BIOENGINEERING BACTERIOPHAGE FOR THE SENSITIVE & RAPID DETECTION OF BACTERIA IN WATER

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ABSTRACT

Access to sanitary drinking water is a fundamental human right required for the continued prosperity of humanity. As a result, the World Health Organization (WHO) has published water safety recommendations stating that drinking water supplies should contain no detectable coliforms in 100 mL of water. Finding a single bacterial colony forming unit in that volume is akin to finding a single grapefruit dropped into Lake Erie, a size difference of approximately 15 orders of magnitude. Only traditional culture techniques are capable of such detection limits, and all require at least 24 to 48 hours. The United Nations Children's Fund (UNICEF) has declared the need for detection devices to accomplish the abovementioned detection limits in much shorter time frames. In response, we developed a series of bacteriophage-based biosensors with

detection limits that approach and achieve the aforementioned detection requirements in fractions of the time required for traditional culture techniques. Our most rapid assay is capable of detecting 10-20 CFU of *E. coli* in 100 mL of drinking water in only 5 hours. Our fully quantitative assay only takes 12 hours and is directly compared to established methods. The sensitivity of our rapid bacteriophage-based detection technology is the result of multiple technologies leveraged together. We created novel reporter enzymes using genetic fusions between highly active enzymes and an affinity binding motif that irreversibly binds to cellulose. These constructs were inserted into phage genomes which served as sensitive biorecognition agents in drinking water detection assays. The novel chimeric reporters specifically bound to the cellulosic filters used in the detection assays, allowing for low detection limits in fractions of the time required for standard methods.

BIOGRAPHICAL SKETCH

Troy Cameron Hinkley, son to Lewis & Dixie Hinkley, grew up on a dirt road in Springville Pennsylvania with two brothers, Kyle & Logan. In 2006, Troy achieved the rank of Eagle Scout with a project that included building a shed for the community church where the Troop held meetings.

In 2007, Troy matriculated in the top five in his class from Elk Lake High School and began studying Chemistry at Wilkes University in Wilkes-Barre Pennsylvania.

In 2011, he graduated with a B.S. in Chemistry and worked in industry two years before deciding on graduate school.

In the Fall of 2013, Troy started a master's program in food science at the University of Massachusetts Amherst. After earning his master's degree under Amanda Kinchla he began work with Sam Nugen in the Nugen Research Group the next fall.

To my family:

I cannot thank you enough for your love, support & encouragement. Without you, none of this work would have been remotely possible. I love you.

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PREFACE

The work contained herein encompasses my work in creating recombinant bacteriophages for bacterial detection. The nomenclature used for the phages described herein has changed from the initial names used in Chapters 2 & 3 to a more systematic naming convention for Chapters 4 & 5. The final naming convention for recombinant phages will remain as NRGp# representing “Nugen Research Group phage” numbered chronologically.

CHAPTER 1

FINDING A GRAPEFRUIT IN A GREAT LAKE: SENSITIVE DETECTION OF BACTERIA IN LARGE WATER SAMPLES USING BACTERIOPHAGE-BASED BIOSENSORS*

*Troy C. Hinkley, Randy Woroboro, Sam R. Nugen

INTRODUCTION

While safe food and water is essential for life, millions throughout the world do not have reliable access to reliable sources. Clean drinking water is a fundamental human right essential for health and well-being.[1] Food and waterborne illnesses affect all populations and all demographics. The CDC estimates that in the United States alone, 48 million people fall victim to foodborne illness each year.[2] That statistic represents an annual cost-of-illness (medical, loss of productivity, mortality) of approximately $51 billion.[3] The global foodborne illness is even more grim with an estimated 600M illnesses and 420,000 deaths each year.[4] While clean water has been determined a fundamental human right, the morbidity and mortality associated with unsafe water is even more alarming. The World Health Organization estimated that in 2016, 829,000 deaths occurred from diarrhea as a result of poor water, sanitation and hygiene.

The importance of safe water for the safety of food has been outlined in the Food Safety Modernization Act (FSMA) which mandates periodic testing of water use to irrigate and rinse produce.[5] Unclean water has been linked to contaminated produce which represents

the largest share for commodities responsible for foodborne illness.[6] Improvements in the ability to rapidly detect food and waterborne pathogens will help reduce the burden of these illnesses. There has been tremendous progress in rapid detection through the development of improved molecular diagnostics, microfluidics/μTAS, and transduction methods. Additionally, there has been an increase in the number of research reports aiming to detect bacteria from food and water.[7, 8] Hopefully these technologies will bring better assurances those in low resource settings and lower the incidences of these burdensome diseases. This review aims at helping assay developers in recognizing the true bottlenecks in developing rapid assays for food and water. Because many of these challenges are imposed by regulatory requirements, we have summarized many of them here. By understanding these requirements, developers can design their assays to fit the necessary constraints, in the goal of more pragmatic and deployable assay design for the rapid detection of bacteria in food and water. This critical review aims not to discourage assay developers from targeting food and water matrices, but inform them of the constraints

so that the assays developed are pragmatic and much closer to commercial deployment. The statistics provided earlier highlight the dismal situation many around the world face and provides stark motivation for the immediate need for rapid and reliable detection in bacteria food and water samples.

WHO Guidelines

The World Health Organization (WHO) establishes many global guidelines for human health and places a significant importance on effective sanitation of drinking water.[9]. Together with the Organization for Economic Co-operation and Development (OECD) and the International Water Association (IWA), the WHO publishes a document titled “Guidelines for Drinking-Water Quality” as a set of regulatory recommendations.[10, 11] These guidelines are intended to serve as a framework for which local governments and municipalities can base their individual drinking water legislation while still overcoming the numerous challenges present in the delivery of clean drinking water to the local populace. The specifics of this effort are detailed in a Water Safety Plan (WSP),

documentation that focuses on comprehensive risk mitigation of all parts of the water supply, from initial collection to consumer.[12, 13]

As more nations adopt the WHO guidelines into official standards, the design of novel devices must achieve single CFU detection to realize any level of efficacy in real world applications. This review serves to outline the necessary goals for the design of novel detection devices.

As nations where the majority of the world's population lives transform the WHO international guidelines into national standards, the demand for detection devices capable of detecting the presence of a single *E. coli* colony forming unit (CFU) in 100 mL will only increase. The WHO guidelines that are increasingly being adopted across the world stipulate a zero-tolerance policy for coliforms in drinking water. Practically speaking, these guidelines require a detection device to reproducibly differentiate between the presence and absence of a single coliform CFU in 100 mL of drinking water. While this challenge is significant, the potential impact the implementation of clean drinking water systems is extraordinary. While there is still a long list of countries around the world with

varying requirements for microbial contamination in drinking water, most countries are trending towards adoption of zero tolerance policies. It is extremely unlikely that a responsible legislative body would relax microbial contaminant standards.

Regulatory limits

Governments around the world have tasked regulatory agencies to meet the aforementioned testing requirements recommended by the WHO water safety guidelines. These guidelines detail testing requirements with regard to frequency, performance, and bacterial limits. In order to establish reproducibly, these regulations set the limits for both bacteria concentrations and sample size. The latter is often the largest obstacle for rapid testing in food and water 100 mL of sample are typically required for water samples. For example, a biosensor that meets these standards must be capable of detecting the presence or absence of a single CFU in a 100 mL drinking water sample with reproducibility.

Challenges of single CFU detection

The physical dimensions of a single *Escherichia coli* cell (diameter 0.5-1.0 um, length 1-3um) equate its volume to be approximately

one femtoliter or 10^{-12} mL.[14] When that single *E. coli* cell is dropped into a 100 mL water sample, it is approximately 15 orders of magnitude smaller than the sample and comprises just 0.0000000000004% of the available space. With all things being equal, a standard bowling ball dropped into Lake Erie would be easier to find. The motivation behind this requirement is that bacteria reproduce asexually through binary fission and can exponentially increase in biomass. Unlike inorganic contaminants like mercury, arsenic, or lead, a single bacterium can quickly increase in concentration provided favorable environmental conditions. In a relatively short period of time, a single bacterial cell can quickly divide into a population of many billion with most descendants being a near identical genetic copy of the original cell. If that original bacteria possessed antibiotic resistance and/or human virulence factors, its proliferation and persistence through water treatment systems presents a major public health concern.

As a result, many legislation bodies have adopted presence/absence criteria for indicators microorganisms as recommended by the WHO[15]. The rationale for this decision

surrounds the fact that the complete absence of bacterial contamination is the only way to ensure future nonproliferation of pathogens.

Indicators

In many cases, the probability of finding a pathogen, even in a contaminated lot, is low. Therefore, rather than searching for the needle in the haystack, a common approach is to identify other analytes which serve as indicators of contamination. The selection of the indicators can be controversial, as none have proven to be perfect. A good example is drinking water, where rather than looking for all of the pathogens which could be in the sample, a search for "generic *E. coli*", which may or may not be a pathogens, could indicate fecal contamination, and therefore the potential of pathogen contamination. Similarly, *Listeria* spp., which contains both pathogenic and non-pathogenic species is a common analyte as opposed to searching for just the pathogenic *Listeria monocytogenes*. The presence of the genus *Listeria*, indicates that contaminating sources and growth conditions exist and that there is a potential of *Listeria monocytogenes* growth.[16] From the point of

view of the food producer, a positive test for an indicator organism *does not* mean that there has been a positive pathogen test, which may require reporting to regulatory agencies. It however, does mean that some form of remediation is required as the risk for pathogen contamination increases. Even though modifications to standard procedures have been developed for the specific isolation of pathogens,[17] sampling strategies only provide a small percentage of final product samples. Therefore, the presence of an indicator organism suggests the environmental conditions are favorable to the growth of bacteria and/or potential pathogens.[18] The most effective assemblage of microbial indicators will vary based on location and is best determined when local and regional factors are considered[19-22]. The simultaneous evaluation of multiple indicators (including *E.coli* as recommended by WHO) provides a more complete picture of the water's microbiological quality, as compared to a single universal indicator.

