no difference in packaging efficiency between mature phage DNA and extraneous genetic material (Rao, 2008). All of the aforementioned bacteriophage life cycle characteristics suggest that an exuberant level of genetic exchange has occurred in the past and continues to transpire not only among phage populations, but between the bacterial hosts that drive viral propagation.

Previous evaluations of gene transfer risks associated with bacteriophage have produced the scientific zeitgeist that acknowledged transduction as a mediator of genetic transfer, but narrow host ranges of phage generally limited the phenomenon to a small fraction of bacterial hosts with which the phage can infect and exchange genes. Evolutionary theory, and ever increasing experimental evidence, suggests that phage are not restricted to a single host species and many phage display broad host ranges.

A sewage phage, SN-T, has been shown to infect six genera of bacteria (Jensen, 1998). Phage isolates from activated sludge showed that 15 of 17 had broad host ranges and 12 could infect across bacterial genera (Thomas, 2002). Another study showed that phage derived from activated sludge could infect both Gram positive and Gram negative bacterial species (Khan, 2002). Broad host range bacteriophage were thought to be misrepresented by enrichment and isolation practices that selected for narrow host range phage. These biased, conventional techniques consist of plating phage lysate on varying lab grown bacterial hosts and noting plaque formation, thereby indicating a limited representation of phage host ranges. This approach grossly underestimates the number of bacterial hosts that the phage may interact with in an uncontrolled environment, giving a very restricted representation of phage host ranges. The technical limitations of phage host range analyses are compounded by phages' abilities to modify their ability to recognize and attach to potential host cells in a rigorously dynamic bacterial environment (Wilkinson, 2001). A fundamental application of evolutionary theory provides logic to the concept of higher frequency broad host ranges in bacteriophage: species in a diverse microbial community occur at dynamically low abundance in relation to environmental challenges, thereby making it selectively advantageous for phage plasticity to allow for infection and propagation in a myriad of genetically divergent hosts. This scenario would increase the prospect of phage mediated gene transfer due to a wide range of hosts with which to interact.

An increased interest in bacteriophage exploitation for biotechnological and economic purposes has been steadily increasing, thereby intensifying the potential for

gene transfer in the environment (Petty, 2006). The increase in antibiotic resistant bacterial strains has produced various bacteriophage applications in hopes of alleviating the problem. Bacteriophage applications to hospital surfaces and objects has been shown to decrease the transmission of *E. coli* infections in children and adults (Drozdova, 1998), burn wounds have been treated with bacteriophage cocktails to prevent *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections (Merabishvili, 2009), and a whole host of other medical-based applications have been described with increasing occurrence (Marks, 2000; Chanishvilli, 2001; Skurnik, 2006). Veterinarian uses of bacteriophage include spraying aqueous bacteriophage solution on litter in calf pens to prevent *E. coli* infections in calves (Smith, 1987), sewage-derived phage cocktails applied to pig skin reduced *Salmonella Typhimurium* infections (Hooton, 2011), *in vivo* treatment of chickens to prevent *Salmonella* (Dahiya, 2006) and *Clostridium perfringens* (Toro, 2005) colonization, *Staphylococcus aureus* mastitis in lactating dairy cattle (Gill, 2006), and control of *Aeromonas salmonicida* infections in brook trout (Imbeault, 2006). As anti-infective agents, phage have been approved by the FDA for use as a spray to control *Listeria monocytogenes* on consumer deli meat products as well as various methods to control pathogens on fruits, vegetables, and dairy products (Hudson, 2005). In addition to the bio-control uses for phage, phage-based biosensors have been developed to detect food borne pathogens (Petty, 2006) and phage-based techniques for treating wastewater bulking and foaming have been deployed (Withey, 2005). This widespread use and exploration of phage utility is resulting in an increased

release of phage into the environment, particularly in wastewater ecosystems, where a better understanding of phage-mediated gene transfer must be achieved.

An approach to measure risk associated with the increase in phage released into the environment and how they interact within natural ecosystems relies on biomolecule tagging and immobilization. Passive adsorption is one of the most straightforward methods for attaching biomolecules to hydrophobic polymeric particles. Biomolecules are commonly immobilized through passive adsorption onto polystyrene tubes and other surfaces with adequate surface modifications. This passive approach is beneficial in many applications of immobilizing biomolecules because it requires little additional treatment of the target molecules and substrate. The passive nature of this approach depends on the formation of numerous weak bonds between targets, which can cause the immobilized molecule's functional configuration to be altered (Wilson, 2001). Biotinylation alleviates this problem by creating a limited number of tighter bonds between the target molecule and the substrate (Hoogenboom, 1998). Biotin, a vitamin and coenzyme vital to fatty acid synthesis and gluconeogenesis, can be covalently linked to DNA or proteins in a phenomenon known as biotinylation. When biotin is bound to a protein, it can be immobilized on avidin-coated surfaces. This technique has been used extensively in a variety of ligand-binding studies (Bayer, 1976; Heitzman, 1974) and avidin is routinely replaced with streptavidin, which has a higher conjugate binding affinity. The biotin-streptavidin bond is one of the strongest non-covalent interactions found in nature, with a dissociation constant of approximately $10^{-14}$ mol/L (Green, 1975). Numerous techniques utilize this strong interaction, which can allow for

the sensitive detection of biotinylated molecules. Biotinylation is usually achieved by modifying protein amino groups with biotin-N-hydroxysuccinimide or other acylating agents. This chemical approach to biotinylation has been applied to phage capsid proteins and coupled with streptavidin-coated bead interactions, it has been shown to increase capturing efficiency over previously described systems (Bennett, 1997).

The only biotinylated protein produced by *E. coli* is the biotin carboxyl carrier protein (BCCP), which is a 22-32 kDa subunit of acetyl-CoA-carboxylase (Fall, 1979). *E. coli* holoenzyme synthetase uses ATP to link biotin to the amino group of ε-lysine 122 in BCCP. Several studies have reported biotinylation of fusion proteins *in vivo* which consisted of constructing biotin acceptor domains similar to BCCP-like acceptor proteins that could be fused to genes for heterogenous proteins (Cronan, 1990; Tatsumi, 1996; Li, 1992; Choi-Rhee, 2003; Yamano, 1992). Additionally, phage T7 has been successfully engineered to display biotinylation peptides on its capsid surface and subsequent *in vivo* host *E. coli* biotinylation of the peptides by biotin-ligase protein, BirA (Edgar, 2006). Streptavidin-coated quantum dots were used to detect, concentrate, and quantify the biotinylated progeny phage after host cell lysis, thereby indicating host *E. coli* infection events.

The work described herein utilized this biotinylating characteristic of *E. coli* and a BCCP fusion with the small outer capsid (SOC) protein of bacteriophage T4 to allow for the retrieval and enumeration of progeny phage after host cell infection in a wastewater ecosystem. The binding site on the SOC protein was not near the N- or C-termini, thereby minimizing the possibility of interrupting the interaction between SOC and the

phage capsid. Additionally, it has been observed that SOC is nonessential for bacteriophage viability, but confers additional stability to the phage capsid structure against osmotic shock, pH fluctuations, and temperature denaturation, thereby making SOC a useful target for potentially benign protein fusions (Comeau, 2008). This recombinant phage has been effectively immobilized onto streptavidin-modified surfaces without observable affects on phage infection efficiency (Tolba, 2009). This technology could allow for the unbiased isolation of tagged phage particles and the quantitative characterization of transduction events through phage genomic analysis targeting host-derived 16S rRNA gene sequences and bacterial antibiotic resistance genes. The isolation techniques utilized in this effort will allow for specific labeling of phage after host infection and swift retrieval from an environmental solution, relying heavily on the strong non-covalent binding interactions of biotin and streptavidin (Figure 1).

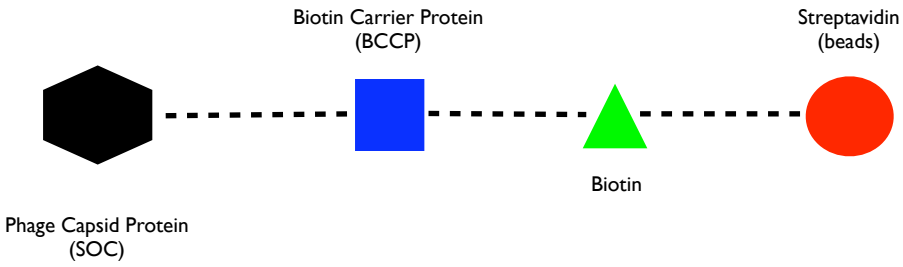


**Figure 1. Overview of bacteriophage biotinylation and retrieval system.**

## Materials and Methods

Genetic Construction of E. coli K12-12017 host

The host *E. coli* K12 (strain 12017) was grown in ½ salt Luria Burtani broth (Table 9) overnight in 100mL of culture medium at 37°C, shaking at 200 rpm. All following bacterial and phage incubations at 37°C were incubated with shaking at 200 rpm, unless noted otherwise. After incubation, 30$\mu$l of culture were transferred into 1.4mL of fresh ½ salt Luria Burtani broth in 1.5mL microcentrifuge tubes and incubated at 37°C for three hours. After incubation, the tubes were transferred to ice and subsequently centrifuged for 30 seconds at 9,000 x *g* at 2°C. The supernatant was removed from the tubes and the pelleted cells were resuspended in 1mL of sterile, deionized $H_2O$. The resuspended cells were centrifuged at 9,000 x *g* at 2°C and the supernatant was removed. The remaining pelleted cells were resuspended in 30$\mu$l deionized $H_2O$. After resuspension, 1$\mu$l of pGlowBS2STOP (Figure 2) plasmid DNA at a concentration of 600ng/$\mu$l was added to the cell mixture and transferred to an electroporation cuvette that was cooled on ice. The electroporation device (Biorad, Hercules, CA) was set to the following settings: T=2.5kV, C=50$\mu$F, R=125 ohms, S=2.45kV. The field strength was calibrated to produce 12.25 kV/cm with a pulse length of 5-6 milliseconds. After electroporation, the mixture was immediately resuspended in 1mL of super optimal broth with catabolite repression (Table 9) and transferred to a sterile microcentrifuge tube. The culture was incubated at 37°C for 70 minutes. After incubation, 100$\mu$l of cell solution were spread on $LB_{amp}$ plates laced with 100mg/mL of ampicillin to act as a

selecting agent. The plates were incubated at 37°C overnight and observed for successful transformation colony growth.

For purification of the pGLOWBS2STOP plasmid that contained the marker genes used in this study, a Wizard+ Miniprep kit (Promega, Madison, WI) was used according to manufacturer protocol. The purified plasmid DNA was subjected to PCR and gel electrophoresis to validate primer functionality and gene presence. Primers were designed to amplify fragments of marker genes found on the plasmid, thereby indicating the possible presence of the entire gene of interest. Three marker genes on the pGlow plasmid were established as being: the ampicillin resistance gene, *ampR*, the evoGlow *Pp1* gene, *pGlow*, and the fluorescent marker gene, *gfp*, all of which were derived from the pGlow-Bs2-stop plasmid (Evocatal, Düsseldorf, Germany). The *ampR* and evoGlow *Pp1* genes required a 56°C annealing temperature, while the *gfp* gene primer set required a 62°C annealing temperature in the thermocycler programming (Table 3). Each PCR was performed using PuRe Taq Ready-To-Go™ PCR Beads (GE Healthcare, Little Chalfont, UK), and primer sets were designed using primer3 software (MIT, Cambridge, MA) and commercially obtained from Operon. All PCR primers were diluted to 100$\mu$M initially, and working stocks were further diluted to 5$\mu$M for PCR applications. Each PCR reaction contained 1$\mu$l of each primer at 5$\mu$M concentrations, 300ng of template DNA, and sterile deionized $H_2O$ to a total volume of 50$\mu$l per reaction. Wide-Range DNA ladder (Takara Bio, Otsu, Japan) was used as a DNA molecular size marker in gel electrophoresis. An Owl Easy Cast gel electrophoresis system (Thermo Scientific, Waltham, MA) was used for casting and running 1% agarose

gels with PCR products. Tris-borate-EDTA buffer was used for agarose gel preparation and also electrophoresis. Ethidium Bromide was mixed with molten agarose by adding 4$\mu$l of EtBr (Fisher Scientific, Hampton, NH) for each 100 ml volume of agarose gel. The gels were run at 60V for approximately 45 minutes at room temperature and visualized in an UV photodocumentation device. All subsequent gel electrophoreses utilized these parameters.

**Table 1. Biological entities utilized in this study.**

| | |
|---|---|
| **Bacterial Host** | Escherichia coli (strain 12017) |
| **Marker Plasmid** | pGlowBS2Stop |
| **Bacteriophage** | BCCP-fused T4 |

**Table 2. Primers used for determining plasmid fidelity.**

| Primer | Sequence |
|---|---|
| AmpR FW | TTGCCGGGAAGCTAGAGTAA |
| AmpR RV | AAGCCATACCAAACGACGACGAG |
| pGlow FW | TGGAAGATAACCCGATCGTC |
| pGlow RV | GATCGATGTTCAGTTCGTTCCA |
| gfp FW | ACGCGTATGGCGTCGTTCCAGTCGTT |
| gfp RV | AGATCTGCAGTCACTCGAGCAGCT |

**Table 3. Polymerase chain reaction thermocycler protocol. *AmpR* and *pGlow* primer sets required 56°C annealing, and *gfp* primers required 62°C annealing.**

| Activity | Temperature | Time |
|---|---|---|
| Denaturation (long) | 95°C | 5 minutes |
| Denaturation (short) | 95°C | 30 seconds (repeated 39X) |
| Annealing | 56°C *or* 62°C | 30 seconds (repeated 39X) |
| Extension (short) | 72°C | 1 minute (repeated 39X) |
| Extension (long) | 72°C | 10 minutes |

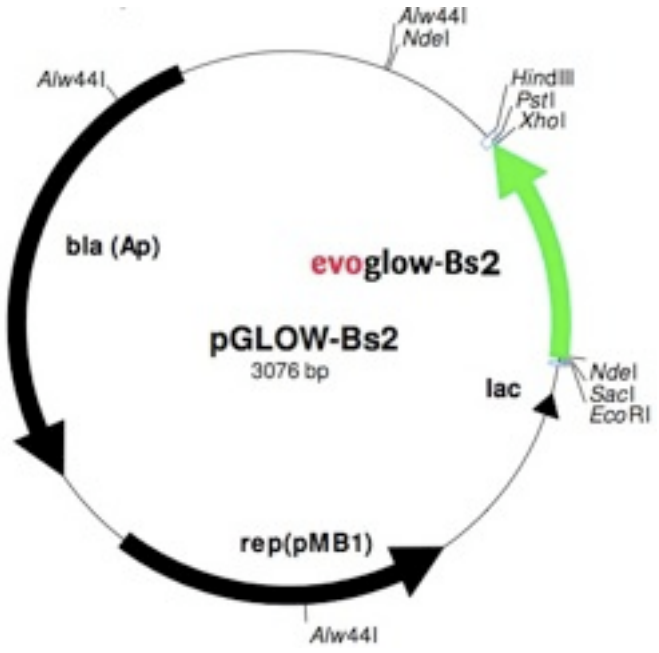


**Figure 2. Map of pGlow-Bs2 plasmid used for marker genes in genetic analysis.**

Genetic Characterization of Biotinylated Bacteriophage T4

The biotinylated bacteriophage T4 was developed by Mona Tolba at the University of Guelph. The phage had been developed to use a biotin-binding peptide as an affinity tag that was displayed on the phage head as fusions with the Small Outer Capsid (SOC) protein. The gene for the capsid-fused peptide was the biotin carboxyl carrier protein (BCCP), from acetyl-CoAcarboxylase of *E. coli*. In addition to BCCP, the T4e lysozyme gene was also used as a marker gene to aid in qualitative and quantitative standards. Polyethylene glycol 8000 (Fisher Scientific, Hampton, NH) and sodium chloride (Fisher Scientific, Hampton, NH) were used for the two-phase precipitation of bacteriophage T4. The host *E. coli* K12 was grown in 100mL of LB medium at 37°C overnight. After incubation, cultures were centrifuged at 5800rpm for 10 minutes at room temperature and the supernatants discarded. The pellets were resuspended in 3mL of SM buffer and phage particles were added to a multiplicity of infection of 0.5. The infected cultures were incubated for 20 minutes at 37°C with intermittent swirling. The cultures were then added to 500mL of 37°C prewarmed LB medium, incubated at 37°C and observed for visible lysis during an 8-12 hour period. After visible lysis, 10mL of chloroform (Fisher Scientific, Hampton, NH) were added to the cultures and incubated for 10 minutes at 37°C. The lysed cultures were then cooled to room temperature and transferred to a sterile 1000mL separation funnel. At room temperature, 32.5g of PEG8000, 1g Dextran Sulfate, and 8.75g of NaCl were added to 500mL of culture, mixed with gentle swirling and stored at 4°C overnight. The liquid supernatant mixture were then transferred to sterile polypropylene bottles and stored on

ice for 1 hour. After cold incubation, the precipitated bacteriophage particles were centrifuged at 8300rpm for 10 minutes at 4°C. The supernatants were discarded and the bottles were allowed to stand inverted at a titled position for 5 minutes. The remaining pellets were rinsed and resuspended with 8mL SM buffer for each 500mL bottle and the bottles were placed on their sides for 1 hour at room temperature to allow for pellet saturation. An equal volume (8mL) of chloroform was added to the bottles to extract the cellular debris and PEG from the suspension. The mixture was vortexed for 30 seconds and centrifuged at 3000*g* for 15 minutes at 4°C, and the aqueous phase was recovered.

Phage plaque titer plates were made using LB agar as the base medium and LB 1% agarose as the top medium. Host *E. coli* K12 was grown at 37°C overnight, and 200*μ*l were transferred to a sterile 15 ml polypropylene tube. The overlay medium, LB 1% agarose was melted and cooled to 42°C to prevent heat shock effects on the bacterial cells. Once cooled, 4 ml of the top agarose was added to the 15 ml polypropylene tubes containing the host *E. coli* K12 and gently mixed. The mixture was poured onto 37°C warmed LB agar plate and allowed to solidify at room temperature. After solidifying, serial dilutions of the phage precipitate were pipetted onto the top layer containing the host *E. coli* K12 in LB agarose. The plates were incubated for 12 hours at 37°C in the dark. Bacteriophage enumeration occurred after the incubation period to determine viral concentration in the solution. This plaque assay was repeated after the following CsCl ultracentrifugation procedure to determine phage concentration and particle loss.

**Table 4. Cesium chloride density gradient concentration calculations and associated refractive indexes.**

| Density ρ (g/ml) | CsCl (g) | Salt Magnesium Buffer (ml) | Refractive Index η |
|---|---|---|---|
| 1.45 | 60 | 85 | 1.3768 |
| 1.50 | 67 | 82 | 1.3815 |
| 1.70 | 95 | 75 | 1.3990 |

For density gradient ultracentrifugation, three CsCl gradients were prepared (Table 4). For each ml of bacteriophage suspension, 0.5 g of CsCl (Fisher Scientific, Hampton, NH) was added and mixed until all of the CsCl was dissolved. The CsCl gradients were added to an Ultra-clear ultracentrifuge tube (Beckman Coulter, Pasadena, CA), 7.6 ml of each CsCl concentration was added in the order of 1.70ρ at the bottom, 1.50ρ in the middle, and 1.45ρ on the top. Additionally, 13 ml of bacteriophage solution was added to the top of the CsCl gradient layers. Each gradient zone was marked on the tube with a permanent marker to indicate the layers. The suspension was centrifuged at 87,000*g* (22,000 rpm Beckman SW28 rotor) for 2 hours at 4°C. After ultracentrifugation, a sterile syringe was used to puncture the side of the tube in the density above the 1.5 g/ml CsCl region where the bacteriophage particles formed a band. The contents were transferred to a sterile ultracentrifuge tube, filled with 1.50 g/ml CsCl, and sealed. The solution was centrifuged at 150,000*g* (41,000 Beckman Ti50 rotor) for 24 hours at 4°C. The band of bacteriophage particles was collected as previously described and stored at 4°C.

For bacteriophage genomic DNA extraction, the volume of the band containing purified bacteriophage particles was measured and 0.1 volume of 2M Tris-Cl, pH 8.5 and 0.2M EDTA (Fisher Scientific, Hampton, NH) were added and inverted to mix. After thorough mixing, 1 volume formamide (Fisher Scientific, Hampton, NH) was added, gently vortexed to mix, and left to stand at room temperature for 30 minutes. Subsequent addition of 2 volumes, each equal to the original bacteriophage band volume, of 100% ethanol was mixed into solution and centrifuged for 2 minutes at 10,000*g* for 2 minutes at room temperature. The supernatant was discarded and the remaining pellet was rinsed with 70% ethanol. All droplets of residual ethanol were removed with a pipette and the moist, genomic DNA-containing pellet was resuspended in TE buffer.

PCR primer sets were designed using primer3 software (MIT, Cambridge, MA) for the BCCP and T4e genes (Table 5). The target amplicon size for BCCP was 146 bp and the T4e amplicon was 139 bp. The PCR protocol followed the same general program as described in Table 3, with the exception of the annealing temperature being set to 58°C for both BCCP and T4e primer sets. PCR reactions tubes, gel electrophoresis, staining, and visualization followed the same parameters previously described.

**Table 5. PCR primer names and sequences for BCCP and T4e gene amplification.**

| Name | Sequence |
|---|---|
| BioFW | CAAGTTTCCCTGTGATGCAAC |
| BioRV | GGGTGCGGTAGAAAGTACCA |
| T4eFW | TTTGCGCGATTAGGTGTTTG |
| T4eRV | GTTCGTCGCTGTGCATTGAT |

After PCR verification of BCCP and T4e presence in the bacteriophage genomic DNA, a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) was used to clone the gene fragments using pcr4 cloning vector and TOP10 *E. coli* cells. Cloning was followed by Wizard+ SV miniprep (Promega, Madison, WI) application to purify the plasmid DNA for further PCR verification. Plasmid DNA was then used as the template for PCR amplification of the BCCP and T4e genes. All PCR parameters were identical to those previously described.

Multiplicity of Infection Optimization

Necessity dictated that the host *E. coli* K12 growth characteristics be determined to allow for effective bacteriophage infection and propagation. The mercurial relationship between bacteriophages and their hosts can result in highly variable infection rates, which can directly affect the optimal multiplicity of infection necessary for bacteriophage propagation. To standardize and streamline the process, optical density

measurements (600 nm) were taken at specific time intervals of bacterial growth and subsequently compared to cfu/ml dilutions on growth medium.

Three cultures containing Luria Burtani broth supplemented with 100 mg/ml ampicillin were inoculated with *E. coli* K12 pGlowBS2Stop from single colonies on $LB_{amp100}$ agar plates and incubated overnight at 37°C. The cultures' $OD_{600}$ were measured after overnight incubation and 1 ml of overnight culture was transferred to 100 ml of pre-warmed to 37°C $LB_{amp100}$ broth. The initial $OD_{600}$ was measured immediately after the transfer and the cultures were incubated at 37°C. The optical density of the cultures were measured every hour and returned to incubation between measurements. In addition to optical density measurements, an additional 1 ml of culture was removed at each time point and serially diluted in $LB_{amp100}$ broth for spread plating on $LB_{amp100}$ agar plates. The viable cell count plates were incubated at 37°C over night in the dark and quantified the next day. The viable cell counts were matched with the optical density measurement time points for an observable correlation and the bacterial growth phases were mapped according to time and growth conditions using Prism 4 statistical software (GraphPad Software Inc, La Jolla, CA).

The optimal multiplicity of infection value was determined by adding a known quantity of infectious viral particles to a known quantity of viable bacterial cells during a specific growth phase in batch culture. This was achieved by growing *E. coli* K12 host overnight at 37°C and transferring 1 ml to 200 ml of fresh LB broth. The culture's $O.D._{600}$ was measured until the density reached approximately 0.4 and bacteriophage T4 at a M.O.I. of 0.5 (one viral particle per two bacterial cells in solution) was added to the

culture. The concentration of the bacteriophage sample added to bacterial culture was quantified through plaque assay enumeration. After the transfer and bacteriophage inoculation, the culture was incubated at 37°C and optical density at 600 nm was monitored during the post-inoculation period and the presence of visible cellular debris accumulation was observed. The lysed culture was treated with the addition of 2 ml of chloroform and centrifuged at 10,000*g* for 5 minutes at room temperature and the supernatant was transferred to a sterile polypropylene tube. The solution was filtered through a 0.45 $\mu$m membrane filter, serially diluted with SM buffer, plated onto top-agarose containing host *E. coli* K12 host and incubated at 37°C overnight in the dark. The top agar plates were examined to determine bacteriophage titers of the infected culture.

Bacteriophage Purification and Concentration

The previously described method of isopycnic CsCl gradient ultracentrifugation for purifying and concentrating bacteriophage samples was found to be inadequate for high throughput processing. Therefore, a liquid two-phase system was employed to precipitate bacteriophage-lysed bacterial cultures. This system was a modified version as described in the literature (Vajda, 1978; Philipson, 1960; Leberman, 1966; Venekamp, 1964).

The host *E. coli* K12 was grown in 15 ml LB broth overnight at 37°C. After incubation, 10 ml of *E. coliI* culture was transferred to 190 ml of pre-warmed (37°C) LB broth and incubated at 37°C until the $O.D._{600}$ measured 0.45, indicating exponential growth phase. At this point, 50 ml of phage stock at $5x10^9$ pfu/ml was added and mixed

with the culture. The infected culture was incubated for 2-3 hours at 37°C until visible lysis occurred. When lysis was observed, characterized by clearing and cellular debris accumulation in the medium, the culture was transferred to a sterile 500 ml separation flask at 4°C. In the cooled separation flask, 4.375 g of NaCl (Fisher Scientific, Hampton, NH) was thoroughly mixed into solution and incubated at 4°C for 1 hour. After incubation, 16.25 g of PEG-8000 m.w. (Promega, Fitchburg, WI) and 0.5 g of Dextran Sulfate (Fisher Scientific, Hampton, NH) were added to the 250 ml total volume culture and the flask was inverted several times until all added compounds were in solution. The culture was kept at 4°C overnight to allow for bacteriophage precipitation. After the precipitation incubation, the bottom layer of the solution containing the cellular debris and other contaminants, was removed via the separation apparatus on the flask and discarded. The supernatant was transferred to a sterile polypropylene tube and centrifuged at 11,000 *g* for 10 minutes at 4°C. The supernatant was discarded, any residual fluid was removed by pipette aspiration, and the tubes were inverted for 5 minutes at room temperature to allow any remaining liquid to drain away. The bacteriophage-containing pellet that was formed on the wall of the polypropylene tube was resuspended in 5 ml of SM buffer by using a wide-bore pipette and the tube was placed on its side for 1 hour to allow the SM buffer to saturate and soak the pellet. Any remaining cell debris and PEG was extracted from the bacteriophage suspension by adding 5 ml of chloroform and gently vortexing the mixture for 30 seconds. The mixture was then centrifuged at 3000 *g* for 15 minutes at room temperature and the bacteriophage-containing aqueous phase was transferred to a sterile glass tube. The

phage stocks were then serially diluted to produce a pfu/ml value for the solution, thereby indicating the quantity of viable phage in solution. Additionally, the chloroform step performed after the PEG extraction process was replaced with a syringe filtration step through a 0.2 $\mu$m filter, which was found to be comparably effective at purifying phage stocks. This was done by transferring the resuspended bacteriophage-containing pellet to a sterile 5 ml syringe with a 0.2 $\mu$m filter disc peripheral attached to the syringe tip. The solution was passed through the filter into a sterile glass tube and stored at 4°C for plaque assay applications.

DNase I Treatment

The endonuclease, DNase I, was utilized as a means for degrading contaminating DNA in samples. The optimization of the enzyme's capabilities was intended to provide a method to purify bacteriophage particles of host DNA in solution. The effective conditions for DNase I activity were investigated by creating a gradient of added metal ions ($MgCl_2$) to the solution after bacterial cultures were lysed and preliminarily filtered. Bacterial cultures of *E. coli* K12 pGlowBs2Stop were grown overnight in 25 ml of $LB_{amp100}$ at 37°C. The culture was then lysed by the addition of 2.5 ml of chloroform and incubated for 10 minutes at 37°C. The lysed culture was then centrifuged for 10 minutes at 10,000*g* at room temperature and the supernatant was subsequently transferred to a sterile syringe and filtered through a 0.2 $\mu$m filter into a sterile polypropylene tube. Aliquots of varying volumes of the supernatant were transferred to 1.5 ml polypropylene tubes for DNase I treatments (Table 6). The variable volumes of supernatant added allowed for final concentrations of $MgCl_2$ to be

precise. To each tube of bacterial culture supernatant, 20 units of RNase- free DNase I (New England Biolabs, Ipswich, MA) was added. A gradient of $MgCl_2$ concentrations of 10 mM, 20 mM, and 30 mM were added to the reaction tubes (Table 6). In addition to these experimental conditions, supernatant with metal ions, supernatant-only, raw culture-only, DNase I-only, and DNase I with metal ion controls were prepared in parallel. The reactions were incubated according to the manufacturer's protocol at 37°C for 10 minutes and heat inactivated by incubating the reactions at 75°C for 10 minutes. The reactions were then used as templates for PCR verification using pGlow and gfp primer sets (Table 2). The PCR thermal cycling conditions and reaction tube volumes were identical to those previously described (Table 3). The PCR products and wide range DNA ladder (Takara) were loaded into a 150 ml volume, 1% agarose gel with 7 $\mu$l ethidium bromide and 60V were applied to the gel electrophoresis reaction for 45 minutes. The gel was viewed under an ultraviolet light and photodocumented.