Alternative indicator microorganisms that have shown merit include coliphages,[23] *Enterococcus,*[24] among others.[18, 19, 25, 26]

Viable But Not Culturable (VBNC)

In many instances, food and water to be consumed may have undergone a mitigation step in order to reduce that chances of pathogens consumption. These methods include chlorination of water, pasteurization of milk, and high pressure processing of foods. If these foods entered their kill step with a high bacterial load, the final product may be free of viable bacteria, but still contain intact bacterial DNA. Therefore, some detection methods which rely on pre-enrichment and growth may result in negative results, while those detecting DNA might provide positive result. There is also the possibility of the bacteria receiving a sub-lethal injury, resulting in bacteria that in the short term, may not display much growth, but following recovery, could cause disease. Therefore, a detection assay aiming for rapid results may miss injured cells which in the short timeframe resemble non-viable cells. However, maintaining clean supplies of drinking water for even small communities is non-trivial. Unfortunately, a visual examination of drinking water does not indicate the absence of pathogens. Therefore, routine microbiological evaluation of drinking water supplies must be

performed to confirm adequate sanitation for public health. The less time required for a detection assay to reach a result means more time for possible remediation and/or supply of clean water to thirsty communities.

Bacterial Injury & Recovery

Wastewater treatment procedures subject many indicator bacteria to chemical and physical processes that serve to be sublethal due to the cell's stress response system.[20, 29]

As a bacterial cell enters the persistor state multiple stress responses cause significant morphological changes to aid the cell navigate its new environment. As opposed to the logarithmic cell growth that occurs when environmental conditions are favorable, bacterial cell growth almost ceases in stationary phase. While the overall cell population remains relatively static, the surviving cells still exhibit relatively large amounts of protein production.[30]

Furthermore, standard culture methods have failed to detect microbial indicators that have been injured through common disinfection techniques.[31] At the higher bacterial concentrations this

result simply leads to an underestimation of colony counts while at low concentrations it produces a false negative, a significant risk.

Detection of cells with GASP mutations

In nature, bacteria are often starved of resources. Even locations where microbial food is relatively plentiful, such as the GI tract of a well-fed mammal, bacterial populations experience intense competition from microbial rivals as they all contend for available resources. As the race to convert available metabolites into biomass continues, nutrients are depleted exponentially as competing populations concurrently experience logarithmic growth. The change in the cellular environment from nutrient rich to nutrient poor triggers a cascade of changes to the cell's proteome. The increased expression of stress related sigma factors (rpoS) further promotes the production of proteins uniquely suited for stressful environments. If nutrients remain sparse over an extended period of time, cells expressing the Growth Advantage in Stationary Phase (GASP) phenotype will dominate the population as the nutrient scarcity creates a strong selection factor for which they are uniquely suited. When bacterial cells do not have the proper environmental

conditions for growth they enter a stationary state where most cells die (~99%) and cell division essentially terminates for the survivors. It has been shown that less than a week is required for the original bacterial population to become dominated with GASP cells better suited for their new desolate environment. [32] It has been shown that upregulation of the *rpoS* sigma factor causes a regulatory shift toward the favored expression of stationary phase genes in *E. coli* [33, 34].

Furthermore, *E. coli* has been shown to maintain plasmid DNA even in the absence of selection.[35] These extrachromosomal DNA elements often provide the cell with new capabilities (virulence factors, antibiotic resistance, etc.) but at the cost of valuable cellular resources. Detection methodologies must take into consideration the myriad of outcomes possible when cells exhibit the GASP phenotype common to injured but not killed bacterial cells.

CONCLUSION

Incredible improvements have been made globally regarding clean drinking water, with the achievement of the millennium development goal 5 years early in 2010.[27] However, many are still forced to go to

great lengths to obtain drinking water without any promise that the water will be free of pathogens. Furthermore, the ingestion of drinking water that appears perfectly safe to drink may still cause significant disease due to the small infectious doses of some pathogens[28]. Therefore, rapid analysis of drinking water for microbial contaminants is critical to maintain public health.

While water is a common analyte for those developing biosensors, it should be noted that detecting bacteria in water presents many more challenges due to practical considerations. Technologies such as lab-on-a-chip has shown tremendous promise in many fields. But if drinking water is selected as a matrix, the challenge of sample size (100 mL) makes miniaturized systems difficult.

Similarly, the push for rapid diagnostics has led many to investigate real-time or near real-time assays. This is met with the problem of the need to differentiate viable, non-viable and injured cells. In order to perform these differentiations, sufficient recovery time must be given to injured bacteria to enable them to be identified as viable vs. non-viable. These processes are biological in nature and difficult to accelerate. Most biosensors for the detection of bacteria

in water samples often need to compromise on some of the current requirements set forth by regulatory agencies. A rapid test (~1-5 hours) which can confidently detect 1 CFU/ 100 mL of viable or sub-lethally injured *E. coli* in drinking water, using minimal equipment, and at low-cost, has not yet been developed. Therefore, while many biosensor developers may consider drinking water to be the “low hanging fruit” of matrices, the constraints that go with it places pragmatic biosensors for bacteria detection out of reach.

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CHAPTER 2

GENETIC OPTIMZATION OF A PHAGE-DELIVERED ALKALINE PHOSPHATASE REPORTER TO DETECT *ESCHERICHIA COLI**

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ABSTRACT

A large fraction of foodborne illnesses are linked to (~46%) leafy green vegetables contaminated by pathogens harbored in agricultural water. To prevent this, accurate point-of-production detection tools are required to identify and quantify bacterial contaminants in produce before consumers are impacted. In this study, a proof-of-concept model was engineered for a phage-based Escherichia coli detection system. We engineered the coliphage T7 to express alkaline phosphatase (ALP) to serve as the signal for E. coli detection. Wild type *phoA* ($T7_{ALP}$) and a dominant-active allele, *phoA* D153G D330N ($T7_{ALP*}$) was inserted into the T7 genome, with engineered constructs selected by CRISPR-mediated cleavage of un- altered chromosomes and confirmed by PCR. Engineered phages and *E. coli* target cells were co-incubated for 16 hours to produce lysates with liberated ALP correlated with input cell concentrations. A colorimetric assay used p-nitrophenyl phosphate (pNPP) to demonstrate significant ALP production by $T7_{ALP}$ and $T7_{ALP*}$ compared to the vector control ($T7_{EV}$) ($p \leq 0.05$). Furthermore, $T7_{ALP*}$ produced 2.5-fold more signal than $T7_{ALP}$ ($p \leq 0.05$) at pH 10.

Due to the increase in signal for the modified ALP* allele, we assessed $T7_{ALP*}$ sensitivity in a dose-responsive manner. We observed 3-fold higher signal for target cell populations as low as ~2 × 10^5 CFU mL^{-1} ($p \leq 0.05$ vs. no-phage control).

INTRODUCTION

Foodborne pathogens cause significant human suffering and their effects potentially extend to loss of life. The United States Center for Disease Control estimates that there are 9.4 million incidences of foodborne illness in the U.S. with approximately 56 000 hospitalizations each year.[1,2] Approximately 46% of these cases of foodborne disease are linked to leafy green vegetables,[3] which are often contaminated from soiled rinse or irrigation water and other poor agricultural practices. In order to reduce the incidence of diarrheal morbidity and mortality and address the considerable public health burden attributed to foodborne pathogens, next generation rapid detection innovations are sought to identify contaminated foods prior to distribution and consumption.

Currently, portable detection technologies target bacterial pathogens such as Escherichia coli, Salmonella spp., Campylobacter jejuni, and Listeria spp. using antibodies that interact with taxon-specific cell surface epitopes.[4] Whereas antibodies efficiently detect target microbes, these platforms are often financially restrictive, unstable, and may require a suboptimal

timeline to identify threats to food systems. In contrast, bacteriophage or phage-based detection platforms can be produced cheaply and often produce expedient results.[5]

Phages are viruses that prey on bacteria and commandeer their fundamental cellular processes in order to replicate. Due to a restricted host range in which phages infect specific targets, phage–host interactions could be exploited to serve as a biological probe to provide rapid and specific pathogen recognition.[6] Phage-based detection platforms commonly utilize genetically engineered phages that encode reporter proteins to be expressed in their targeted hosts in order to amplify what would be a weak detection signal.[7,8] These reporter proteins include firefly luciferase,[9] green fluorescent protein,[10] tobacco etch virus protease,[11] and alkaline phosphatase (ALP; EC 3.1.3.1).[11] For the latter marker, there are several sensitive mechanisms for alkaline phosphatase detection: colorimetric,[12] fluorescent,[13] chemiluminescent,[14] and electrochemical.[15] Here we report modification of bacteriophage T7, a double-stranded DNA virus with a broad-host range in E. coli,[16] as a vector that overexpresses ALP upon infection of its target.

MATERIALS & METHODS

Bacterial strains and growth conditions

Bacteria and bacteriophage strains used in this study are listed in Table 1. Bacterial strains were propagated in lysogeny broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar when appropriate) and supplemented with ampicillin (100 µg mL–1), kanamycin (50 µg mL–1), chloramphenicol (35 µg mL–1), and/or streptomycin (50 µg mL–1) as needed. Cultures were grown aerobically at 37 °C and on an orbital shaker (225 rpm) for broth cultures.

ALP-encoding constructs

The three ALP constructs that were engineered within T7 were obtained through direct synthesis (GenScript, Piscataway, NJ).

Table 1 Strain and plasmid list

	ID	Source
Bacteria and bacteriophage		
	Escherichia coli DH5α	Invitrogen
	Escherichia coli JM109	Promega
	Escherichia coli BL21	Invitrogen
	Escherichia coli BL21 pWUR397, pWUR400, pWUR477-anti-T7	This study
	Escherichia coli O6	ATCC 25922
	*Escherichia coli*O157:H7	ATCC 43890
	*Escherichia coli*O157:H16	PSU 750140
	*Escherichia coli*O2:H7	PSU 880592
	*Escherichia coli*O26:H11	CDC 3047-86
	Bacteriophage T7 wild type	ATCC BAA-1025-B2
	$T7_{EV}$; empty vector in class 1 region	This study
	$T7_{ALP}$; *phoA* with C-term 6× HIS tag in class 1 region	This study
	$T7_{ALP*}$; *phoA* D153G D330N with HIS tag	This study
Plasmids		
	pBAD18	17
	pEV; pBAD18 + T7 expression construct (T7EC)	This study
	pALP; pEV with wild type *phoA* in T7EC	This study
	pALP*; pEV with *phoA D153G D330N*in T7EC	This study
	pWUR397	18
	pWUR400	18
	pWUR477	18

pWUR477-anti-T7; pWUR477 with T7 targeting spacer	This study

This includes a construct that does not contain ALP referred to as the empty vector (pEV), a construct that incorporates the *E. coli* BL21 (Thermo Fischer, Waltham, MA) ALP gene (*phoA*) with a C-terminal polyhistidine (6×) affinity purification tag referred to as (pALP), and a construct with a modified variant of *phoA* that expresses two amino acid substitutions (i.e. D153G and D330N) referred to as (pALP*). In order to insert the constructs into the phage genome via homologous recombination, we placed the synthetic genes into a plasmid backbone (pBAD18-Amp.17). The inserts were amplified with the following primers: For-ACCCGTTTTTTTGGGCTTGTTACTTAGCGGCAGTGTG. Rev-CTA- GAGGATCCCCGGGGAGCGCAAGGCATCCTA). The PCR product was ligated into NheI-HF and KpnI-HF digested pBAD18-Amp using the In-Fusion HD Cloning Kit (Clontech Laboratories, Inc, Mountain View, CA). Ligated vectors were transformed into Clontech Stellar chemically competent cells with transfor- mants selected on LB with ampicillin. Transformants were screened by purifying plasmids with the QIAprep Spin Mini- prep kit (Qiagen, Valencia, California), and confirmed via PCR and DNA sequencing.