**Table 6. DNase I treatment conditions for PCR verification**

| Reaction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Supernatant (volume) | 14.5 µl | 14 µl | 13.5 µl | 25 µl | 24.5 µl | 24 µl | 23.5 µl | 25 µl (raw culture) | 15 µl |
| DNase I (volume) | 10 µl 20 Units | 10 µl 20 Units | 10 µl 20 Units | 0 µl | 0 µl | 0 µl | 0 µl | 0 µl | 10 µl 20 Units |
| $MgCl_2$ (volume) | 0.5 µl (0.5 M stock) | 1 µl (0.5 M stock) | 1.5 µl (0.5 M stock) | 0 µl | 0.5 µl (0.5 M stock) | 1 µl (0.5 M stock) | 1.5 µl (0.5 M stock) | 0 µl | 0 µl |
| MgCl2 (final concentration) | 10 mM | 20 mM | 30 mM | 0 mM | 10 mM | 20 mM | 30 mM | 0 mM | 0 mM |

Realtime Quantitative Polymerase Chain Reaction

Quantitative PCR (QPCR) was used to measure transduction events in controlled batch cultures. The batch cultures represented a grossly simplified interaction scenario between bacteriophage T4 and a single host, *E. coli* K12, that possessed the pGlowBs2Stop plasmid harboring marker genes. The previously described primer sets for ampR, pGlow, and gfp amplicons were used for QPCR analysis.

The host *E. coli* K12 pGlow Bs2Stop was grown in 500 ml of $LB_{amp100}$ to an $O.D._{600}$ of 0.3 at 37°C. When optimum optical density was achieved, biotinylated bacteriophage T4 was added at multiplicity of infection (MOI) of 1.0. The infected culture was incubated at 37°C for 4 hours. After bacteriophage-induced lysis of the culture, the previously described CsCl density gradient coupled with PEG precipitation method was applied. The bacteriophage-containing band was aseptically extracted from the ultracentrifuge tube and serially diluted to quantify infectious bacteriophage particles in the solution. The plaque assay served as the enumerating agent in standardizing bacteriophage T4 genome copies in QPCR due to the stringent conditions found in the density gradient. The density gradient theoretically collects only intact viral particles in the designated region above 1.50 g/ml of CsCl. This purified bacteriophage sample was then used for genomic DNA extraction via the formamide protocol, as previously described.

The phage genomic DNA was serially diluted and used as template DNA in QPCR for the marker genes. Brilliant II QPCR Master Mix (Stratagene, La Jolla, CA) was used as the fluorescent agent according to the manufacturer's protocol. Each

QPCR set was prepared in white, 96-well plates in a laminar fume hood. The reaction components are described in Table 7, with primers being interchanged for each marker gene primer set (Table 2). The QPCR thermocycler was programmed according to Table 8, and all data were analyzed using Numbers software (Apple Inc, Cupertino, CA).

**Table 7. QPCR reagent distribution.**

| Reagent | Volume per Well |
|---|---|
| Master Mix | 12.5 $\mu$l |
| Bovine Serum Albunim (BSA) | 1.0 $\mu$l |
| Forward Primer (5 $\mu$M) | 1.0 $\mu$l |
| Reverse Primer (5 $\mu$M) | 1.0 $\mu$l |
| Sample Template | 2.5 $\mu$l |
| dd$H_2$0 | 7 $\mu$l |

**Table 8. QPCR thermal cycling protocol for pGlow, ampR, and gfp primer sets.**

| Step | Temperature | Time |
|---|---|---|
| 1 | 50°C | 2 minutes |
| 2 | 95°C | 15 minutes |
| 3 | 95°C | 1 minutes |
| 4 | 60°C | 30 seconds |
| 5 | Plate Read | n/a |
| 6 | 72°C | 30 seconds |
| 7 | Plate Read | n/a |
| 8 | 85°C | 30 seconds |
| 9 | Plate Read | n/a |
| 10 | Repeat Steps 3-9, 45 times | n/a |
| 11 | 65°C-95°C melting curve | read every 1°C, holding 5 seconds b/w reads |

Streptavidin Paramagnetic Particle Recovery of Biotinylated Bacteriophage T4

The biotin-binding protein that was fused to the small outer capsid of bacteriophage T4 theoretically allowed for the virus' recovery via the strong non-covalent bond between avidin, or its biological relative, streptavidin, and biotin molecules. Thus, commercially available Dynabeads® MyOne™ Streptavidin C-1beads (Invitrogen, Carlsbad, CA) were used as a means to recover the biotinylated bacteriophage T4 particles from solution due to their higher binding capacity for biotin. Phage stocks that were produced using the aforementioned two-phase polymer

precipitation system at a concentration of $9x10^{10}$ pfu/ml were applied to the Dynabeads® MyOne™ Streptavidin C-1 beads according to the manufacturer's recommended protocol with a minor modification with regard to the number of washes the beads were exposed to. The beads were prepared by resuspension in the original vial through rotating and vortexing. The washing buffer, 1 ml sterile PBS pH 7.4, was mixed with 100 $\mu$l of resuspended beads (1 mg of beads) in a sterile polypropylene tube and subsequently placed on a Dynal® MPC™ magnet for 2 minutes at room temperature. A pipette was used to remove the supernatant while the tube remained on the magnet. The tube was removed from the magnet and the beads were resuspended in 100 $\mu$l of sterile PBS (pH 7.4). This washing process was repeated two additional times to further remove preservatives from the bead solution. After the final wash, the supernatant was removed and replaced with 1 ml of phage stock containing $9x10^{10}$ pfu/ml and incubated for 30 minutes at room temperature with gentle rotation. The beads were collected on the magnet for 3 minutes at room temperature and the supernatant removed with a pipette. The tube containing the collected beads was then removed from the magnet and the beads were resuspended in 100 $\mu$l PBS containing 1% BSA. This washing and resuspension method was repeated eight times and the washing buffer was transferred to a sterile polypropylene tube each time for plaque assay analysis. Following the final wash, the beads were resuspended in 100 $\mu$l of $ddH_2O$ and incubated at 70°C for 3 minutes to break the biotin-streptavidin bond, releasing the biotinylated bacteriophage particles. The beads were immediately collected using a magnet and the supernatant was transferred to a sterile polypropylene tube for plaque

assay analysis. An identical experiment was performed with the modification of adding four additional wash steps, for a total of eight washes, to observe any changes in binding and recovery characteristics of streptavidin-bound bacteriophage. The same phage stock solution, buffers, and streptavidin bead type were used in this modified extension of the previously described experiment.

All supernatant wash solutions collected during the experiment were serially diluted and plated onto host *E. coli* K12-containing LB top agarose. The $ddH_2O$ solution containing the released bacteriophage particles were subjected to the same serial dilution and plaque assay treatment. The plates were incubated at 37°C in the dark overnight and plaque titers were determined by counting pfu's on the top agarose plates. All samples were processed in triplicate and the data were analyzed using Prism 4 software (Graphpad Inc).

Western Blot Analysis of Biotinylated Bacteriophage T4

Western blots were performed to verify the BCCP gene's functionality and presence in the T4 bacteriophage. The biotinylated bacteriophage T4 samples were measured to be at concentrations of $1x10^{10}$ pfu/ml and used as the experimental samples in the protein analysis. A *S. cerevisiae*-derived biotinylated protein, T199 PBEC2, was used as the positive control (30 μg/lane) due to its confirmed biotinylated status and highly-concentrated samples (Becker Lab, Knoxville, TN). The *wild-type* lambda phage ($2x10^9$ pfu/ml) acted as the negative control. Under two conditions, 65°C for 10 minutes and 90°C for 5 minutes, 1 ml of each phage solution was denatured and membranes were solubilized in 10% sodium dodecyl sulfate (SDS) sample buffer. Two

volumes of each denaturation condition were loaded into the gel lanes: 30 $\mu$l and 60 $\mu$l (Figure 15). Proteins were fractioned by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted. Blots were probed with Pierce Neutravidin +HRP conjugate (Thermo Scientific, Waltham, MA) and imaged on a ChemiDoc-XRS photodocumentation system (BioRad, Hercules, CA). The protein bands were measured using PageRuler™ prestained protein ladder (Fermentas, Glen Burnie, MD).

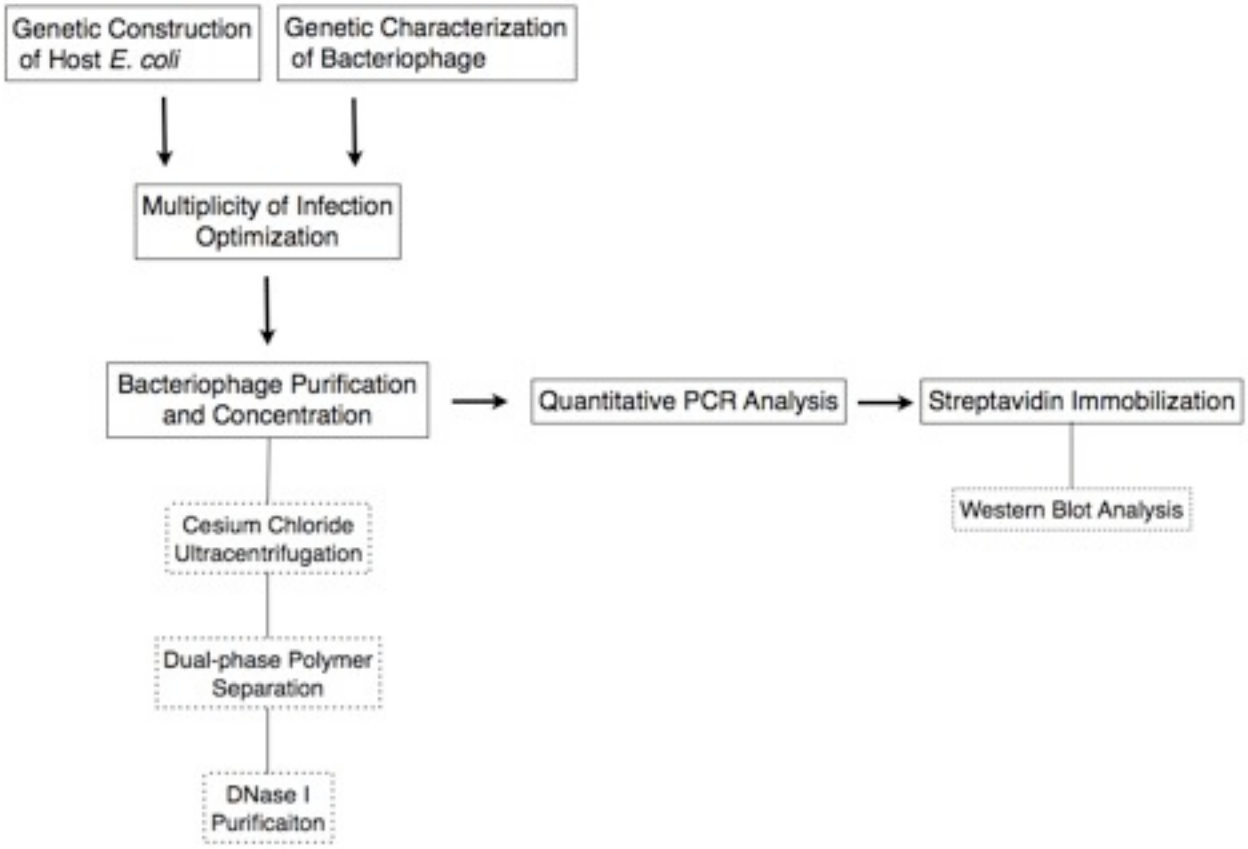


**Figure 3. Overview of experimental methods utilized in the study.**

**Table 9. Chemical solutions used in sample processing and growth media. All chemicals were obtained from Fisher Scientific (Hampton, NH).**

| Chemical Solution | Concentration | Chemical Solution | Concentration |
|---|---|---|---|
| Luria-Bertani Broth | 10 g/L Tryptone | Super Optimal Broth with Catabolite Repression | 20 g/L Bacto tryptone |
| | 5 g/L Yeast extract | | 5 g/L Yeast extract |
| | 10 g/L NaCl | | 0.5 g/L NaCl |
| | $ddH_2O$ to 1 L | | 0.186 g/L KCl |
| ½ salt Luria-Bertani Broth | 10 g/L Tryptone | | 0.952 g/L $MgCl_2$ |
| | 5 g/L Yeast extract | | 3.063 g/L Glucose |
| | 5 g/L NaCl | | $ddH_2O$ to 1 L |
| | $ddH_2O$ to 1 L | | |
| Tris-borate EDTA Buffer (5X) | 54 g/L Tris base | | |
| | 27.5 g/L Boric acid | | |
| | 20 ml 0.5 M EDTA | | |
| | $ddH_2O$ to 1 L | | |
| SM Buffer | 5.8 g/L NaCl | | |
| | 2.0 g/L $MgSO_4*7H_20$ | | |
| | 50 ml/L 1M Tris-HCl (pH7.5) | | |
| | $ddH_2O$ to 1 L | | |
| TE Buffer | 10 ml/L 1M Tris-HCl (pH 8.0) | | |
| | 2 ml/L 0.5M EDTA (pH 8.0) | | |
| | $ddH_2O$ to 1 L | | |
| PBS Buffer (pH 7.4) | 0.26 g/L $NaH_2PO_4*H_2O$ | | |
| | 1.44 g/L $Na_2HPO_4* H_2O$ | | |
| | 8.78 g/L NaCl | | |
| | $ddH_2O$ to 1 L | | |

# Results

Genetic Construction of E. coli K12 host

The marker genes ampR, gfp, and pGlow were confirmed to be present on the plasmid pGlowBS2STOP through mini-prep purification and PCR confirmation (Figure 3). The plasmid pGlowBS2STOP, containing marker genes (GFP, pGlow, and AmpR), was electroporated into *E. coli* K12, producing over 300 colonies, and 24 colonies were isolated for PCR analysis. Mini-prep purification produced pGlowBS2STOP DNA concentrations of 35ng/$\mu$l in 50$\mu$l volumes, resulting in a total of 1.75 $\mu$g of plasmid DNA purification. The success of the electroporation was indicated by the PCR and gel electrophoresis results (Figure 3). The reference ampicillin resistance amplicon (ampR) was 104bp in size, gfp marker gene (gfp) was 483bp, and the evoGlow (pGlow) marker gene was 219bp long.

Genetic Characterization of Bacteriophage T4

The presence of the biotin carboxylase carrier protein (BCCP) and T4e lysozyme genes in the BCCP-fused bacteriophage T4 were confirmed by PCR and gel electrophoresis (Figure 4). The template phage genomic DNA used for molecular confirmations was found to be at concentrations of 317ng/$\mu$l in 50$\mu$l volumes after formamide DNA extraction. The PEG/NaCl precipitation protocol produced phage titers of $10^{11}$ pfu/mL and ultracentrifugation through CsCl gradients produced a visible band of viral particles, resulting in final plaque titers of $10^{8}$ pfu/mL. The formamide genomic DNA extraction resulted in 15.85$\mu$g of total genomic DNA. The subsequent TOPO-TA cloning reaction to produce plasmid standards for QPCR analysis resulted in successful

integration of the BCCP and T4e genes into a pcr2.1 cloning vector and TOP10 *E. coli* cells (Figure 5). The PCR and gel electrophoresis results showed no amplification in the negative, no-template controls for both primer sets. The positive control, containing 295ng BCCP-fused T4 genomic DNA showed visible gel bands for both T4e and BCCP genes, indicating successful amplification of the target genes (Figure 5).

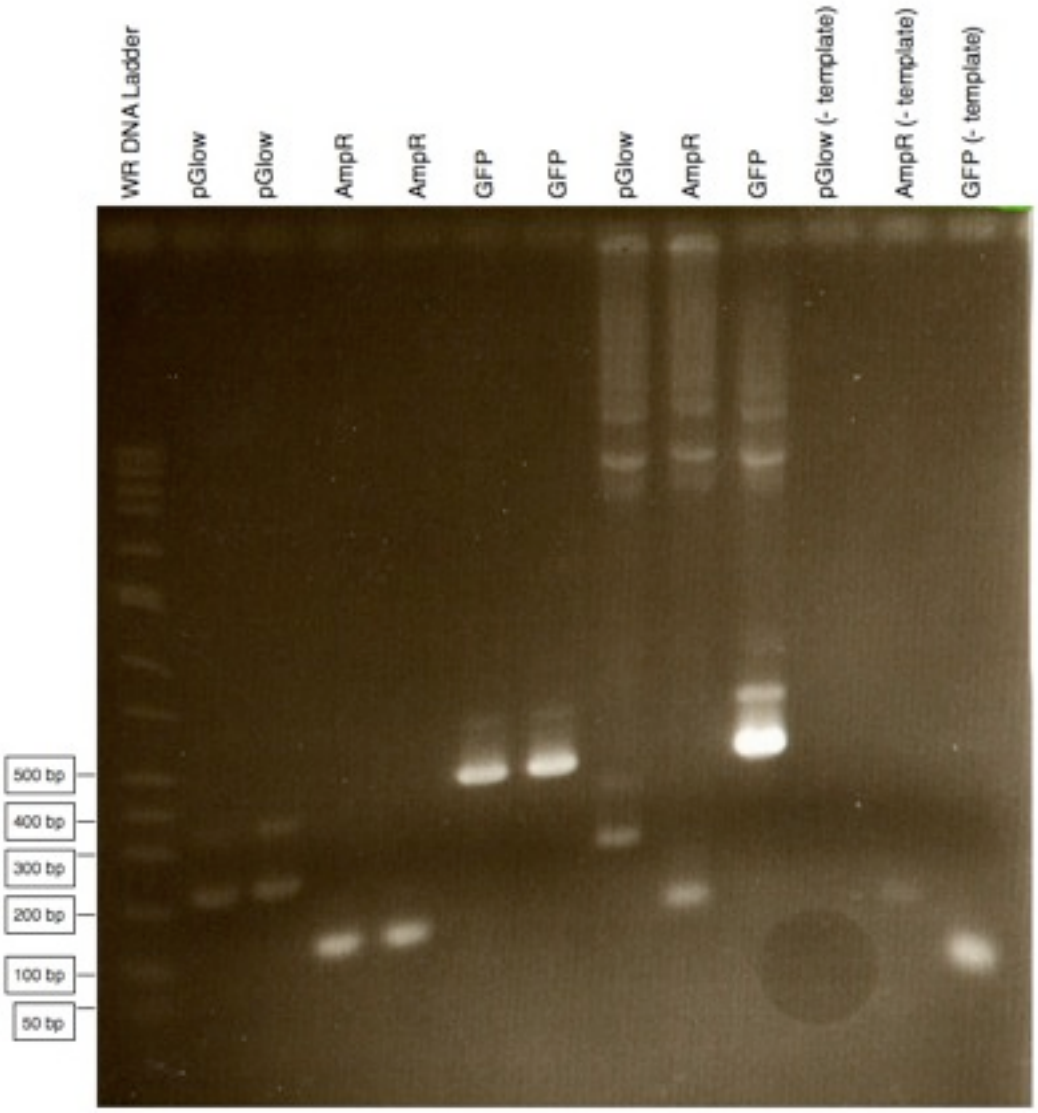


**Figure 4. Marker gene confirmation of pGlow BS2 Stop. Template DNA consisted of mini-prep purified pGlow BS2 Stop plasmid, aliquoted at 300 ng per reaction. Negative controls consisted of template-free reactions.**

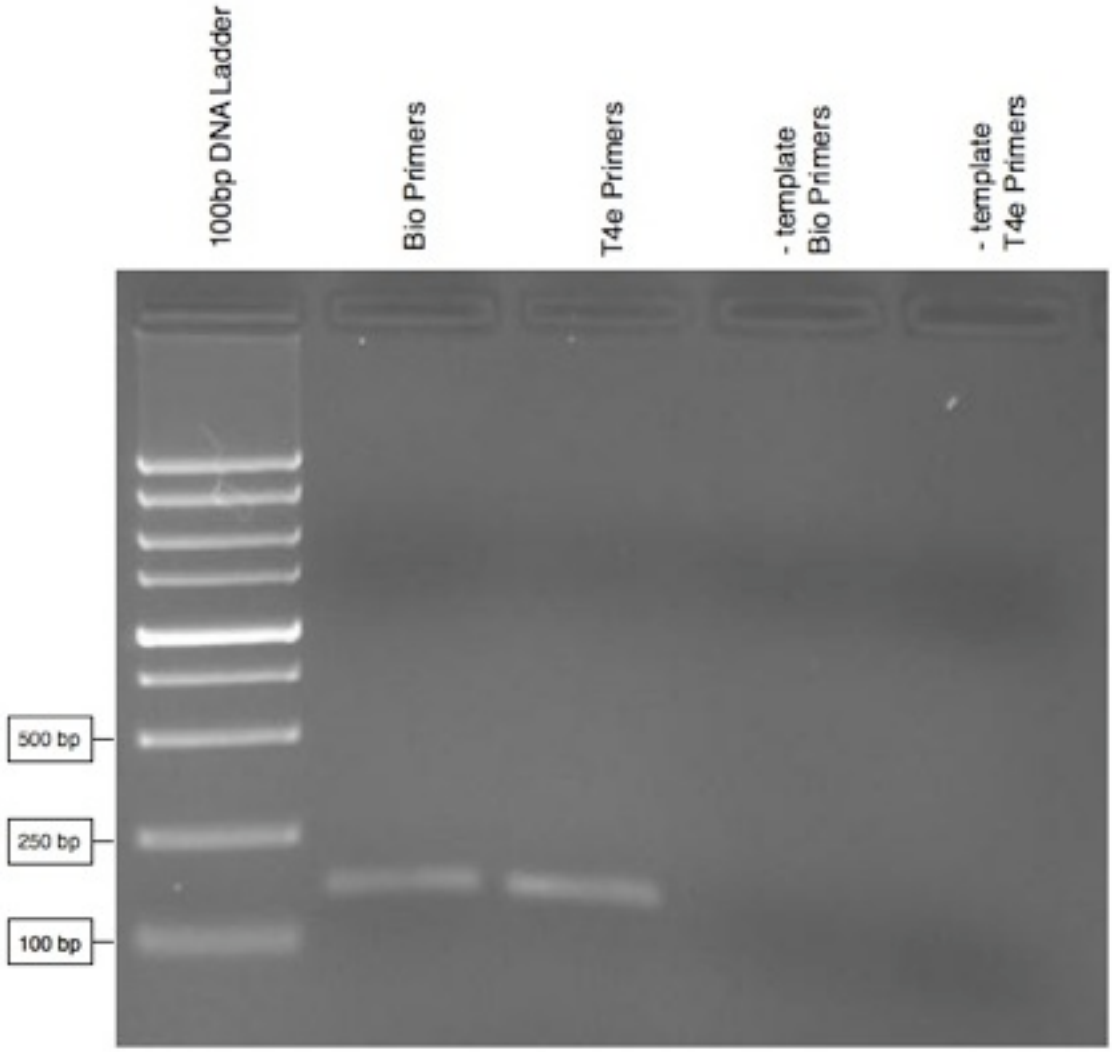


**Figure 5. Gel electrophoresis of PCR product for BCCP (Bio) and T4e genes from biotinylated bacteriophage T4 genomic DNA. Both positive reaction lanes were loaded with 317 ng template DNA, while the negative reactions indicate template free, negative controls.**

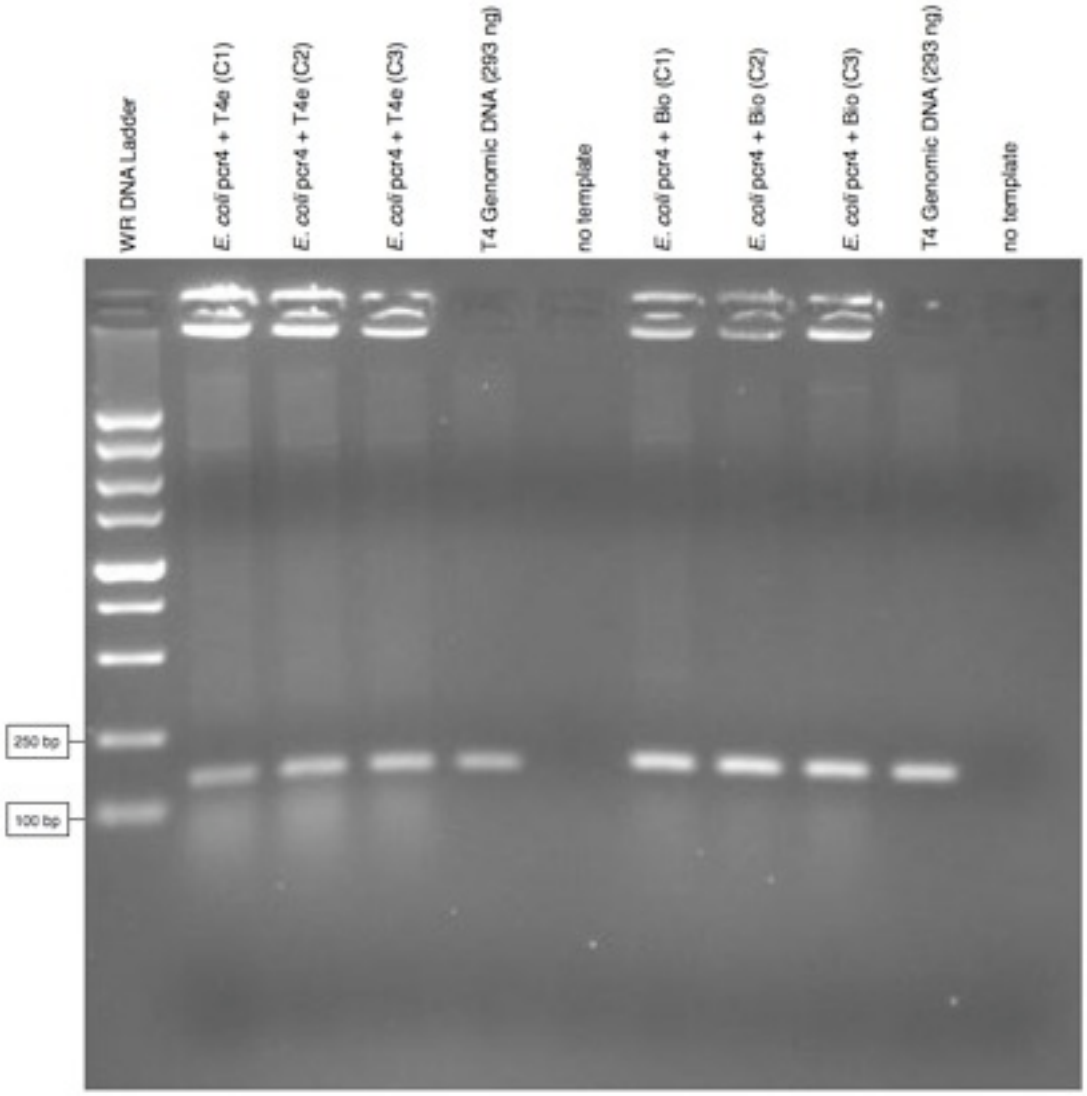


**Figure 6. Gel electrophoresis of PCR products resulting from TOPO-TA cloning BCCP (Bio) and T4e genes into pcr4 plasmid hosted in *E. coli* TOP10 cells. Three colonies are presented for each cloning reaction, each of which indicates successful cloning when compared to positive controls using biotinylated bacteriophage T4 genomic DNA. Both no-template controls for each primer set support the PCR results from the cloning reactions.**

Multiplicity of Infection Optimization

The growth characteristics of the host *E. coli* K12 were observed by comparing the optical density of growth cultures, time, and colony forming unit quantification (Figures 6-7). These results show that after seven hours of growth at 37°C with shaking at 200 rpm, 1mL of overnight culture added to 100 mL of fresh LB medium produced an $O.D._{600}$=0.4, which is indicative of the host *E. coli* being in exponential growth phase (Sezonov, 2007). The most effective period of bacterial batch culture growth for phage production has been shown to be during logarithmic growth phase (Hadas, 1997). The optimal multiplicity of infection (M.O.I.) was found to be 0.5 based on observed phage propagation. Effective M.O.I. ranges may vary based on environmental and growth conditions, however, the initial M.O.I. value of 0.5 provided productive results for this study. Overnight culture was grown to an $O.D._{600}$=0.98 prior to phage inoculation. After dilution and phage inoculation at a M.O.I. of 0.5, the $O.D._{600}$=0.45. After three hours of co-incubation, the $O.D._{600}$ had dropped to 0.125 with visible lysis defined by an increased accumulation of cellular debris in the medium solution. The subsequent plaque assay to determine bacteriophage propagation produced titers of $2x10^9$ pfu/ml in 200 ml solution.