Phage-based detection platform engineering

Generation of crude phage lysates. To produce T7 phage with the EV (T7EV), ALP (T7ALP), or ALP* (T7ALP*) insert, 1.0 × 108 plaque forming units (PFU) mL–1 of T7 phage were added to exponentially growing E. coli BL21 containing the plasmids pEV, pALP, and pALP*. Co-incubation proceeded overnight (16 h) at 37 °C to allow lysis to occur. The co-cultures were then centrifuged at 13 000 rpm for 10 minutes and filter steri- lized (0.45 μM) (Millex; EMD Millipore, Billerica, MA).

CRISPR-based selection of mutant phage. We selected for mutant phages by adapting a previously published protocol to modify the T7 genome.8 This method exploits the type I-E CRISPR-Cas system18 to preferentially digest non-edited genomes as directed by spacers encoding the native genome sequence. The native spacer for T7 phages was generated by PCR amplification of plasmid pWUR477 that contains proto- spacers while adding 31 bp sequences from the class 1 T7 locus to target this site (FWD-GGAGGTACACACCA-

TGGAGTTCCCCGCGCCAGCGGGGATAAACCGCAGCCGAAGC

C- AAAGAATTC REV-TTTAGTGAGTCGTATTCGGTTTATCCCCGCT-GGCGCGGGGAACTCTCTAAAAGTATACATTTGTT). The PCR product was phosphorylated (T4 Polynucleotide Kinase, NEB), self-ligated, and chemically transformed into E. coli BL21 com- petent cells. The companion CRISPR-encoding plasmids pWUR397 and pWUR400 were co-transformed resulting in a BL21 derivative that cleaves unmodified T7 following infection (BL21 anti-T7). A plaque assay was performed with 100 µL of crude lysate from "Generation of crude phage lysates" was mixed in molten top agar (0.8%) with BL21 anti-T7 and spread on pre-warmed LB plates. Following overnight incubation at 37 °C, plaques were counted and assessed for their size. Plaques larger than 0.4 cm were screened by PCR using primers specific for the expression construct FWD-ACCCGTTTTTTTGGGCTTGTTACTTAGCGGCAGTGTG Rev-CTAGAGGATCCCCGGGGAGCGCAAGGCATCCTA.

Alkaline phosphatase assay

BL21 anti-T7 was propagated in LB + Cm/Kan/Str overnight at 37°C and subcultured at OD600 nm 0.05 to exponential phase (~3 h).

Cultures were infected with the indicated phage and were co-incubated until lysis of bacterial culture. Lysates were centrifuged at 13 000 rpm for 10 minutes and filter sterilized (0.45 µM) (Millex). Lysates were adjusted to pH 8.2 ($T7_{EV}$ and $T7_{ALP}$), or pH 10.0 ($T7_{ALP*}$) with NaOH and supplemented with 1 mM $MgCl_2$ in order to detect optimal activity.[19] To measure ALP activity, lysates from $T7_{EV}$, $T7_{ALP}$, and $T7_{ALP*}$ co-cultures were incubated with p-nitrophenyl phospate (pNPP, NEB) as a colorimetric indicator using a method adapted as described previously.[19] Colorless pNPP was hydrolyzed to yellow p-nitro- phenol (pNP) in the presence of ALP and was quantified at 405 nm (molar extinction coefficient 1.62×10^4 M^{-1} cm^{-1}).[19–21] 25 µL of the lysate was added to an equal volume of 50 mM pNPP and monitored for 60 minutes in a 96-well microplate fluorometer (BioTek; Synergy H4) according to the instructions of the manufacturer. pNP release was measured by the change in absorbance at 405 nm at 37°C. Assay calibration was performed evaluating serial dilutions of p-nitrophenol in lysogeny broth to generate a standard curve.

Dose response assay

Overnight cultures of *E. coli* strains were serially diluted 10-fold to produce 10^8 to 10^5 CFU mL^{-1} solutions. Aliquots of 10 µL from each dilution were plated to determine cell density. Subsequently, the target cell dilutions were inoculated with 1×10^7 PFU mL^{-1} $T7^{ALP*}$ and co-incubated at 37 °C for 16 h. pNP formation was assayed as described. As a control, the $T7_{ALP*}$ susceptible strains were serially diluted in sterile water.

T7ALP* host range

The *E. coli* strains listed in Table 1 were propagated overnight in LB at 37 °C with 200 rpm shaking. An aliquot (100 µL) of overnight culture was co-incubated with 1×10^7 PFU mL^{-1} $T7_{ALP*}$ in fresh LB broth to determine the infectivity profile of the recombinant phage. pNP conversion was measured in positive strains in a dose response manner as described.

ALP purification

HisPurTM Cobalt Resin Purification columns (ThermoFisher Scientific) were used to purify the ALP expressed from the engineered bacteriophage. The recombinant enzymes $T7_{ALP}$ &

$T7_{ALP*}$ both contain a 6× poly-histidine residue that selectively binds to a cobalt-charged chelator immobilized onto a 6% agarose crosslinked resin. A protease inhibitor was added to the phage lysate applied to the column and all purification steps were performed on ice or at 4°C. Wash and elution buffers both contained 300 mM sodium chloride and 50 mM sodium phosphate buffer, pH adjusted to 7.4. Elution was facilitated with imidazole, which exhibits stronger binding affinities to the cobalt chelators than the poly-histidine tags. The wash buffer contained 10 mM imidazole to displace non-specifically bound proteins whereas the elution buffer releases the bound proteins from the column with 150 mM imidazole. However, the concentration of imidazole in the elution buffer is high enough to strongly inhibit alkaline phosphatase[22] and must be removed from solution prior to pNPP activity assays. Zeba Spin Desalting Columns (ThermoFisher Scientific) were used to remove the excess imidazole by employing a size exclusion resin with a molecular weight cut-off at 20 kDa. The samples were dialyzed against a storage solution containing 20 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$ as a cofactor, and 0.02% (w/v) NaN_3. Long term storage of the purified enzyme

was done at –20°C in a 50% glycerol/storage buffer solution to minimize solvent crystallization.

Enzyme quantification

The total concentration of purified enzyme was determined by the Micro-Bradford assay (Thermo Scientific) in which 150 µL of a 10% purified alkaline phosphatase sample was introduced to 150 µL BCA working reagent in a 96-well microplate and shaken continuously at 37 °C for 120 minutes. The dynamic range of the Micro-Bradford assay has been reported to be 0.5–20 µg mL^{-1}.[23] Absorbance values at 562 nm were measured using a 96-well microplate fluorometer (BioTek; Synergy H4) and compared to a calibration curve produced from bovine serum albumin (0.4–200 µg mL^{-1} BSA).

Enzyme kinetics

The purified alkaline phosphatase enzyme samples were subjected to biochemical characterization at 37°C by measuring p-nitrophenol production every 60 seconds at 405 nm. Varying concentrations of substrate (0.25 mM–100 mM pNPP) were used to elucidate the K_{cat} and K_m values from Michaelis–Menten enzyme kinetics where saturation occurs for both the enzyme (100 mM pNPP) and the

substrate (0.25 mM pNPP). At a constant enzyme concentration, the rate of reaction products (μmol pNP s^{-1}) was plotted with respect to substrate concentration (mM pNPP). One unit of alkaline phosphatase activity was defined as the concentration required to produce 1 μmol of p-nitrophenol in 60 seconds. Non-linear curve fitting was employed to calculate K_m and K_{cat} values for the respective wild type and mutant alkaline phosphatase enzymes.

General statistics

Biological triplicates were averaged for the alkaline phosphatase assays. Resultant means were compared in a two-sample *t*-test assuming equal sizes but unequal variance. Significant differences were reported as $p \leq 0.05$.

RESULTS

In order to engineer T7 to produce a heterologous reporter protein, we designed several gene expression constructs that were inserted into the T7 genome in the class 1 region. Because genes in this region are expressed before those in class 2 or 3,[24] we predicted that expression of the reporter would be higher in this region. Fig. 1 depicts the features of the expression construct in the absence of

the *E. coli* ALP gene (i.e. *phoA* insert). The empty vector construct lacks *phoA* and serves as the negative control for subsequent ALP expression assays. The sequence of the control construct and experimental constructs is provided in the appendix. The construct was engineered to incorporate gp1.2 from bacteriophage T3 (ATCC 11303-B3) and a multiple cloning site (MCS) with seven