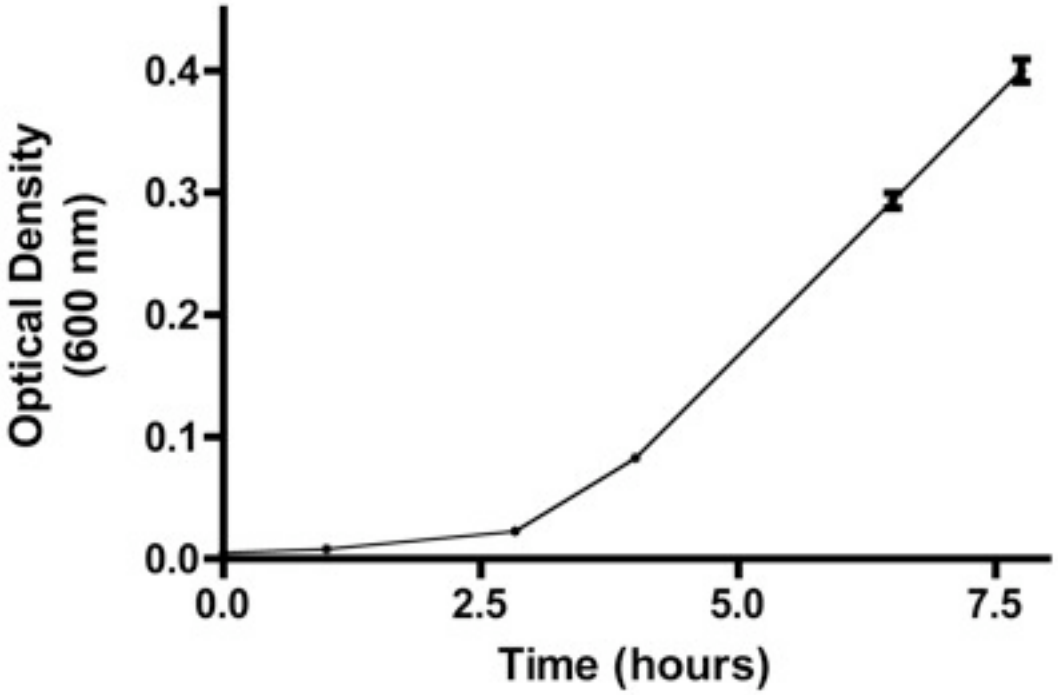


**Figure 7. Optical density measurements compared with time of incubation for host *E. coli* K12 pGlow BS2 Stop. Source cultures were grown in triplicate and measured simultaneously using the same spectrophotometer. Statistical analysis was performed with Prism 4 (GraphPad Software Inc, La Jolla, CA).**

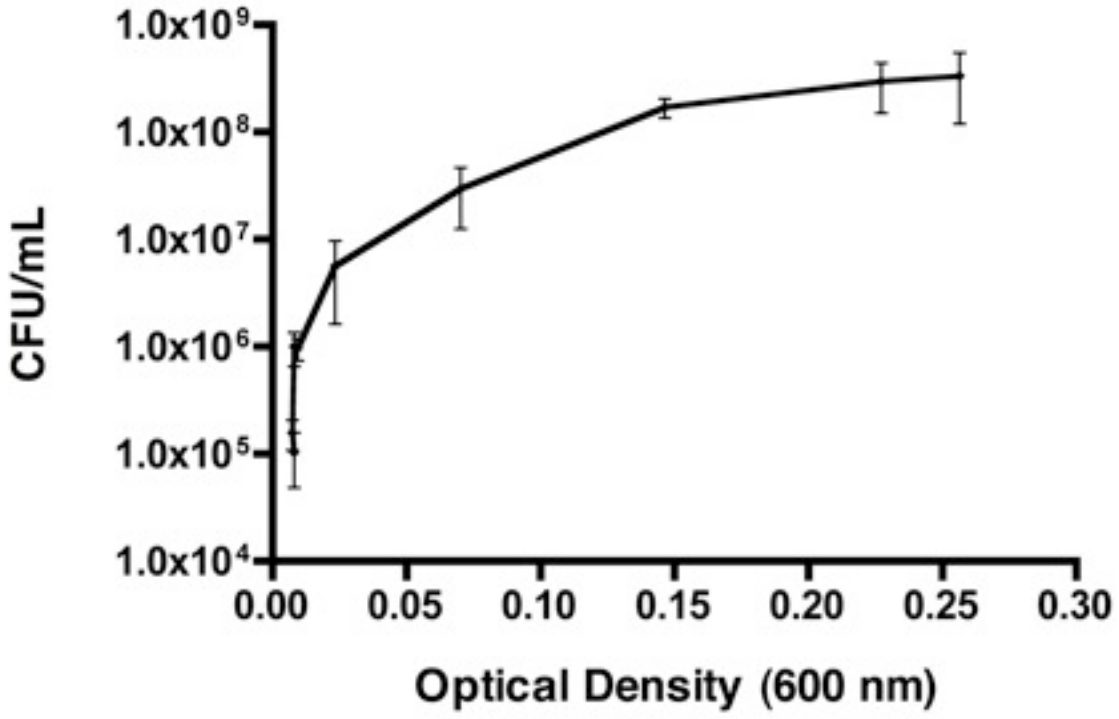


**Figure 8. Graphical representation of optical density (600 nm) measurements of *E. coli* K12 pGlowBS2Stop growth characteristics compared to viable cell counts in quantities of colony forming units (CFU) per mL. A correlation between optical density measurements and CFU/mL was observed and factored into the optimization for multiplicity of infection experiments. Statistical analyses were performed using Prism 4 software (GraphPad Software Inc., La Jolla CA).**

Bacteriophage Purification and Concentration

The most efficient results for T4 bacteriophage purification and concentration were obtained through the polyethylene glycol-dextran sulfate, two-phase system. In addition to the two-phase PEG-Dextran Sulfate polymer system of bacteriophage precipitation, filtration through 0.2$\mu$m filter produced exceptional phage yields. The primary advantage of the two-phase polymer system was the concentration effect. Cultures of *E. coli* K12 that were infected with bacteriophage T4 at M.O.I.=1 that were treated with chloroform, centrifuged at 10,000x*g* and filtered through 0.2$\mu$m filters produced phage stocks containing $2x10^8$ pfu/mL. The PEG-Dextran sulfate two-phase system produced phage stocks that contained $1x10^{11}$ pfu/mL. The concentration effect was observed in the two-phase system of phage precipitation due to the total volume being reduced by half during processing and removal of the phage-containing phase. The replacement of the chloroform precipitation of PEG and cellular debris with filtration through a 0.2 $\mu$m filter after re-suspending the phage-containing pellet produced no observable phage loss after performing plaque assays on the resulting solutions and no bacterial contamination was shown to occur in either product. The chloroform and filtration-only treatment resulted in the same volume of phage stock, albeit at lower pfu/ml values, as the original culture due to minimal removal of any liquid during processing. In addition to the previous two approaches to bacteriophage purification, the CsCl density gradient with ultracentrifugation method resulted in erratic phage titers and observable bacterial colony growth on plaque assay plates, indicating possible bacterial contaminant residue. Ultracentrifugation through CsCl density gradients was

determined to be low-throughput and inefficient for producing consistently pure phage solutions.

DNase I Treatment

Deoxyribonuclease I was found to be most enzymatically active in the presence of metal ions, specifically $MgCl_2$. PCR and gel electrophoresis performed after DNase I treatment of *E. coli* K12 pGlowBS2STOP lysed cells initially showed that DNase I degraded DNA efficiently at 5mM $MgCl_2$ concentration (Figure 8). Concentrations of $MgCl_2$ that exceeded 10mM showed lower enzymatic activity and metal-only controls that were not exposed to DNase I indicated that the metal ion did not affect the PCR to produce false negative results (Figures 8-9). Additionally, the reaction that was exposed to DNase I, but did not contain additional $MgCl_2$ produced the same gene amplification as the metal-only controls and the reactions with higher $MgCl_2$ concentrations (Figures 8-9). Both figures (Figure 8-9) represent lysed *E. coli* cultures containing the pGlowBS2 STOP plasmid after DNase I treatment with varying $MgCl_2$ concentrations after PCR amplification of the gfp marker gene amplicon.

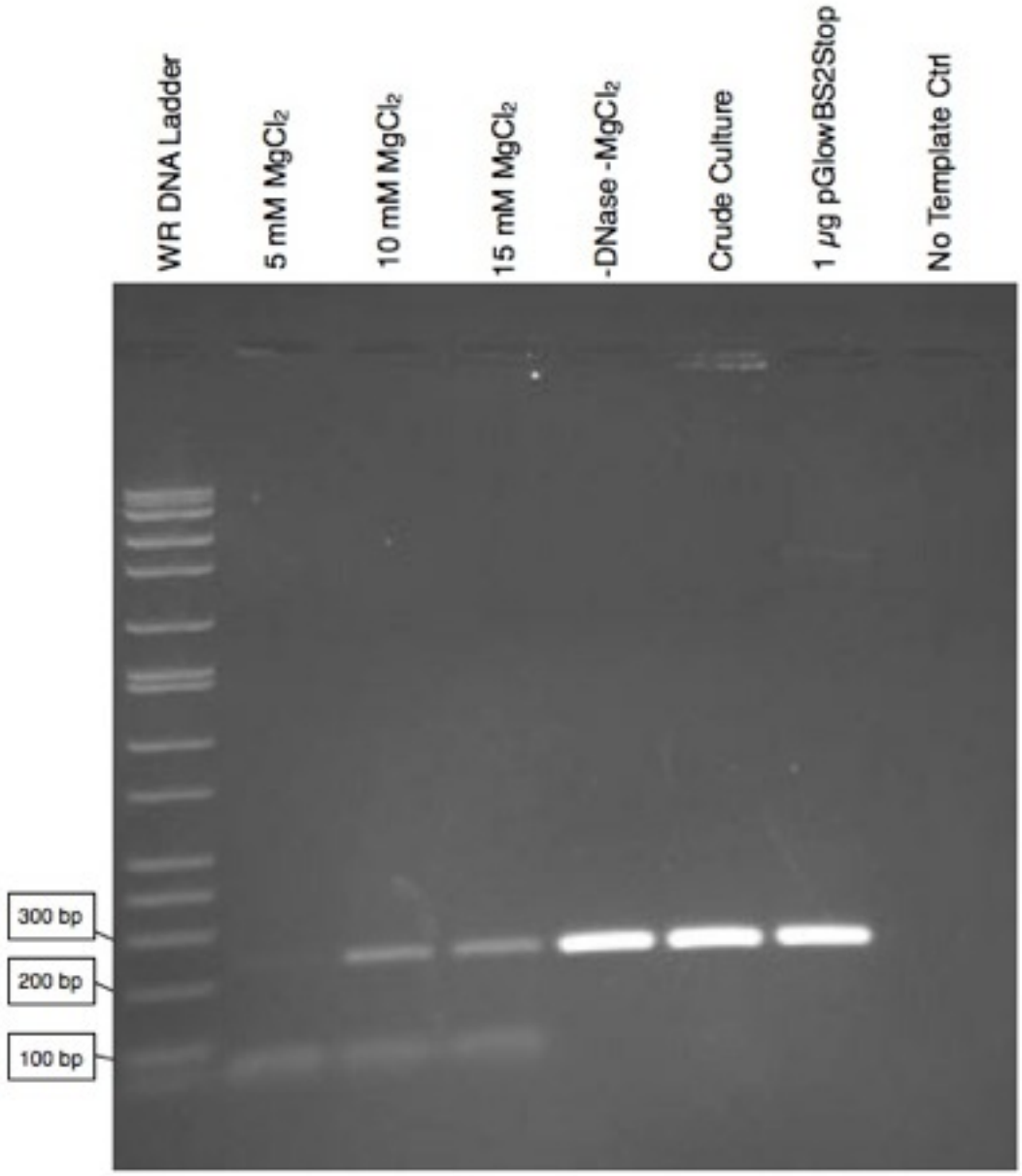


**Figure 9. Gel electrophoresis of the gfp amplicon PCR product after lysed *E. coli* culture after exposure to DNase I. These findings indicated that 5 mM $MgCl_2$ was optimal for DNase I activity, leaving observable amounts of template DNA to be amplified in the subsequent PCR. All reactions utilized the gfp primer set for PCR and compared metal ion gradients with crude *E. coli* K12 pGlowBS2Stop culture, no DNase I, purified pGlowBS2Stop plasmid, and no template controls to alleviate numerous affecting factors.**

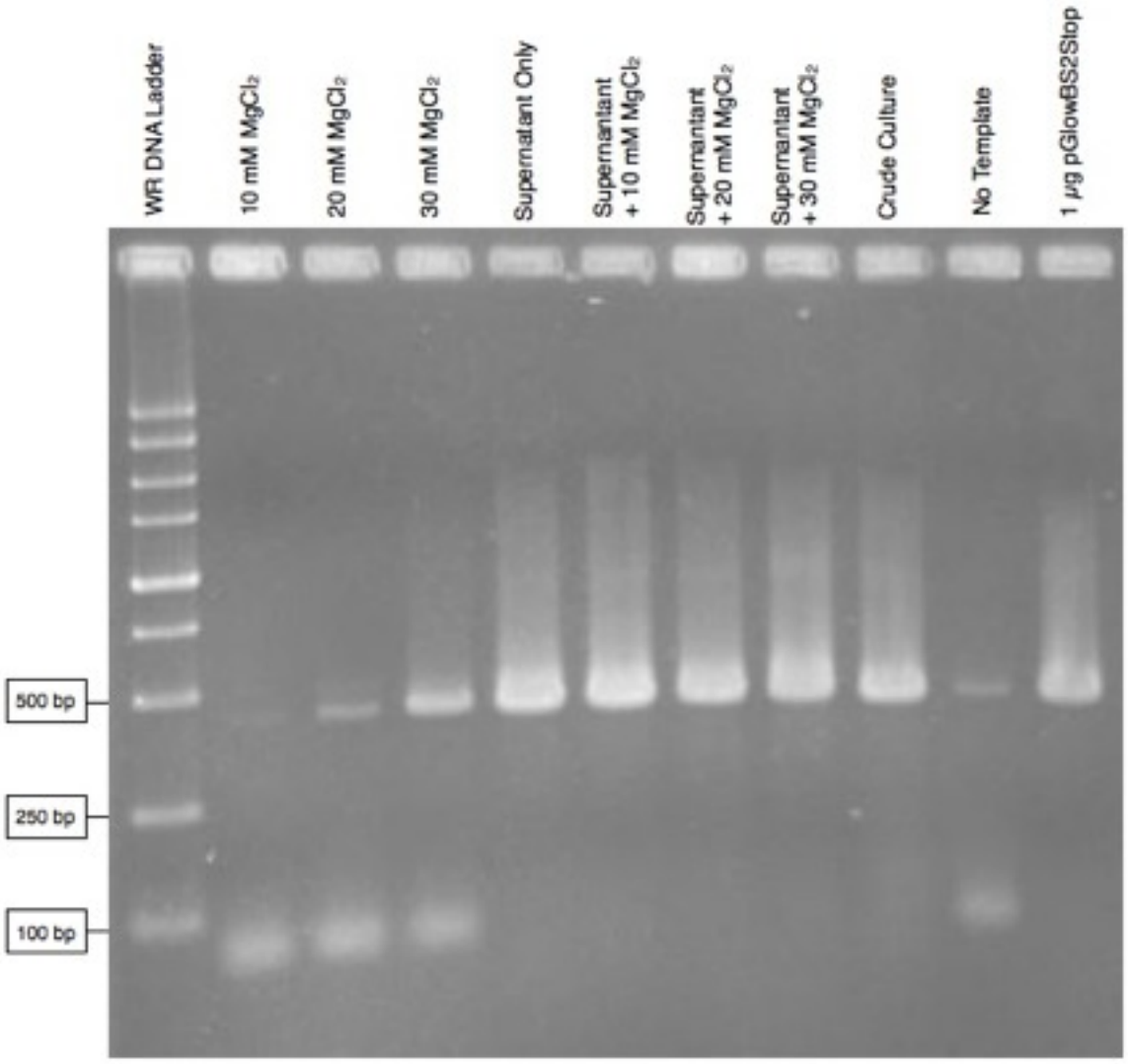


**Figure 10. Gel electrophoresis of the gfp amplicon PCR product after lysed host cell supernatant exposure to DNase I and varying concentrations of $MgCl_2$. The optimal concentration of $MgCl_2$ was shown to be at 10 mM, while higher concentrations were indistinguishable from the positive controls. Metal-only controls included in the PCR batch produced no observable interference in the polymerase reaction and positive controls indicated proper primer function.**