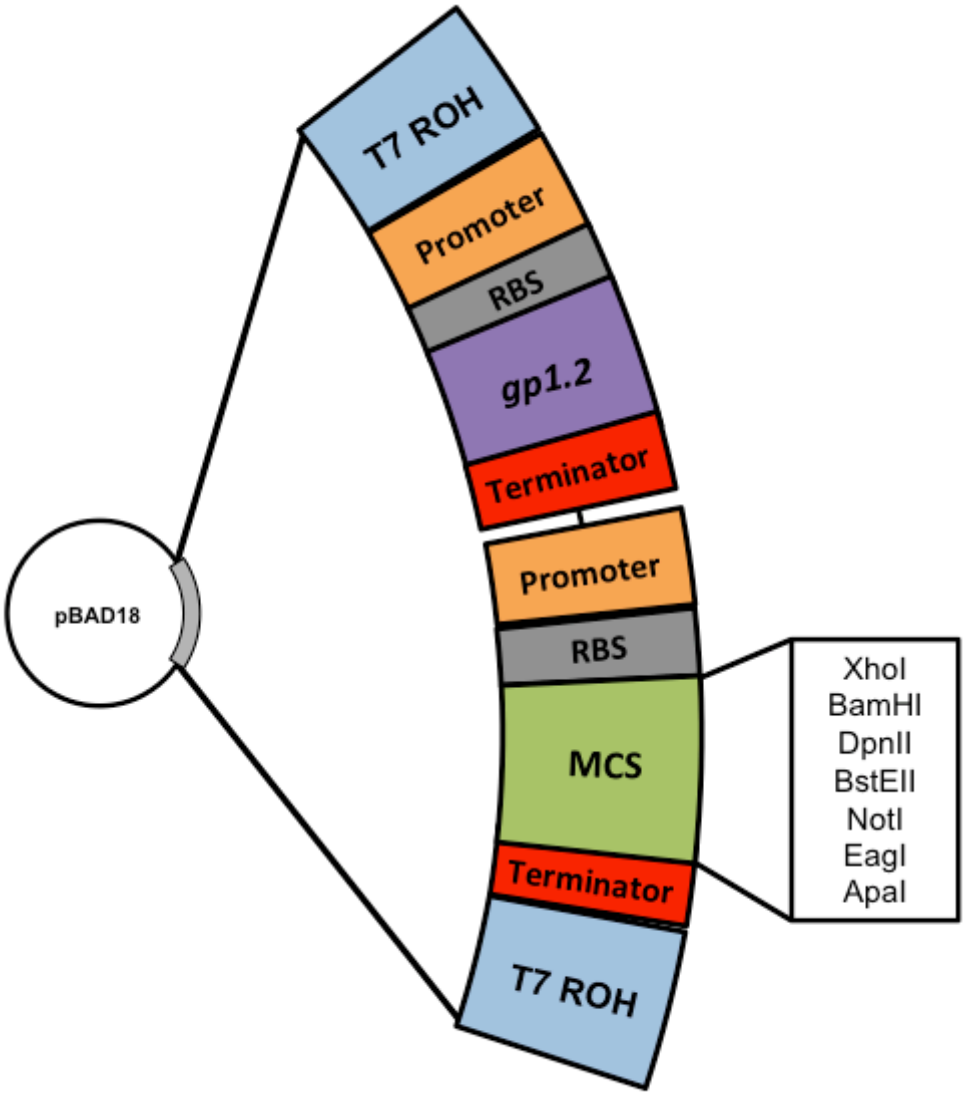

Figure 1. T7 Expression Construct Design. Depicted is the control construct, pEV. It features (1) Bacteriophage T3 *gp1.2* under control of the T7 promoter, ribosome binding site (RBS), and terminator from pET-3a (EMD Millipore). (2) An MCS sequence with 7 unique single-cutter restriction sites (XhoI, BamHI, DpnII, BstEII, NotI, EagI, ApaI) under the control of the T7 promoter, RBS, and terminator. The construct is flanked by 66 bp segments of the T7 genome. pALP and pALP* have *phoA* and *phoA D153G D330N* inserted into the MCS.

unique restriction sites under the control of the T7 promoter, ribosome binding site (RBS), and terminator. Our previous work demonstrated that these promoter and RBS sequences enables efficient overexpression of downstream genes in T7.[25] The construct is flanked by 5′ and 3′ regions of homology to the class I region of the T7 genome to facilitate homologous recombination. The gp1.2 gene enables bacteriophage T3 to successfully infect F-plasmid containing E. coli, thus broadening the host-range of the T7 detection platform.[26]

In order for the infecting phage to express ALP, wild type *phoA* (ALP) and a dominant-active allele, *phoA* D153G/D330N (ALP*)[19] were inserted into the MCS site. The variant allele encodes for a protein that maintains conformation and stability.[27] Use of the dominant-active allele was preferable because ALP* activity was previously demonstrated to be stronger with these two amino acid substitutions.[19] Therefore, we investigated the potential for the ALP* allele to provide elevated activity in a phage-based detection scheme.

Accordingly, these constructs were synthesized and cloned into an *E. coli* expression vector.[17] As designed, engineered phages were generated in a series of propagations on target cells containing anti-native T7 CRISPR plasmids (i.e. BL21 anti-T7) as detailed in materials and methods. Candidate phage vectors were confirmed by PCR before ALP detection assays were performed.

Production of alkaline phosphatase by engineered T7 strains

To characterize the capacity of the engineered phage to detect *E. coli,* the concentration of ALP produced following infection was assessed. Exponentially growing target *E. coli* cells were incubated at 2.0×10^8 CFU mL^{-1} with the three engineered phages: $T7_{EV}$, $T7_{AP}$, and $T7_{ALP^*}$. We assayed media alone and the $T7_{EV}$ as controls to ascertain background ALP phosphatase activity. Lysates were collected after 16 hours of co-incubation, and analyzed for ALP activity based on pNPP hydrolysis.

We assayed pNP production after 10 minutes and observed insignificant basal ALP expression in lysates generated with $T7_{EV}$ (Fig. 2).

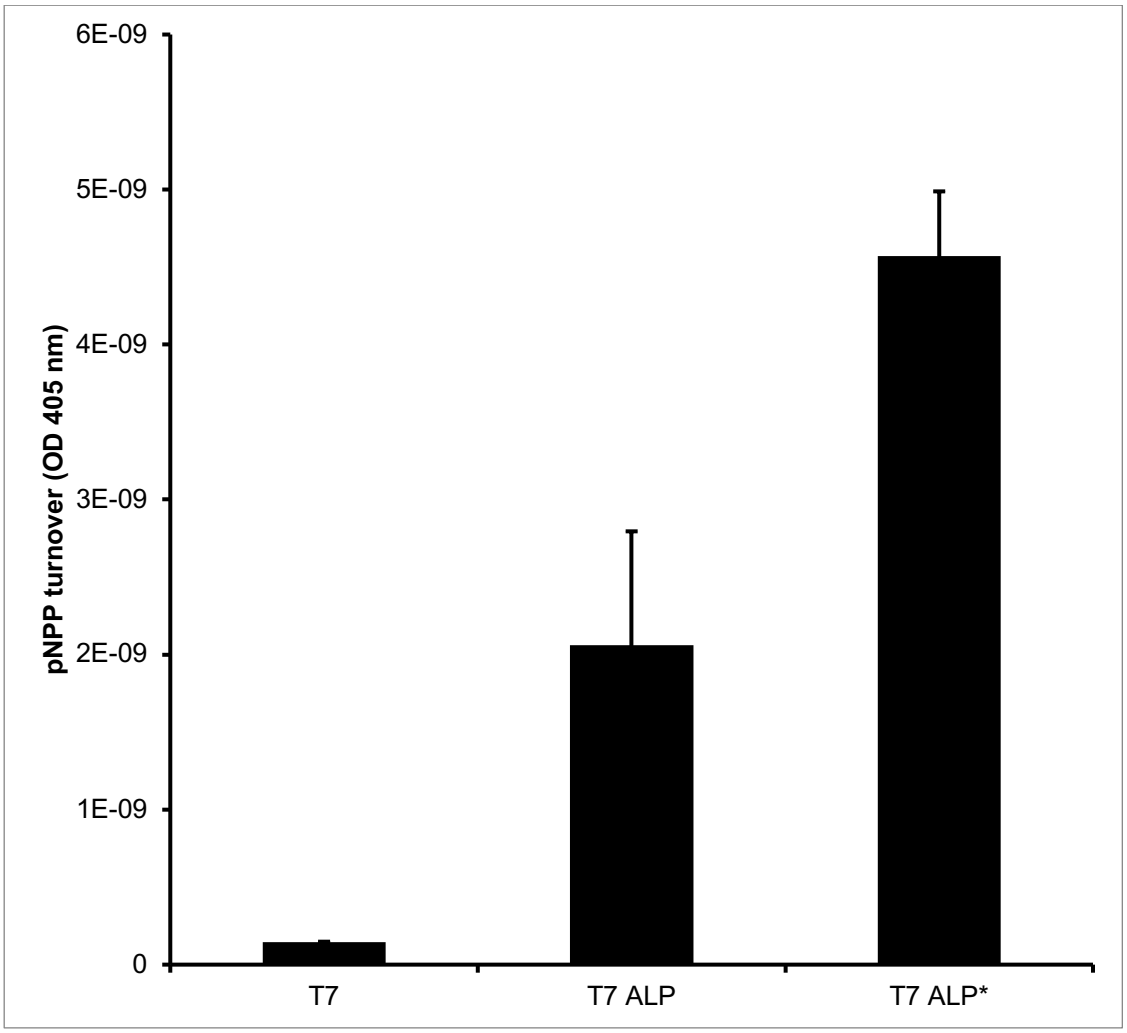

Figure 2. Alkaline Phosphatase is expressed in engineered T7 phage. Engineered T7 phage were co-incubated for 16 h at 37˚C to generate lysates. Lysates were measured for ALP production by the hydrolysis of 50 mM pNPP in 30 minutes. Data was read over time at OD_{405} nm. The data are the means of three biological replicates. *, $P \leq 0.05$ compared to empty vector control

This low level (1.4×10^{-4}) of ALP activity may be attributed to constitutive expression within the *E. coli* target strain. However the $T7_{ALP}$ phage promoted a 5.5-fold increase in pNP signal over baseline ($p = 0.08$). Whereas the native *E. coli* ALP protein (i.e. *PhoA*) is active at pH 8.0, the ALP* variant is most active at pH 10.0 (ref. 19) thus assays were conducted at this optimal alkaline environment. Interestingly, $T7_{ALP*}$ provided 15-fold more signal compared to basal conditions with $T7_{EV}$ ($p < 0.02$), and 2.7-fold more signal than the wild type ALP allele ($p < 0.05$). These data demonstrate successful engineering of the T7 genome as well as a clear improvement in signal production by the modified ALP* allele.

Biochemical characterization of ALP & ALP* purified enzymes

To characterize the differential rates by which ALP and ALP* hydrolyzes substrate, both recombinant proteins were purified to have their kinetics evaluated. These HIS-tagged proteins were purified from a cobalt-column prior to determining concentrations. The enzymatic kinetics was determined using a range of substrate concentrations (100 mM–0.25 mM) that allowed for full saturation of substrate (100 mM pNPP) and full saturation of enzyme (0.25 mM

pNPP). The turnover number (K_{cat}) was determined by normalizing the V_{max} value to the concentration of enzyme present. As a result, K_{cat} was calculated to be $30s^{-1}$ for the wild type enzyme and $1344s^{-1}$ for the D153G/D330N double mutant. K_m corresponds to the substrate concentration at which the reaction rate is half of its maximum and was calculated to be 14.2 µM for the native ALP and 354.1 µM for the modified ALP* allele. The catalytic efficiency of 2.1 for ALP and 3.8 for ALP* was evaluated by taking the ratio of K_{cat} : K_m to describe the efficiency pNPP hydrolysis.

ALP intensity is dependent on target cell density

As $T7_{ALP*}$ provided the strongest signal intensity, this vector was used for determining the optimal density of target cells with a dose–response assay. *E. coli* BL21 cells that had achieved mid-exponential growth were serially diluted 10-fold with 1×10^9 PFU mL^{-1} $T7_{ALP*}$ added and co-incubated for 16 h. Fig. 3 depicts the range of target bacterial densities were detected using $T7_{ALP*}$.

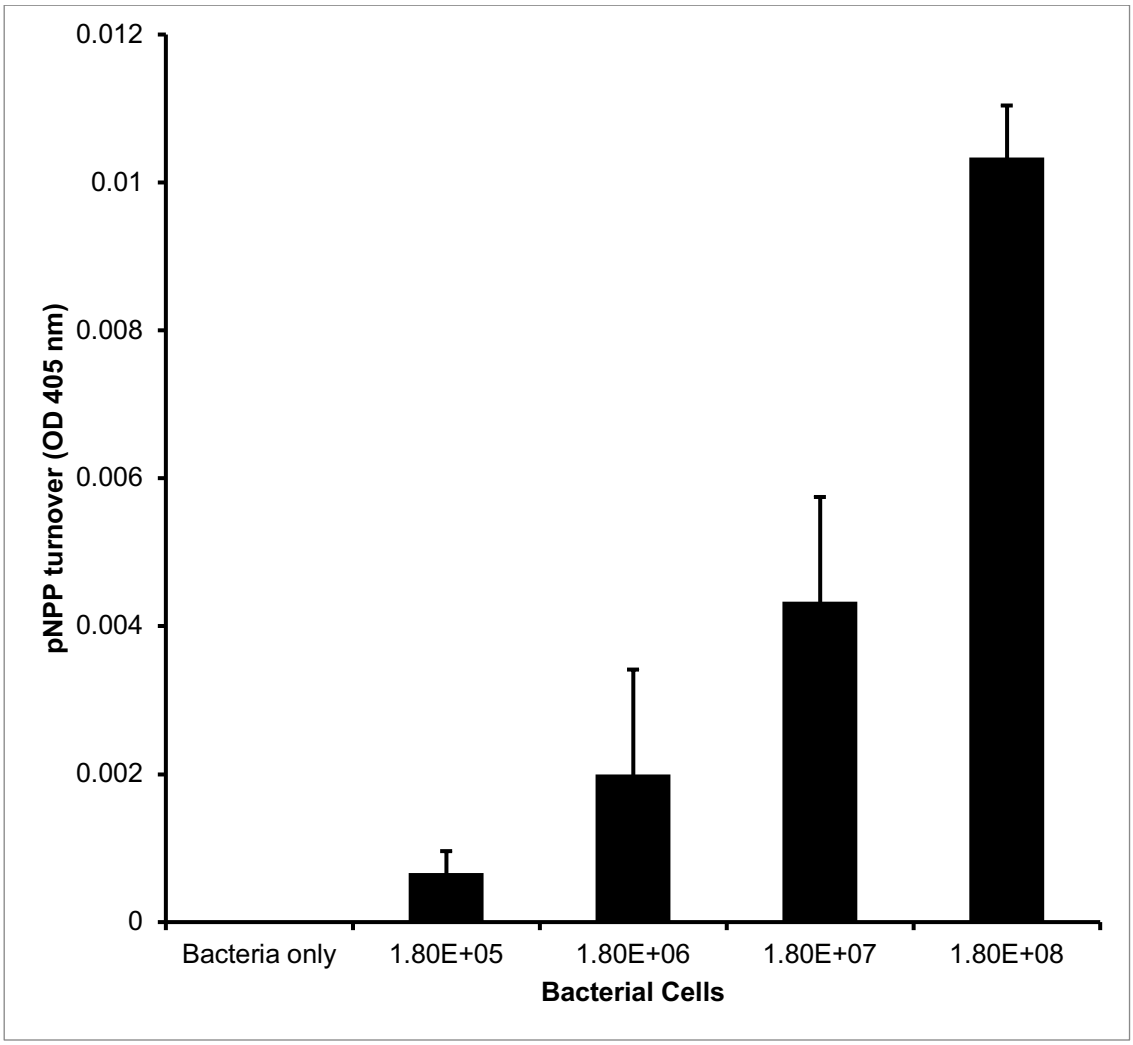

Figure 3. ALP activity signal increases in a dose-dependent manner. $T7_{ALP*}$ was co-incubated with varying concentrations of *E. coli* BL21 until culture lysis. ALP expression was measured by the hydrolysis of 50 mM pNPP in 60 mins. A culture without $T7_{ALP*}$ served as the base-line control. The data are the means of three biological replicates. *, $P \leq 0.05$ (compared to empty vector control).

A positive signal was obtained above background for 1.8×10^5 CFU mL–1 ($p < 0.05$). In addition, we observed an increase in signal (~2.5-fold) coinciding with increased target cell density. These data indicate that the sensitivity for $T7_{ALP*}$ is ~1×10^5 CFU mL^{-1} under proof-of-principle conditions.

E. coli host range of the engineered $T7_{ALP*}$ phage

In order to determine the host range breadth of the recombinant phage, *E. coli* strains were co-incubated with a high titer LB solution of $T7_{ALP*}$ (Table 1). Cell lysis was observed in the three laboratory strains (i.e. BL21, DH5α, and JM109) and did not occur in the strains possessing the O or H antigens tested. The limit of detection (i.e. positive signals above background) for DH5α and JM109 were 6.5 $\times 10^5$ CFU mL^{-1} and 5.4×10^5 CFU mL^{-1} respectively. No significant differences in detection limits were observed when dilutions were performed in sterile water as opposed to LB (data not shown).

DISCUSSION

We have developed an effective model to modify broad-host range bacteriophages to express heterologous reporters. The engineered phage successfully delivered the reporter protein to target cells to enable *E. coli* detection. Furthermore, the signal intensity following modification of the ALP gene to generate the $T7_{ALP*}$ phage represents a significant improvement (2.6- fold, $p < 0.05$) than that of the wild type allele. We have demonstrated that the engineered ALP* is ~45 fold more active than the wild type ALP, in close agreement with previous work.

In addition, this 45-fold increase in alkaline phosphatase activity in assays of purified ALP D153G/D330N protein in the absence of cell debris. This is likely due to assessing purified enzyme activity rather than expression within the complex biochemical environment of a lysate. Moreover, increasing the concentration of divalent cations may improve phage adsorption and could potentially improve ALP* intensity, thus providing another opportunity to optimize this detection platform.[28] Affinity purification and subsequent kinetic characterizations of the enzymes in the absence of cell debris

provided a method to accurately compare the activity of different enzymes.

Previously, the two amino acid substitutions to the ALP* catalytic pocket yielded a highly active, thermo-resistant enzyme similar to bovine ALP in purified assays.[19] This is due to the higher turnover number (k_{cat}) of this "mammalianized" alkaline phosphatase along with a stronger substrate affinity (K_m).[29] This represents a significant improvement for the $T7_{ALP*}$ vector to be applied as a preferred on-farm diagnostic. Second, the variant ALP* previously exhibited the ability to maintain activity after extended storage period of 1 year.[19] This suggests that it is recalcitrant to environ- mental challenges that would inactivate antibody-based detection schemes.

Genetic manipulation of the T7 phage did not modify structural components that may interfere with host cell binding (e.g. tail fibers). As such, and consistent with previous results,[30] T7ALP* did not infect all *E. coli* serotypes tested in this study. Future applications may necessitate deploying several phages simultaneously to cover the broadest possible host range.[31]

E. coli BL21 was detected at a concentration of 1.8×10^5 CFU mL^{-1} following 16 hours of co-incubation. This limit of detection was comparable to other strains tested (i.e. DH5α, JM109). A lower detection limit of detection could be conceivably achieved with further assay optimization.

CONCLUSION

This phage-based system represents a facile template to innovate analogous platforms to detect and quantify bacterial pathogens in food systems, clinical settings, and the environment. This is consistent with future goals of rapidly engineering multiple detection platforms in parallel to address emerging bacterial threats. Phage propagation can be accom-plished relatively quickly, making it much more cost- and time-effective than raising antibodies. Thus, this approach could be extended towards pathogenic threats by modifying cognate phages including Salmonella, Listeria, and Campylobacter.[32–34] Together these data demonstrate that the bioengineered phage-based detection platform is a viable alternative to current bacterial detection systems.

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CHAPTER 3

THE REPORTER BACTERIOPHAGE $T7_{NLC}$ UTILIZES A NOVEL NANOLUC::CBM FUSION FOR THE ULTRASENSITIVE DETECTION OF *ESCHERICHIA COLI* IN WATER*

* T. C. Hinkley, S. Singh, S.Garing, A-L. M. Le Ny, K. P. Nichols, J. E. Peters, J. N. Talbert, and S. R. Nugen

ABSTRACT

Rapid detection of bacteria responsible for foodborne diseases is a growing necessity for public health. Reporter bacteriophages (phages) are robust biorecognition elements uniquely suited for the rapid and sensitive detection of bacterial species. The advantages of phages include their host specificity, ability to distinguish viable and non-viable cells, low cost, and ease of genetic engineering. Upon infection with reporter phages, target bacteria express reporter enzymes encoded within the phage genome. In this study, the T7 coliphage was genetically engineered to express the newly developed luceriferase, NanoLuc (NLuc), as an indicator of bacterial contamination. While several genetic approaches were employed to optimize reporter enzyme expression, the novel achievement of this work was the successful fusion of the NanoLuc reporter to a carbohydrate binding module (CBM) with specificity to crystalline cellulose. This novel chimeric reporter (*nluc*::*cbm*) bestows the specific and irreversible immobilization of NanoLuc onto a low-cost, widely available crystalline cellulosic substrate. We have shown the possibility of detecting the immobilized fusion protein in a filter plate

which resulted from a single CFU of *E. coli*. We then demonstrated that microcrystalline cellulose can be used to concentrate the fusion reporter from 100 mL water samples allowing a limit of detection of <10 CFU mL^{-1} *E. coli* in 3 hours. Therefore, we conclude that our phage-based detection assay displays significant aptitude as a proof-of-concept drinking water diagnostic assay for the low-cost, rapid and sensitive detection of *E. coli*. Additional improvements in the capture efficiency of the phage-based fusion reporter should allow a limit of detection of <10 CFU per 100 mL.