Realtime Quantitative Polymerase Chain Reaction

The results for quantifying horizontal gene transfer in batch culture controls indicated transduction frequency was measurable. The ampR indicator gene was found to be in equal quantities to the calculated number of phage genome copies in the reactions. The standard deviation for the phage genome quantities of $10^5$ was $2.6x10^4$, however, the measurements for phage genomes at quantities of $10^4$ were shown to have a much smaller standard deviation value at $5.8x10^3$. The QPCR results for the pGlow primer set indicated that the number of gene copies was slightly lower than the number of calculated phage genome copies. At phage genome numbers at $10^5$, the number of pGlow copies was shown to be $2.3x10^4$, with a standard deviation value of $1.4x10^3$. At phage genome copies of $10^4$, the number of pGlow gene copies was $1.5x10^3$, with a standard deviation of $1.2x10^2$. The third indicator gene used was gfp, which showed that the gene was measured at slightly lower levels than the phage genome. At phage genome copy numbers of $10^5$, gfp was shown to be quantified at levels of $1.1x10^4$, with a standard deviation of $1.4x10^3$. At phage genome numbers of $10^4$, gfp was measured to be $9.3x10^2$, with a standard deviation of $2.9x10^2$. The standard curves for the QPCR analyses indicated accurate quantification, with $r^2$ values all surpassing 0.994 (Figures 10-12).

| ampR | | | | |
|---|---|---|---|---|
| Template | C(T) | Copies | Average | Standard Deviation |
| T4 $10^5$ | 16 | 1.28 x $10^5$ | 1.46 x $10^5$ | 2.67 x $10^4$ |
| T4 $10^5$ | 16 | 1.30 x $10^5$ | | |
| T4 $10^5$ | 16 | 1.85 x $10^5$ | | |
| T4 $10^5$ | 16 | 1.40 x $10^5$ | | |
| T4 $10^4$ | 19 | 1.15 x $10^4$ | 1.64 x $10^4$ | 5.88 x $10^3$ |
| T4 $10^4$ | 19 | 1.15 x $10^4$ | | |
| T4 $10^4$ | 18 | 2.32 x $10^4$ | | |
| T4 $10^4$ | 19 | 1.95 x $10^4$ | | |

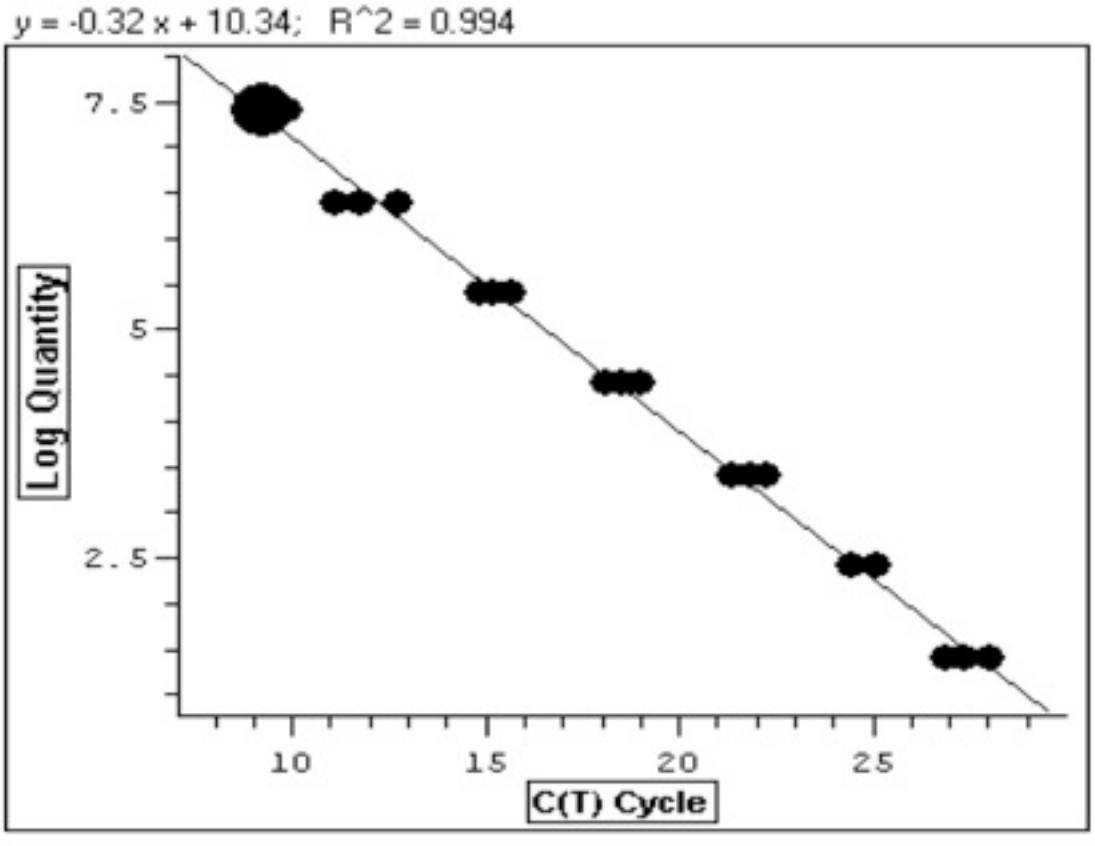


**Figure 11. QPCR data for ampicillin resistance gene (ampR) standards.**

| pGlow | | | | |
|---|---|---|---|---|
| Template | C(T) | Copies | Average | Standard Deviation |
| T4 $10^5$ | 19 | 2.44 x $10^4$ | 2.29 x $10^4$ | 1.44 x $10^3$ |
| T4 $10^5$ | 19 | 2.36 x $10^4$ | | |
| T4 $10^5$ | 19 | 2.24 x $10^4$ | | |
| T4 $10^5$ | 19 | 2.11 x $10^4$ | | |
| T4 $10^4$ | 23 | 1.47 x $10^3$ | 1.51 x $10^3$ | 1.20 x $10^2$ |
| T4 $10^4$ | 23 | 1.60 x $10^3$ | | |
| T4 $10^4$ | 23 | 1.60 x $10^3$ | | |
| T4 $10^4$ | 24 | 1.35 x $10^3$ | | |

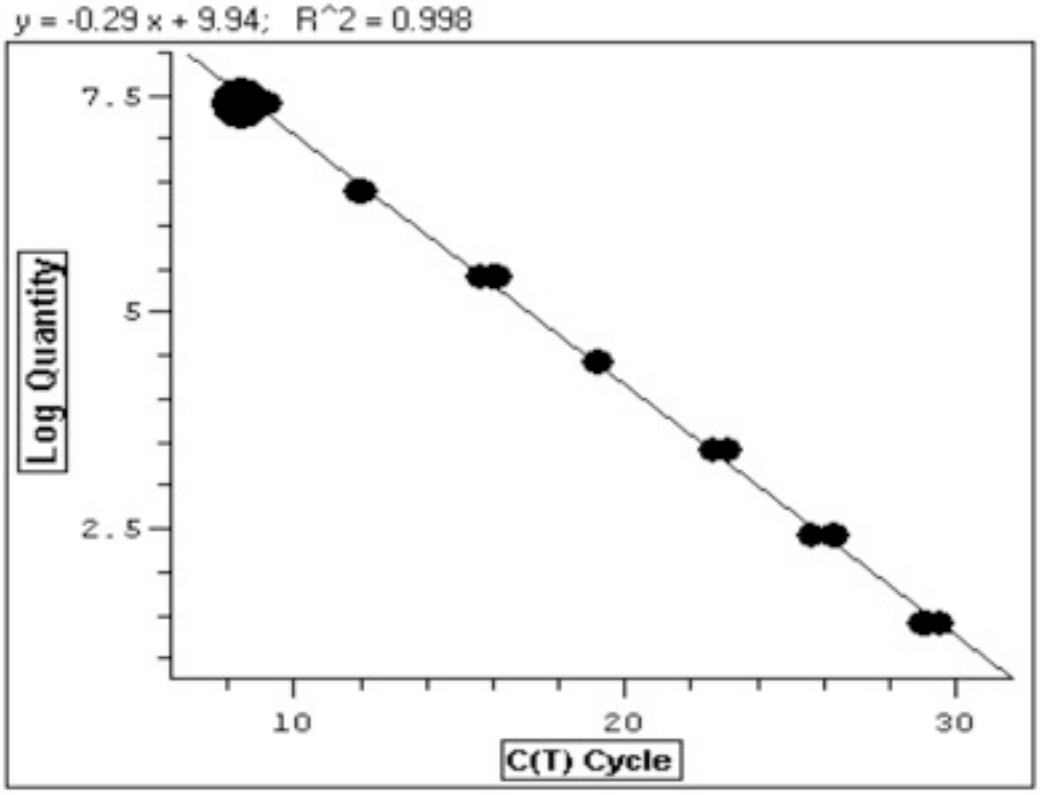


**Figure 12. QPCR data for evoglow gene (pGlow) standards.**

| gfp | | | | |
|---|---|---|---|---|
| Template | C(T) | Copies | Average | Standard Deviation |
| T4 $10^5$ | 18 | 9.78 x $10^3$ | 1.08 x $10^4$ | 1.41 x $10^3$ |
| T4 $10^5$ | 18 | 9.56 x $10^3$ | | |
| T4 $10^5$ | 18 | 1.12 x $10^4$ | | |
| T4 $10^5$ | 18 | 1.26 x $10^4$ | | |
| T4 $10^4$ | 21 | 1.23 x $10^3$ | 9.25 x $10^2$ | 2.88 x $10^2$ |
| T4 $10^4$ | 22 | 6.67 x $10^2$ | | |
| T4 $10^4$ | 22 | 6.91 x $10^2$ | | |
| T4 $10^4$ | 21 | 1.11 x $10^3$ | | |

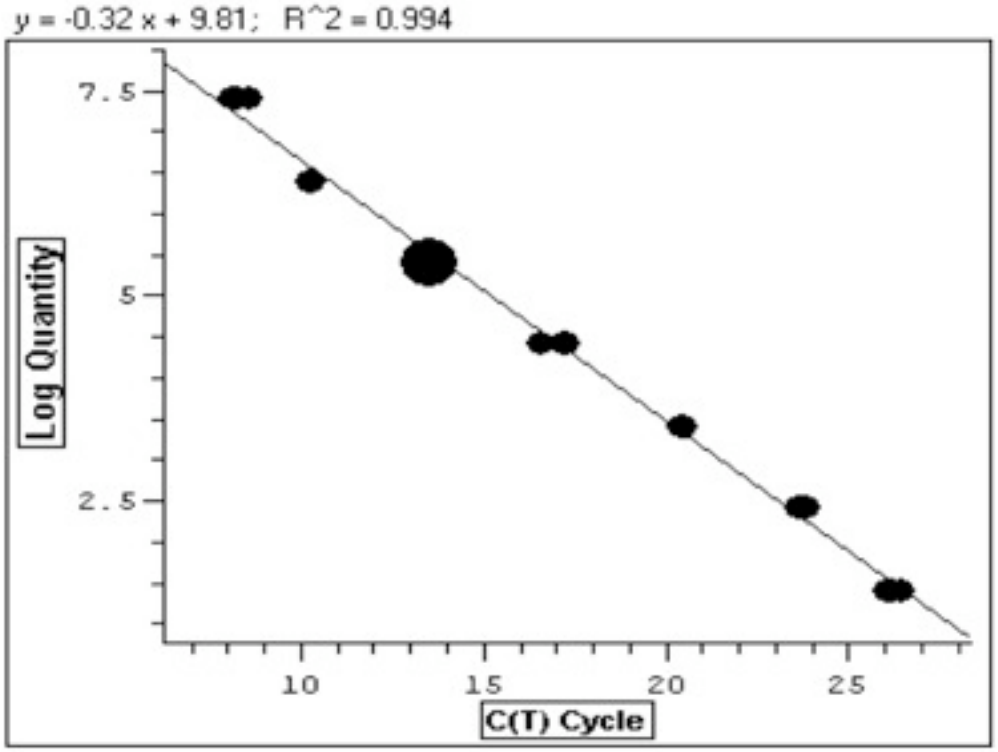


**Figure 13. QPCR data for fluorescent protein gene (gfp) standards.**

Streptavidin Paramagnetic Particle Recovery of Biotinylated Bacteriophage T4

The recovery efficiency for biotinylated phage from streptavidin paramagnetic particles was found to be highly inefficient. From a starting stock of biotinylated bacteriophage T4 at concentrations of $9x10^{10}$ pfu/mL, 1mg of Dynabeads© MyOne™ Streptavidin recovered $7x10^{7}$ pfu/mL with a standard deviation of $1x10^{8}$. Each of the four washes with PBS buffer was used in a plaque assay for quantifying phage contents in solution. The PBS wash buffer from the first wash procedure produced $6x10^{9}$ pfu/mL with standard deviation of $1x10^{9}$. The second wash with PBS contained $8x10^{8}$ pfu/mL and a standard deviation of $2x10^{8}$. PBS buffer from the third bead rinse produced $3x10^{8}$ pfu/mL with standard deviation of $1x10^{8}$. The final wash contained $7x10^{7}$ pfu/mL and a standard deviation of $1x10^{8}$. After exposure to 70°C $H_2O$ for five minutes, the streptavidin beads produced phage titers that were reduced by two orders of magnitude, indicating significant loss of phage particles during binding and washing the streptavidin-bound bacteriophage particles (Figure 13). An additional eight-wash experiment produced corresponding results in that each wash contained diminished quantities of bacteriophage particles for each plaque assay performed on the streptavidin bead supernatant. A minor increase was observed in the later washes (wash 8 and pre-heat wash), however, staying within one order of magnitude. The heat-released bacteriophage titer produced a final concentration of $2x10^{8}$ pfu/mL, an increase of one order of magnitude from the pre-heat solution, but a total loss of two orders of magnitude, or approximately 1% recovery, from the original phage stock (Figure 14).