INTRODUCTION

Drinking water contaminated with pathogenic bacteria is a major public health concern, both in the United States1–4 and worldwide.[5] *Escherichia coli (E. coli)* is a major cause of global morbidity and mortality. While the WHO estimates approximately 63,000 annual deaths are due to foodborne *E. coli* infections, there is an added consequence of 5 million years of life lost (YLLS) and 5 million disability adjusted life years (DALYS).[5] In addition to gastrointestinal infections, *E. coli* is the most common causative agent for urinary tract infections, 8.9% of sepsis cases, and 29% of early onset neonatal sepsis cases.[6,7] "Generic" *E. coli* is often used as an indicator for contamination or improper sanitation of water or food.[8,9] Found in high concentrations in the feces of most mammals, the presence of *E. coli* is considered the best biological indicator for fecal contamination in drinking water.[10]

While the identification of indicators or potential pathogens often involves the culturing of serological, food, or environmental samples, new technologies have been introduced with the promise of bringing assay times from days, to hours, and even minutes. While some of

these advanced technologies (e.g. optical nanostructures, surface enhanced Raman spectroscopy) have shown promise as sensitive detection methods, these methods typically require a relatively clean sample in a small volume. It is clear that the true bottleneck to rapid detection remains with the separation, concentration, and cleanup steps of the initial sample preparation. An ideal separation method should (1) remove the analyte from the matrix, (2) remove any possible inhibitors to a downstream detection system, and (3) reduce the sample size while maintaining a high capture efficiency.[11] Unfortunately, there does not presently exist a pragmatic system that integrates sample preparation and pathogen detection from large volume samples. While the vast majority of research focuses on improving the sensitivity of pathogen detection rather than separation/concentration, an ideal detection platform would incorporate all these components as well as the ability to determine cell viability.

Furthermore, bacterial species causing foodborne disease have been shown to rapidly gain virulence factors as well as antibiotic resistance markers, further necessitating the need for rapid

detection methods to successfully mitigate the population's exposure to potentially harmful pathogens. The increasing prevalence of *E. coli* strains that are resistant to all known antibiotics is considered one of the most serious risks to public health in the near future.[12] This relatively rapid rise of antibiotic resistance in pathogens has been confirmed and is actively monitored by the Centers for Disease Control (CDC) and the National Antimicrobial Resistance Monitoring System (NARMS).[13,14]

Bacteriophage (phage) based detection methods have demonstrated a high potential to detect, mitigate and control the causative bacterial agents of foodborne illness.[15–21] Phages are a natural viral predator of bacteria whose total population outnumbers any other biological entity on the planet.[22] Infection begins with binding to host cell surface receptors and then injecting their genome into the cell through specific and localized degradation of the bacterial cell wall.[23] This highly specific and irreversible interaction is the first major determinant of a phage's range of potential hosts. While other genetic factors contribute to a phage's ability to infect a host (restriction enzymes, CRISPR, etc.), directed genetic

modifications to phage tail components involved in the initial binding event have successfully expanded the host range.[24]

Upon infection, the injected phage DNA has been shown to dramatically alter bacterial gene expression and metabolism. For example, some phages encode factors that alter the promoter specificity of the host RNA polymerase to selectively force overexpression of phage DNA.[25] Furthermore, other phages have been shown to cause the complete cessation of bacterial macromolecular synthesis, allowing for the specialized allocation of cellular resources for phage expression and reproduction.[26]

Reporter phages are created when an exogenous gene is added to a phage genome, causing expression of a readily detectable enzyme concurrent with phage infection.[19] The concentration of enzyme in solution can be correlated with the susceptible bacterial population within the sample. Commonly used phage reporters include alkaline phosphatase (phoA),[27–29] beta-galactosidase (lacZ),[28,30,31] green fluorescent protein (gfp),[32,33] and bacterial luciferases (luxAB or luxCDABE),[34–36] among others.[23,37,38]

Recently engineered and commercialized by Promega, a highly active luciferase (NanoLuc or NLuc) and substrate (furimazine) system have generated luminescent signals orders of magnitude greater than that of other commonly used luciferases.[39] Furthermore, the small size of NanoLuc (19 kDa), coupled with its high activity, makes it an ideal candidate for a phage reporter where any genetic insertion must be small enough to fit the expanded genome into the phage capsid.[40,41]

In order to concentrate and purify engineered proteins, numerous epitope tags such as His-tag,[42] AviTag,[43] and FLAG,[44] among others[45] are commonly used. These tags bind to cobalt,[46] biotin,[43] and/or antibody substrates, respectively, all of which are expensive, complex or both. The type of affinity tag used in this work, a Carbohydrate Binding Module (CBM), has been widely employed as protein fusions[47] to provide immobilization onto low-cost, widely available substrates. Carbohydrate binding modules are commonly found within carbohydrate active enzymes that fold independently of the larger protein structure and display specific binding to carbohydrate substrates.[47] The specific ligand used in this work

(CBM2a from Cellulomonas fimi) displayed irreversible binding to crystalline cellulose when expressed as a fusion protein.[48] A CBM is a crucial component of many carbohydrate active enzymes, especially for insoluble substrates where enzyme diffusion would limit substrate availability.

In this paper, we present a novel recombinant phage that has been constructed and implemented into a detection system capable of detecting low concentrations of viable *E. coli* cells. The novel fusion reporter phage $T7_{NLC}$ employs the highly active NLuc luciferase fused to the irreversibly binding carbohydrate binding module CBM2a to sensitively detect *E. coli* cells in drinking water samples. While a cocktail of phages may be required for the detection of an entire bacterial species, we targeted *E. coli* BL21 as a proof-of-principle model to demonstrate the potential sensitivity of our system.

This work outlines the successful (i) modification of the T7 phage genome to contain *nluc* or *nluc::cbm* reporter genes, (ii) expression of NanoLuc or NanoLuc::CBM in *E. coli* from infection of $T7_{NL}$ or $T7_{NLC}$, respectively, (iii) estimation of *E. coli* concentration required

to generate a detectable signal, and (iv) demonstration of a low-cost and rapid method to detect *E. coli* in 100 mL of drinking water using a phage-based detection strategy.

MATERIALS & METHODS

Materials

Unless otherwise noted, all chemicals were reagent grade and purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St Louis, MO, USA). DNA synthesis was provided by Integrated DNA Technologies (Coralville, Iowa, USA). Restriction enzymes and DNA polymerases were purchased from New England Biolabs (Ipswitch, MA, USA). *Escherichia coli* BL21 (ATCC® BAA-1025TM) and *E. coli* Castellani and Chalmers (ATCC 13706), routinely used in this study as hosts for phage T7, were grown aerobically in Luria Bertani (LB) at 37 °C with continuous shaking agitation. Transformation of recombinant phage DNA was performed in electrocompetent *E. coli* DH10B cells (MegaX, Invitrogen, USA). Genomic phage DNA to be used as a cloning vector was purified from T7Select 415–1 (Novagen, USA) propagated in BL21. Extraction of phage DNA was performed with the Qiagen Genomic

Tip 100/G (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Routine DNA purifications were performed using glycogen for nucleation and precipitated using sodium acetate (3 M) and ethanol (100%).[49]

Phage stock preparation

An overnight *E. coli* BL21 culture (2 mL) was added to LB media (200 mL) and incubated (37 °C, 250 rpm, 2–3 hours) until an OD_{600} of 0.4–0.6, suggesting steady state growth.[50] Recombinant T7 phage was added at a multiplicity of infection (MOI) of 0.1 and incubated (37 °C, 250 rpm, 1.5–2 hours) until a significant decrease in OD_{600} was observed. Chloroform (200 µL) was added directly to the lysate before centrifugation (12,000g, 5 min) to clear bacterial debris. The supernatant was sterile filtered (0.22 µm) and phage titers were determined by standard double overlay plaque assays. Phage samples with titer's exceeding 10^9 PFU mL^{-1} were further concentrated with the addition of PEG6000 (0.4%) & NaCl (0.3 M) and incubated (4 °C, 12–16 hours) to precipitate phage particles. The precipitated phage samples were centrifuged (35,000Xg, 2 hours, 4 °C), the supernatant was discarded, the concentrated

phage pellet was re-suspended in Tris-HCl pH 7.4 (~5 mL), sterile filtered (0.22 µm) and stored at 4 °C until needed.

Bacteriophage genome preparation

Aliquots (5 mL) of concentrated phage samples (>10^{11} PFU mL^{-1}) were mixed with 4% SDS (5 mL) and incubated at 70°C for 20 minutes. After cooling on ice, sodium acetate (2.55 M, 5 mL) was added before centrifugation (10,000 x g, 10 min). The supernatant was passed onto the Qiagen 100/G Genomic DNA column (Qiagen, Hilden, Germany) and phage genomic DNA was purified according to the manufacturer's instructions. DNA concentration was determined on a NanoDrop One (Thermo Scientific, Wilmington, DE, USA).

Genetic engineering of bacteriophage T7

A reporter enzyme expression construct was inserted into the phage T7 genome to create the reporter phage $T7_{NLC}$ (Fig. 1). In an effort to increase expression levels of NLuc, the strongest wild type T7 promoter sequence[51] was used to maximize transcription and a custom ribosome binding site (RBS) was designed to optimize translation.[52] An N-terminal leader sequence (*pelB*) was added

immediately upstream of *nluc* as it has been shown to significantly increase soluble heterologous enzyme expression in *E. coli*[53,54] by directing newly made proteins to the periplasmic space. Cleavage by a signal peptidase in the periplasm eliminates the 22 amino acid *pelB* sequence from the mature NLuc enzyme and confers no loss in enzymatic activity.[55] The *nluc* and *nluc::cbm* genes were codon optimized with respect to the *E. coli* species within the T7 host range.