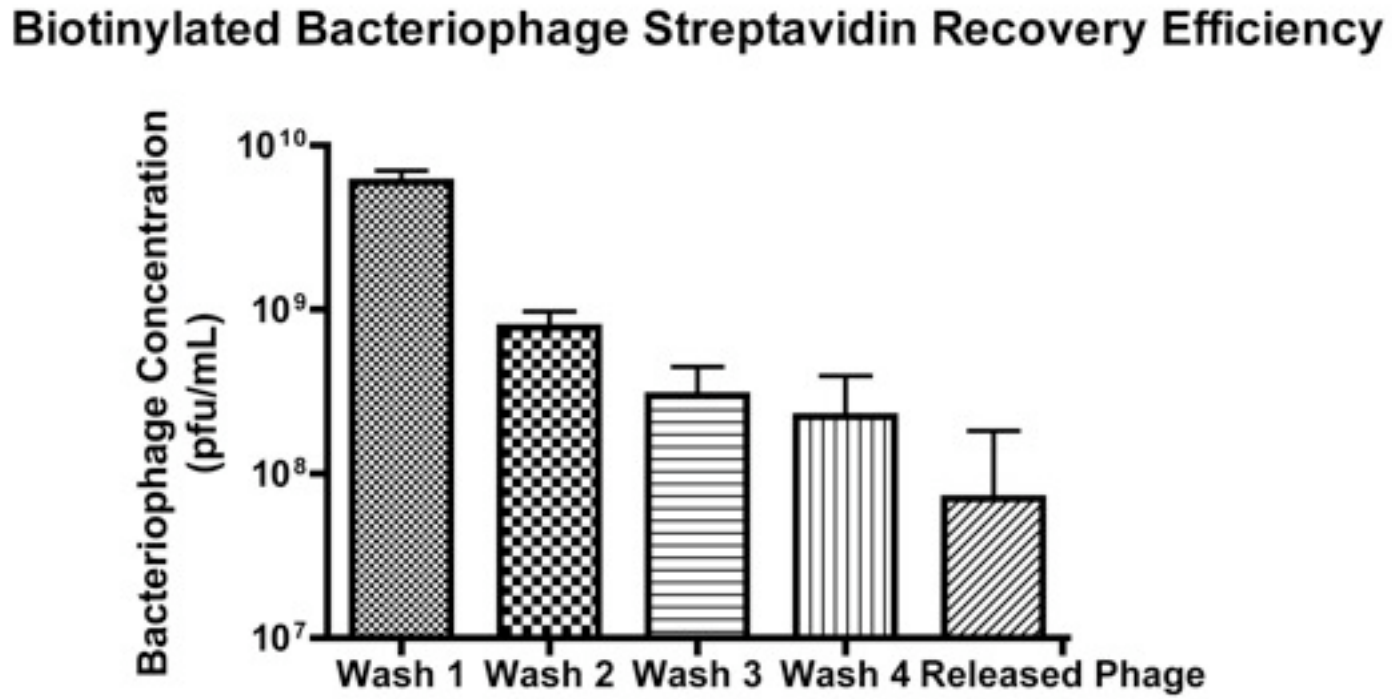


**Figure 14. Biotinylated bacteriophage titers of four washes after exposure to streptavidin particles and subsequent phage titer of heat-released bacteriophage solution.**

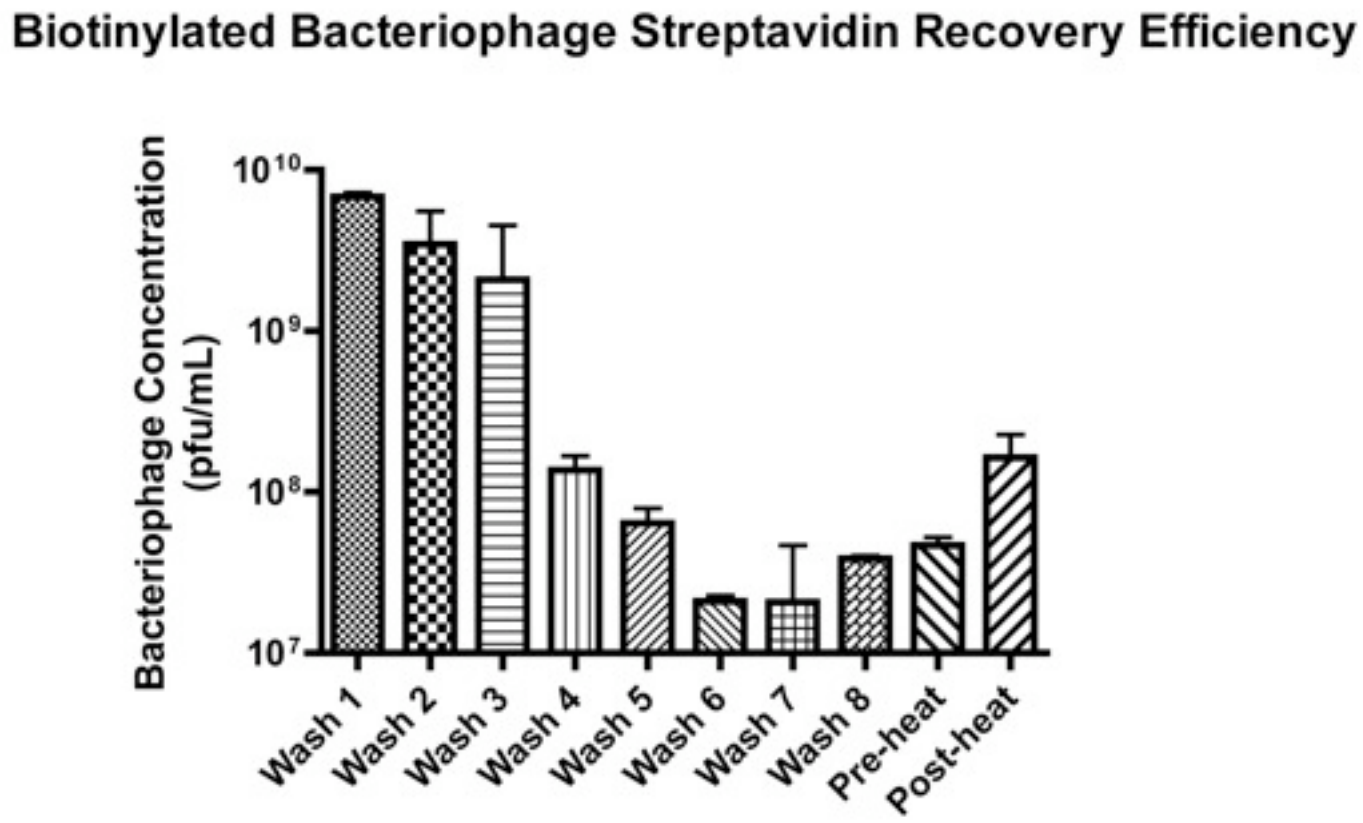


**Figure 15. Biotinylated bacteriophage titers of eight washes after exposure to streptavidin particles and final viable bacteriophage recovery efficiency after heat-induced release.**

Western Blot Analysis of Biotinylated Bacteriophage T4

The status of biotinylation for the bacteriophage T4 sample indicated no reaction to Neutravidin (+ horse radish peroxidase) as a result of Western blot analysis. The band representative of biotinylated BCCP bound to Neutravidin was not visible in the desired ~82-92 kDa region of the protein ladder, whereas the 30 $\mu$g of biotinylated T199 PBEC2 protein showed a very strong reaction. The volumes of bacteriophage samples loaded into the gel represent approximately $10^7$ pfu per lane. All temperature and exposure time denaturation steps for the biotinylated T4 and *wildtype* λ phage produced negative results. The T4 bacteriophage did not produce qualitative evidence that supports the hypothesis that it is biotinylated with regard to established avidin reactions (Figure 15).

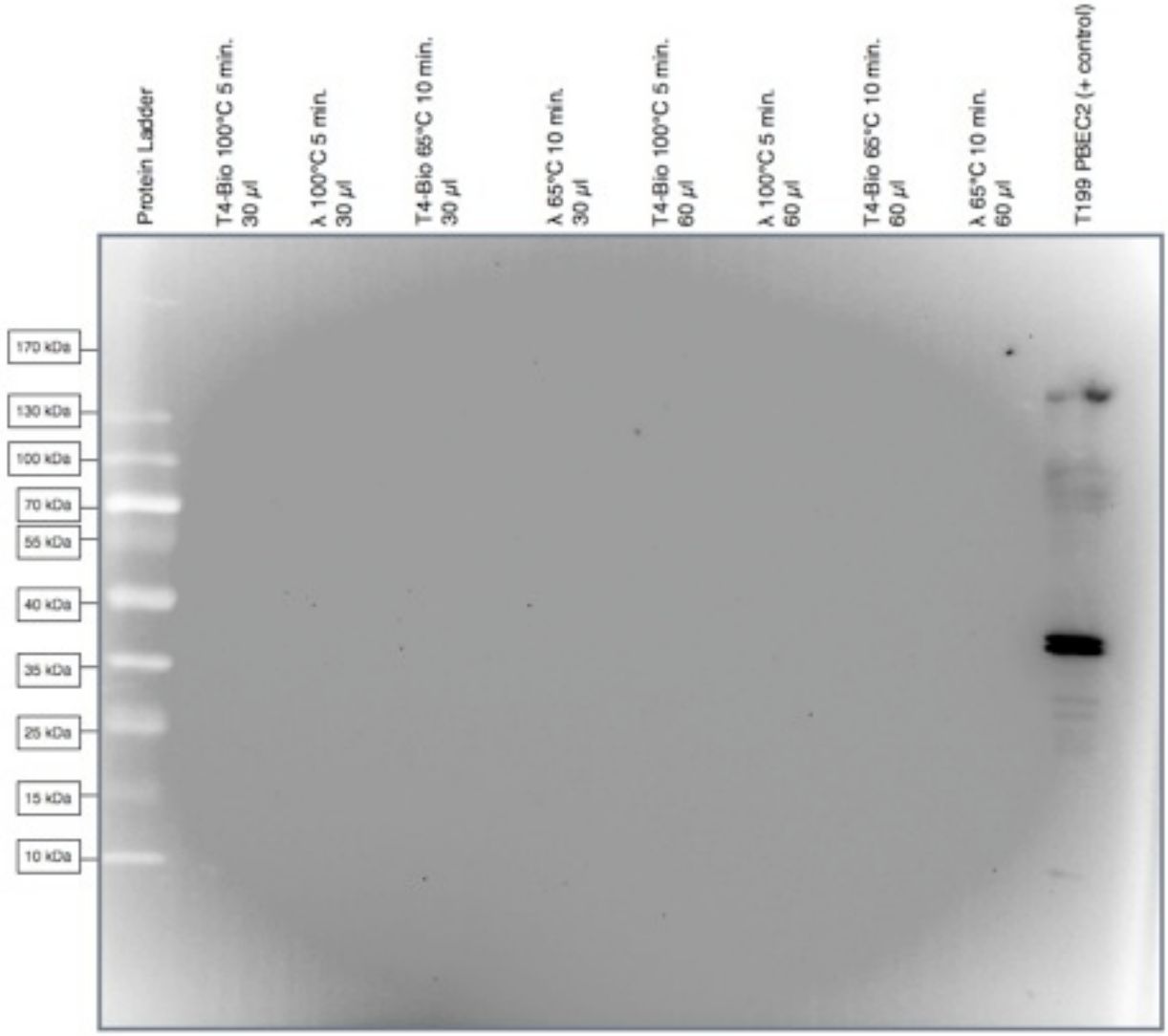


**Figure 16. Western blot of biotinylated bacteriophage T4 coupled with avidin homolog after heat-induced lysis of phage solutions. Parallel controls with *w.t.* λ phage (negative control) and T199 pBEC2 (positive control).**

## Discussion

The ability for biotinylated bacteriophage to act as biotechnological models for exploring their role in microbial community transduction events has been the focus of this work. The technical approaches used to exploit modified phage biophysical characteristics as a window into gene transfer risk assessment have been thoroughly examined and refined. This study has employed numerous molecular techniques in manipulating host genetics as well as genetic diagnostics to elucidate and confirm analytical progress toward the major goal of exploring the role of phage as gene transfer agents in natural systems. A comprehensive examination of bacteriophage propagation and purification methods has been undertaken during this work, which resulted in various modifications central to the advancement of this technology. The information produced from these experimental conglomerations provides a framework for a deeper understanding of bacteriophage as instruments of evolutionary and genetic analyses.

The host bacterium, *E. coli* K12-12017, was used as a model host system for the biotinylated bacteriophage T4. This host *E. coli* system allowed for the successful electroporation of a plasmid, pGlowBS2Stop, which contained three discrete marker genes that were dually used as confirmation of successful molecular protocols and as initial indicators for calibrating quantitative genetic analyses on phage genomic DNA. Additionally, targeting genes located on plasmid DNA within the host bacterium held potential for deciphering the transduction rates of genes distinct from the transduction events derived from host genomic DNA. The pGlowBS2 STOP plasmid is produced in high copy numbers within the host bacteria, thus providing a parallel for high copy

number plasmids found in natural ecosystems. This feature of the plasmid being in higher copy numbers than the genome theoretically allows for an increased probability of plasmid-derived transduction event occurrences. While the increased copies of plasmid-based DNA may allow for an increased likelihood of transduction, studies have shown that the transduction rate discrepancies between genomic DNA and plasmid DNA in marine environments is negligible (Jiang, 1998). Standard electroporation, plasmid DNA purification, and polymerase chain reaction (PCR) techniques were effective for characterizing the modified host bacterial strain as well as genetic verification of the biotinylated bacteriophage T4 sample. All PCR primer sets produced substantial evidence for the observable presence of the genes of interest through gel electrophoresis of PCR products, whether in the bacteriophage or host bacterium (Figures 3-5). As numerous studies have concluded, amplification of PCR products does not provide definitive evidence for the presence of functional genes, however, the *E. coli* possessing pGlowBS2Stop did exhibit sufficient ampicillin resistance to allow for bacterial culture growth to occur in selective media. The PCR evidence coupled with the functionality of the ampicillin resistance gene present on the electroporated plasmid supports the accuracy of PCR products as indicators of gene fidelity in the host bacteria. While no selective chemical agents were applied directly to bacteriophage cultures, the wild-type T4 *e* lysozyme amplicon was successfully PCR amplified. The successful amplification of a fragment of the T4 lysozyme gene coupled with successful bacterial host lysis provides evidence that the bacteriophage strain under examination effectively utilizes lysozyme to lyse host cells. While the amplification of the T4

lysozyme gene supported the hypothetical presence of the enzyme in its functional form, it has been shown that T4 holin gene can act as a substitute for bacterial lysis during T4 infection under special circumstances (Kao, 1980). The fidelity of the BCCP gene is contingent upon the aforementioned logic applied to PCR's ability to act as a successful indicator of the presence of a functional gene. The interpretation of these initial molecular results indicate the limitations of using PCR as a tool for genetic analysis and provides conceptual arguments for how the reliability of the results of the reaction can be improved upon. However, the PCR results produced in this study provided positive genetic evidence for the presence of these genes in the bacteriophage under investigation.