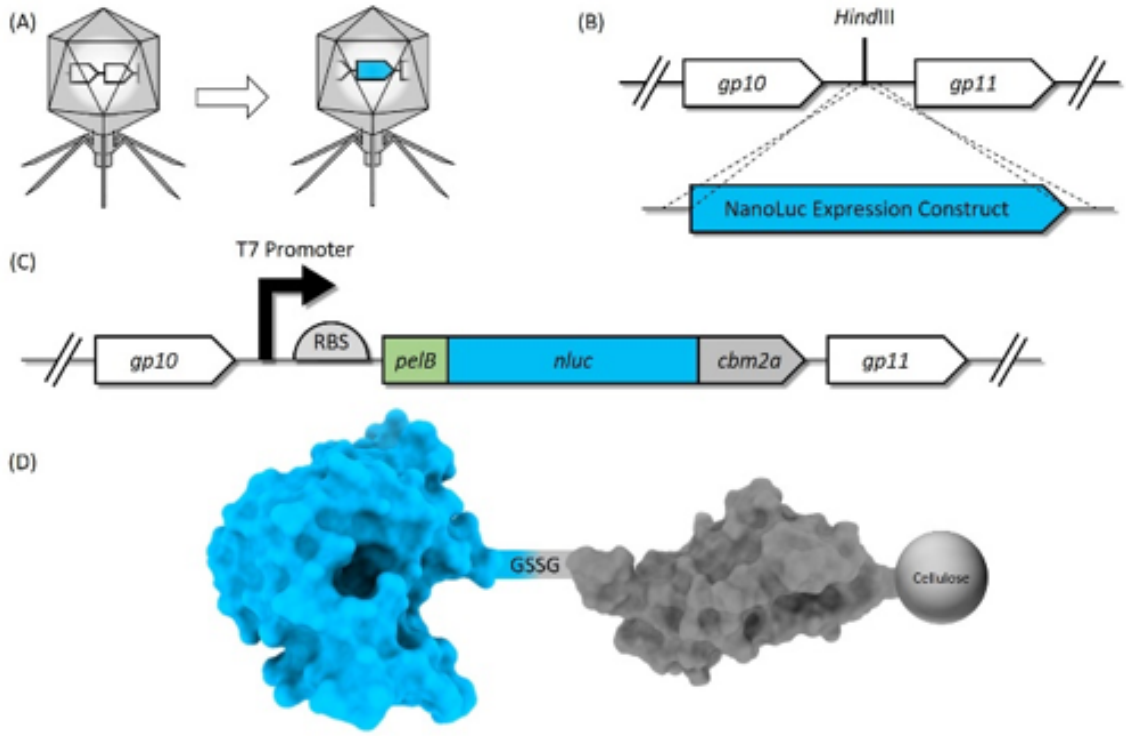

Figure 1: Schematic representation of phage genetic engineering. (A) The ~1 kb NanoLuc expression construct was successfully integrated into the T7 phage genome (B) at a highly expressed region immediately downstream of the capsid gene. (C) Expression construct details: A T7 promoter (black arrow), a ribosome binding site (RBS; half circle), and a pelB leader sequence. The carbohydrate binding module (*cbm2a*) was fused to the C-terminus of nluc with a short flexible linker. (D) Schematic of the novel reporter fusion displaying binding affinity to cellulose. Genes/proteins/phages not drawn to scale for purposes of illustration.

The *nluc* & *nluc::cbm* expression constructs, used to make $T7_{NL}$ (accession number MH651797) and $T7_{NLC}$ (accession number MH651798) respectively, were expected to add 642 bp and 984 bp to the phage genome, respectively. Based on previous work with T7 reporter phages bearing much larger reporters,[31,56] the 1.7% ($T7_{NL}$) and 2.6% ($T7_{NLC}$) increases in genome size were expected to bear no significant reduction in fitness.

A standard restriction digest was performed on the purified phage genome using HindIII-HF to create double strand DNA breaks at the insertion site. The NanoLuc reporter enzyme expression cassette was synthesized as a linear double stranded DNA molecule with 50 bp of phage homology at each terminus. The insert was added to the digested phage genome at a 2:1 molar ratio and assembled with NEBuilder® Hifi DNA Assembly Master Mix (NEB, Ipswitch, MA), in accordance with the manufacturer's specifications. Insert confirmation was performed by PCR and DNA was purified using standard methods and resuspended in nuclease free water prior to transformation. The recombinant phage DNA was electroporated into *E. coli* DH10B cells (MegaX, New England Biolabs, Ipswitch,

USA). Sterile SOB media was added, and the transformed cells were incubated (37 °C, 2 hours) until visible lysis occurred. Chloroform (1–2 drops) was added to lyse any remaining cells and the mixture was centrifuged at 10,000 x g for 1 minute to clear bacterial debris. Following sterile filtration (0.22 μm), the transformation lysate was plated on a lawn of *E. coli* BL21 where individual plaques were isolated, resuspended in broth and insert confirmation done by PCR. Positive plaques were isolated, propagated and submitted for full genome sequencing.

Bacteriophage $T7_{NL}$ & $T7_{NLC}$ stock preparation

Due to their high sensitivity, it was necessary to remove excess reporter enzyme from the phage stock solutions so as to minimize background signal that could significantly impair the assay sensitivity. The concentrated phage sample ($\sim 10^{11}$ PFU mL^{-1}) was first diluted in sterile LB to a concentration of 10^9 PFU mL^{-1}. For phage $T7_{NLC}$, powdered microcrystalline cellulose (0.5 g) was added and the sample was placed on a rotational shaker for 30 minutes before sterile filtration (0.22 μm). The resulting phage stock was

tested for enzymatic activity and the cellulose sequestration procedure was repeated if detectable signal was generated.

Reporter enzyme binding to cellulose

The lysates resulting from infections between the engineered phages and *E. coli* were prepared as described above. In order to test the ability of the CBM to allow binding of the reporter enzyme to cellulose, the lysates were spotted onto regenerated cellulose filters, washed to remove unbound reporters, and then imaged following the addition of the NanoGlo substrate.

Detection of log phase *E. coli* in broth

Log phase *E. coli* 13706 cultures were obtained by adding overnight culture (200 µL) to fresh broth (5 mL). The culture was incubated (37 °C, 250 rpm, ~1 h) until an OD_{600} value of 0.8 was reached. Phage were added to serial dilutions of bacteria and incubated (37 °C, 250 rpm, 90 min) for infection to occur.

Following phage infection, an aliquot of each sample was added to a 384 well cellulose filter plate (AcroPrep 384 BioTrace NT, Pall, Port Washington, USA). Vacuum was applied according to the manufacturer's specifications until no liquid remained. NanoGlo

substrate was applied to each well (20 µL) and luminescence was measured on a plate reader (BioTek Synergy H1) with a 0.1 s integration time. While the vacuum filter plate provides a highly efficient capture mechanism for small sample volumes (~200 µL), higher sample throughput becomes prohibitory when the large sample (100 mL) volumes required for regulatory testing are considered.

Detection of stationary phase *E. coli* in lake water

Lake water was collected from Lake Sammamish (WA) and used as a representative environmental sample matrix. Samples were filtered sterilized (0.22 µm), inoculated with *E. coli* 13707 (ATCC) and left at room temperature for 2 days. After 48 hours, samples were diluted in LB and incubated (37 °C, 250 rpm) for various time intervals. Following enrichment, $T7_{NLC}$ phage (10^7 PFU mL^{-1}) was added and luminescence was measured using 384 well filter plates as described previously.

Drinking water assay

A phage-based drinking water assay was developed as a proof of principle to detect *E. coli* in 100 mL of drinking water using the

recombinant $T7_{NLC}$ reporter phage (Fig. 2). Drinking water samples (100 mL) were autoclaved and inoculated with various concentrations of stationary phase *E. coli* BL21. Sterile concentrated 5× LB media (25 mL) was added to the sample and incubated (37 °C, 225 rpm, 60 min) to allow resuscitation of injured or stressed *E. coli* cells. Following pre-enrichment, phage stock (1 mL of 10^9 PFU mL–1) and microcrystalline cellulose (0.05 g) were added and incubated (37 °C, 225 rpm, 90 min) to allow for phage infection and reporter enzyme production. NanoLuc-CBM complexed with the microcrystalline cellulose was pelleted by low speed centrifugation (3000g, 5 min). The bulk lysate was decanted, and the cellulose pellet was resuspended directly in NanoGlo substrate and immediately evaluated for luminescence.

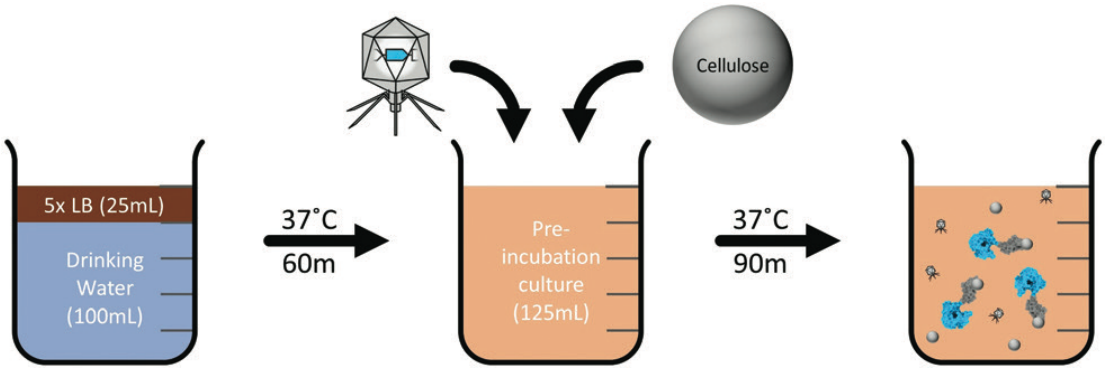

Figure 2: Schematic representation of detection assay. The water sample is first supplemented with concentrated growth media (5× LB) to allow resuscitation of *E. coli*. After an incubation period, the phage and cellulose are added to the sample and the infection incubation period begins, during which expression of NanoLuc-CBM occurs. The reporter enzyme then binds to the cellulose which is collected for analysis.

Bulk lysate luminescence was evaluated for both $T7_{NL}$ and $T7_{NLC}$ phages by adding equal volumes of the corresponding lysate (100 µL) to undiluted NanoGlo substrate (100 µL), in accordance with the manufacturer's specifications. Controls included uninfected bacterial cultures with no phage and phage alone. Measurements were performed in opaque white 96 well plates on a luminescent plate reader (Biotek, Winooski, VT, USA). Luminescent intensity was reported as relative luminescence units (RLU) using an integration time of 0.1 s. Samples were evaluated in triplicate and reported as the mean ± standard deviation. The limit of detection was determined using the sum of the negative control and three standard deviations (0 + 3SD) as the lowest detectable signal.[57–60]

RESULTS & DISCUSSION

Reporter phage construction & isolation

The *nluc* & *nluc::cbm* reporter genes were synthesized within expression constructs and successfully inserted into phage T7 to generate reporter phages $T7_{NL}$ & $T7_{NLC}$, respectively. Plaques were screened initially by the direct addition of substrate followed by PCR. Plaque morphologies for both recombinant phages similar identical to wild type phages. The burst size and lysis times were also comparable to the wild type, suggesting no measurable loss of fitness (data not shown). Correct insertion of the *nluc* & *nluc::cbm* expression constructs were confirmed via PCR using external screening primers. Sanger sequencing results revealed the correct 642 bp & 984 bp insertions, for $T7_{NL}$ and $T7_{NLC}$ respectively, without

other significant mutations, insertions or deletions within the insertion site.