The multiplicity of infection (MOI) is the ratio of infectious phage particles to host cell targets and can vary considerably between phage and host species. Understanding host growth dynamics under defined conditions is critical to observing optimal MOI's for a corresponding phage. The elucidation of a relationship between the growth state of the host culture and its measurable optical density during incremental times of incubation provides a means to define the optimal conditions for desired phage infection. As each batch experiment varies due to deviations in growth medium components and incubation conditions, related scientific literature only provides a framework for designing such an experiment. A thorough investigation included defining the growth state of the host cell culture by comparing viable cell count assays with defined *E. coli* K12 growth properties (Sesenov, 2007). The optical density measurements were used to define a relationship between incubation conditions and

the growth state of the host cell culture. This experimental approach produced positive results for determining the ideal growth state of the host cell culture for phage propagation. The desired MOI was determined through monitoring host cell culture optical density for decreasing turbidity, thus indicating cellular lysis. The successful propagation of phage was semi-quantitatively analyzed through plaque assays to determine fluctuations in infectious particle numbers. This system for determining the ideal growth phase of the host cell culture through optical density measurements and the subsequent phage inoculation produced favorable phage-induced lysates. The applied MOI of 0.5 likely resulted in superinfection of some host cells, which has been shown to not only delay lysis, but increase the number of progeny phage released indicated by larger burst sizes (Bode, 1967). Not only did this approach indicate an effective method for propagating the viral particles specific to this study, but provided positive evidence for the efficiency with which the recombinant bacteriophage T4 can infect the experimental host *E. coli* K12 model under specifically defined incubation conditions.

Deciphering the most efficient and effective means with which to concentrate and purify the BCCP-fused bacteriophage T4 was a central component to this study (Figure 3). Historical published methods for virus purification were extensively explored with emphases on cesium chloride density gradients, polyethylene glycol precipitations, ultracentrifugation, physical filtration, and endonuclease activity. This work has examined these experimental techniques as isolated means for viral purification as well as their combinatory effects.

The principal concept of the cesium chloride (CsCl) density gradient is to isolate viable, highly concentrated, host nucleic acid-free phage samples by targeting phage buoyant densities through isopycnic centrifugation (Yamamoto, 1970). This technical approach is commonly reliant on the tendency of phage to congregate at the interface of two-phase systems employing the polyether compound, polyethylene glycol (PEG), and either high salt (NaCl) concentrations or dextran sulfate additions (Bachrach, 1971; Vajda, 1978). In addition to CsCl density gradients and PEG precipitations, the endonuclease, DNase I, was integrated into this study to alleviate residual host DNA contaminants. Due to the inefficiencies produced by these methods in combination with one another, this study systematically isolated each technique to determine avenues for streamlining and increasing efficiency. Cesium chloride gradients provided an inefficient and unreliable approach for purifying the subject bacteriophage in the laboratory. The observed fluctuations and bacterial contamination provided evidence for the delicate nature of this protocol. The mechanics of overlaying CsCl concentration gradients provided numerous opportunities for mixing, thus robbing the gradient of its density-based concentrating capabilities. Additionally, the transfer of collected phage bands among ultracentrifuge tubes, as required by the speed limitations of the tubes used for isopycnic ultracentrifugation, and the means of collecting the final phage band from the ultracentrifuge tube through penetration with a syringe needle had the potential to expose the samples to gradient perturbations and possible biological contaminants. Thus, the established methods utilizing CsCl density gradients and isopycnic

centrifugations were deemed to be rife with unsanitary conditions and subsequently abandoned as a means to purify bacteriophage particles for this work.

The two-phase precipitation system provided satisfactory results in the quest for an effectively aseptic mechanism for concentrating phage. This system allowed for every constituent to be sterilized with virtually no exposure to biological contaminants other than the phage and lysed bacterial host culture. When applied under the previously described conditions, PEG and dextran sulfate exhibited the most advantageous route to bacteriophage concentration. While the theoretical lack of purification properties for this technique are well founded, its concentrating effectiveness was easily supplemented with other purification processes. The optimization and development of the use of an endonuclease provided an effective measure of compensating for the lacking purification properties of PEG concentration. An assay was designed to test the efficiency of DNase I as a means to degrade bacterial DNA in phage solution. An essential tenet of this application, and subsequent optimization effort, was founded on the observation that DNase I was considered a metal-activated enzyme, specifically enhanced at specific metal ion concentrations (Eichhorn, 1969). The results of this study showed evidence that magnesium chloride ($MgCl_2$) ions at concentrations of 10 mM activated enzyme activity to a degree of effectiveness that resulting solutions produced no observable PCR amplification of target genes contained in the pre-endonuclease treated samples. Due to the specificity of DNase I activity on polynucleotides, phage capsid proteins provide ample protection from enzymatic degradation of phage genetic material. The requirement of metal ions

at specific concentrations is of particular significance to the use of endonucleases in phage purification processes. Coupled with PEG precipitation and physical filtration, the findings of this technical application provided a useful method for concentrating and purifying bacteriophage samples.

The QPCR analysis performed in this study provided an ample framework for the use of this technology as a tool for enumerating phage-mediated transduction events in a natural ecosystem. The significance of these QPCR findings should be understood as preliminary standards with an indication of positive evidence supporting hypotheses pertaining to transduction activity in bacteriophage T4 communities. An evaluation of these QPCR standards unveiled an exploratory relationship between the amplified marker genes derived from the host *E. coli* K12 plasmid (e.g. ampR) and the calculated phage genome numbers, which suggested evidence for phage uptake of host gene sequences in batch culture bench experiments. While it should be understood that these data primarily provide a source of comparison for samples of unknown gene copy numbers, it can be concluded that the prepared components of the QPCR protocol performed adequately and that future applications would provide more definitive evidence regarding the efficacy of this technique for purposes related to transduction event quantification. Indirect measurements of phage genome numbers were calculated in this study based on phage genome size, molecular weight, and DNA concentrations. These variables can differ within phage populations due to genomic variations produced from transduction events and contaminating DNA, thus skewing the projected number of phage genomes based on these values. Additionally, amplifying

only a portion of a maker gene through QPCR does not provide definitive evidence of successful transduction of a functional gene, indicating the significance of amplifying entire genes through this approach. The application of DNase I as a means to rid the phage particles of contaminating host DNA required extensive optimization for this study and the possibility of incomplete degradation of contaminating DNA should be noted. Furthermore, the plasmid containing the marker genes used for this study was produced in the host bacterium in high copy numbers. This plasmid characteristic likely led to phage transducing DNA fragments derived from the high copy number plasmid more often than the host genome, due to the increased number of copies of the marker genes. QPCR analysis targeting genes located on the host bacterial genome would provide data specific to transduction rates associated with genomic DNA. The QPCR data produced from this study provides evidence for transduction rates related to plasmid DNA, which also play a critical role in natural ecosystems. While the application of QPCR technology suggests further utility in the interest of measuring phage-mediated transduction events in the context of this study, the technical limitations should be carefully examined in order to avoid dissociated or convoluted evidence (Smith, 2008).

The interactions between biotin and streptavidin were a central component of this investigation. Streptavidin-coated paramagnetic particles were used in an attempt to recover biotinylated bacteriophage particles from solution, while excluding viruses or other biological entities that did not possess conjugated biotin. The hypothetical concentrating effects of this assay did not produce the desired results of recovering high

titers of biotinylated phage. A significant loss of phage numbers was observed after following the manufacturer's treatment protocol. The plaque assays performed on supernatants from each wash step provided convincing evidence for when the phage losses occurred. These observations could be a result of high levels of free biotin in solution decreasing the efficiency of the streptavidin beads by occupying available binding sites, thereby competing with biotinylated phage for access to the sites. The established protocol of breaking the biotin-streptavidin bond through elevated temperatures (70°C) in water was shown to produce only slight increases in phage titers after an increased number of wash steps, indicating little effect on the final released phage quantities. While this concept has been shown to produce repeatable results for a multitude of biotinylated molecules, evidence for the sustained viability of bacteriophage after this treatment has not been explored (Holmberg, 2005). The decreased numbers produced by the plaque assays performed after the heat treatment may be the result of heat-inactivation of the phage or negative osmotic effects produced from the phage transfer to deionized water, however, the significant quantities of phage released during the washing steps supports the hypothesis that the phage were not efficiently bound to the streptavidin beads. The combinatory effects of phage particles being exposed to washing solutions (PBS buffer), elevated temperatures, and the inherent osmotic changes inherent in water resuspension could contribute to a decrease in phage viability as measured by plaque assays. Thus, the potential cleavage of the biotin-streptavidin bond through elevated temperatures in water should be further

examined in the context of how these parameters can effect the viability of bacteriophage.

The exploration of measuring streptavidin recovery efficiency of the biotinylated phage was complimented by another avenue of inquiry, based on confirming the fidelity of BCCP functionality in the recombinant phage through Western blot analysis. As the proposed streptavidin paramagnetic bead assay suggested undesirable phage recovery, Western blot analysis provided an alternative window into clarifying the functional state of BCCP through detecting its reaction with an avidin homolog (Neutravidin) acting as the primary antibody equivalent. The results of this analytical survey strongly suggested the deactivation of BCCP as a functional protein in the bacteriophage stock samples. While the phage titers used in this assay were measured to be approximately 1 x $10^{10}$ pfu/ml, the quantification assay was biased for viable phage particles, thereby not taking any immature or dysfunctional phage into account. Therefore, the titer values described in this assay should be considered an underestimation of actual phage particles present in solution. Additionally, the targeted protein, BCCP, was previously confirmed to be fused to the small outer capsid (SOC) protein (Tolba, 2009). This detail, regarding the protein to which BCCP was fused (SOC), provides further evidence that the actual quantities of viable phage is a gross underrepresentation of BCCP presence because between 810 and 960 copies of SOC are present in T4 phage capsids (Leiman, 2003; Baschong, 1991). Therefore, it could be estimated that up to 960 BCCP molecules were present for each bacteriophage particle, thus providing further evidence to support the interpretation of these results as

indicating the loss of functionality of BCCP on the bacteriophage capsid. Due to the genetic evidence supporting the presence of the BCCP gene in phage samples, a structural deviation within the target protein is a probable cause of these negative Western blot results. The development and application of a BCCP-specific antibody could illustrate the presence of BCCP as an immunoblotting technique to circumvent the biotin-streptavidin binding utilized in this study. This approach would not be reliant on the functionality of BCCP and host enzymes for biotinylation, but would be indicative of an immunoblotting reaction to the protein itself, thereby providing details as to where the biotinylation process malfunctioned. Additionally, replicating the previously described immunoblotting procedure used in this study with the host *E. coli* K12 strain would provide evidence as to whether the host BCCP and related biotinylating enzymes were functional within the host bacterium without phage infection. Due to the phage reliance on host biotinylating capabilities, the functional status of the host biotinylation machinery could provide additional evidence as to how infecting phage biotinylation failed.

The intrinsic malleability of any biological entity, even within a closed system, plays a crucial role in understanding the results of this investigation. It is difficult to determine the factor responsible for a mutation event or alteration of protein structure within any living organism, however, extrapolations can be made as to what may have contributed to the loss of phage biotinylation. While a single, original phage stock was maintained throughout this study, the optimization of phage propagation and purification techniques may have subjected the phage samples to unintended evolutionary pressures. For example, during batch growth experiments for phage propagation,

biotinylated phage may have been at a slight evolutionary disadvantage due to minor increases in protein synthesis requirements related to BCCP production. This minor extra protein synthesis burden on the BCCP-possessing phage may have been significant enough to produce a selective advantage in any progeny phage that had a more efficient version, albeit dysfunctional, of the biotin-binding protein. Additionally, SOC, which was the capsid protein that was fused with BCCP, is not required for bacteriophage T4 survival and propagation (Comeau, 2008). The evolutionary advantages that SOC provides the phage include improved capsid stability from osmotic shock, alkaline pH, and temperature denaturation. However, the growth media that were used for phage propagation in this study did not possess any of these environmental challenges. The absence of these factors could produce a selective advantage for phage that had relinquished their replication burden of SOC production due to its nonessential nature, thus making BCCP expression on the phage capsid unsuccessful. This scenario could be further compounded by any other selective advantage that the absence of SOC on phage capsids may have presented due to the rapid and efficiency-oriented goal of optimizing the multiplicity of infection assay. The primary factor that was selected for in the phage propagation assays was the ability for phage to lyse bacterial host cultures effectively, thereby selecting for phage that were highly efficient with regard to replication and lysis.

This work presents a distinct repertoire of analytical techniques that can benefit further investigations into the role of bacteriophage in horizontal gene transfer events. While numerous investigations have used biotinylation technology as a means to label a

variety of biomolecules, the limitations of biotinylating living microbes is less common and understood to a lesser degree. The findings presented by this exploration into phage utility as a practical subject of biotinylation aids in understanding the deficiencies of this approach to genetic engineering. Further success of this investigation may include isolating evolutionary successful bacteriophage from an environment in which the study is to be performed, thereby providing some alleviation to the selective pressures applied to a recombinant phage. While selecting a reproductively successful phage from the environment that is under analysis may contribute to increased success with this biotinylation application, alternative sites of BCCP fusion should also be considered due to the dispensability of SOC in bacteriophage T4. The intrusiveness and evolutionary effects of fusing BCCP to the SOC of bacteriophage has not been fully investigated, therefore, additional bacteriophage tagging mechanisms can be explored to enable a further understanding of phage-mediated transduction events in natural systems. The results of this investigation have provided additional evidence supporting the investigator's intellectual tendency to refrain from attempting to alter biological entities without considering the evolutionary repercussions of producing a selectively disadvantaged organism that is expected to maintain genetic integrity. Genetic analysis of any DNA-based organism must avoid the myopic approach of trivializing the importance of environmental factors on human-based engineering efforts on all life forms, even in theoretically controlled experiments. Understanding and developing genetic tools for furthering biological discoveries can only be properly exercised if the

subject environment is thoroughly understood, which has been properly exemplified in this work.

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# Vita

Thomas Mead was born on September 18, 1984 in Memphis, Tennessee. He graduated from Powell High School in Knoxville, Tennessee in 2002. In 2007, Thomas graduated from the University of Tennessee, Knoxville, with a B.S. in Biological Sciences with a concentration in Ecology and Evolutionary Biology. He began work under Dr. Gary Sayler and Dr. Jennifer Debruyn at the Center for Biotechnology as an undergraduate at the University of Tennessee, Knoxville in 2007. He then began studying for a Master of Science degree in Microbiology under Dr. Gary Sayler and Dr. Steven Ripp in 2009.