Characterization of phage infection

Infection of *E.coli* BL21 with recombinant reporter phage transduced the *nluc::cbm* gene into the target bacterial cell where the reporter enzyme was successfully expressed. As seen in Fig. 3, a characteristic phage infection was performed to evaluate the phage's ability to lyse target cell populations and express reporter enzyme by measuring luminescence intensity and optical density at regular intervals. Post phage addition, a plateau in the OD_{600} occurred after approximately 30 minutes.

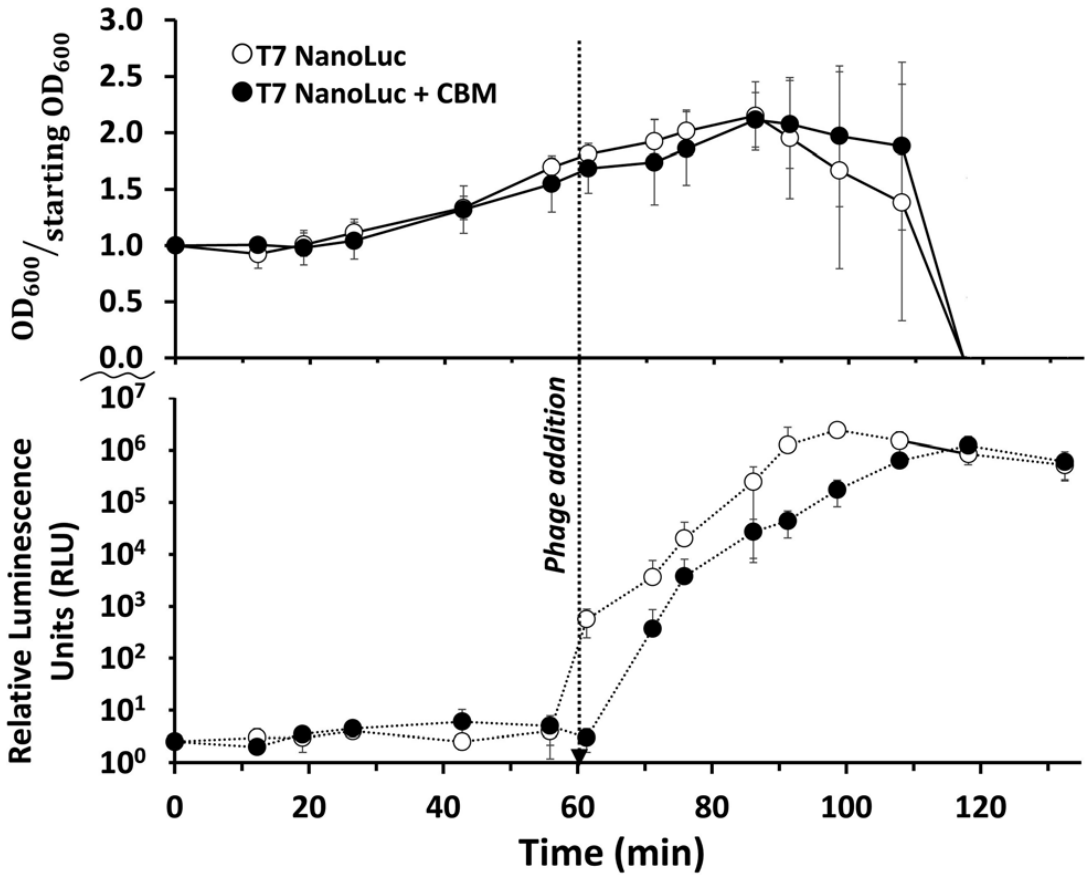

Figure 3: Optical density and luminescence of *E. coli* samples before and after the addition of the phages. The optical density briefly increased following the addition of reporter phage (indicating steady state growth) then rapidly dropped as cells lysed. Data points represent the average of three replicates and error bars represent the standard deviations.

The bioluminescent signal generation was relatively fast following phage addition. While some of the rapid signal generation is due to the carryover of reporter enzyme from the phage lysate, the plateau of the signal occurs 40–50 minutes after phage addition. The final phage stock concentration (10^7 PFU mL^{-1}) was used to maintain an MOI > 1 and therefore minimize the time required for the assay. Lysis times are shortest when phages outnumber bacteria because phage adsorption times are near instantaneous and co-infections could possibly deliver multiple copies of reporter enzyme DNA.[61,62]

Reporter enzyme binding to cellulose

In order to confirm the ability of the CBM to immobilize the enzyme onto cellulose, lysate resulting from the respective infections of $T7_{NL}$ and $T7_{NLC}$ with *E. coli* BL21 were spotted onto regenerated cellulose filter papers. NanoGlo was then directly added to some of the filters and imaged, while another set of filters was washed prior to the addition of substrate. The results demonstrated a spreading of the bioluminescence in relation to the original deposition spot for the $T7_{NL}$ on unwashed filters, while the $T7_{NLC}$ luminescence remained limited to the original lysate spot. Additionally, the washed filters had

a significant drop in bioluminescence for the $T7_{NL}$, while the $T7_{NLC}$ was similar to the unwashed filters (data not shown). This suggested that the CBM facilitated to the immobilization of the reporter enzyme onto cellulose.

Performance of $T7_{NLC}$ for *E. coli* detection

The ability of $T7_{NLC}$ to overexpress reporter enzymes in target bacterial hosts in different stages of growth was evaluated. Exponentially growing cells in broth were infected to evaluate the minimum number of cells required to produce a detectable signal and thus establish a limit of detection. The resulting lysate was vacuum filtered through a cellulose membrane on a filter plate to immobilize the reporter enzyme. Fig. 4 indicates that when coupled with a highly efficient capture mechanism, a detectable signal can be generated from an infection between $T7_{NLC}$ and <10 mid-log phase bacteria.

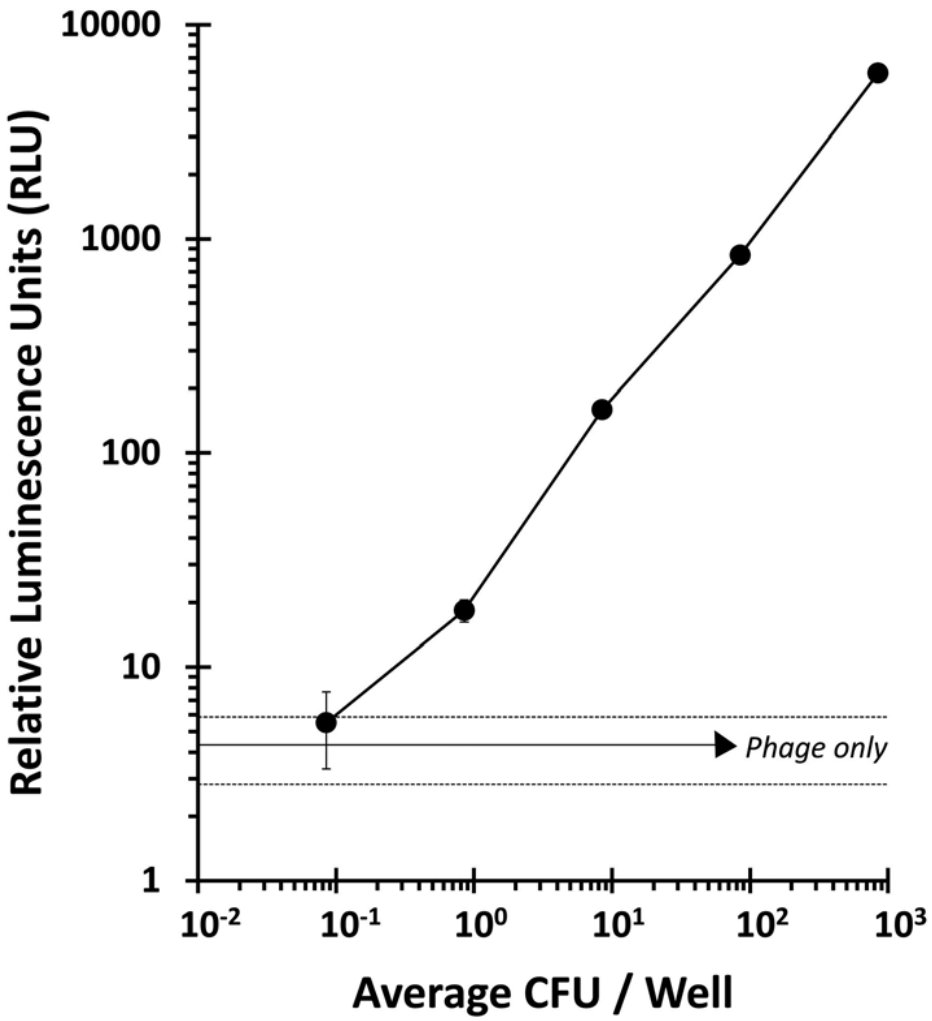

Figure 4: The number of *E. coli* CFUs needed to produce a detectable signal was determined by phage infecting serial dilutions of *E. coli* and passing them through a filter plate. The "phage only" negative control (solid horizontal line) is represented ±standard deviations (dashed lines). Data points represent the average of six replicates and error bars represent standard deviation.

Furthermore, an apparent linear relationship between the *E. coli* population and the resulting bioluminescent signal was observed, indicating that the $T7_{NLC}$ phage can be used to approximate bacterial concentrations from a single CFU to over 10^3 CFU mL^{-1}.

To approximate realistic analytical conditions, *E. coli* were left in sterile filtered lake water for 48 hours to ensure they reached stationary growth phase. Cells were then enriched and infected with $T7_{NLC}$ to evaluate the phage's effectiveness in representative drinking water samples. As seen in Fig. 5, *E. coli* rapidly reached steady state growth allowing a starting concentration of <10 CFU *E. coli* to become detectable after only 3 hours of enrichment.

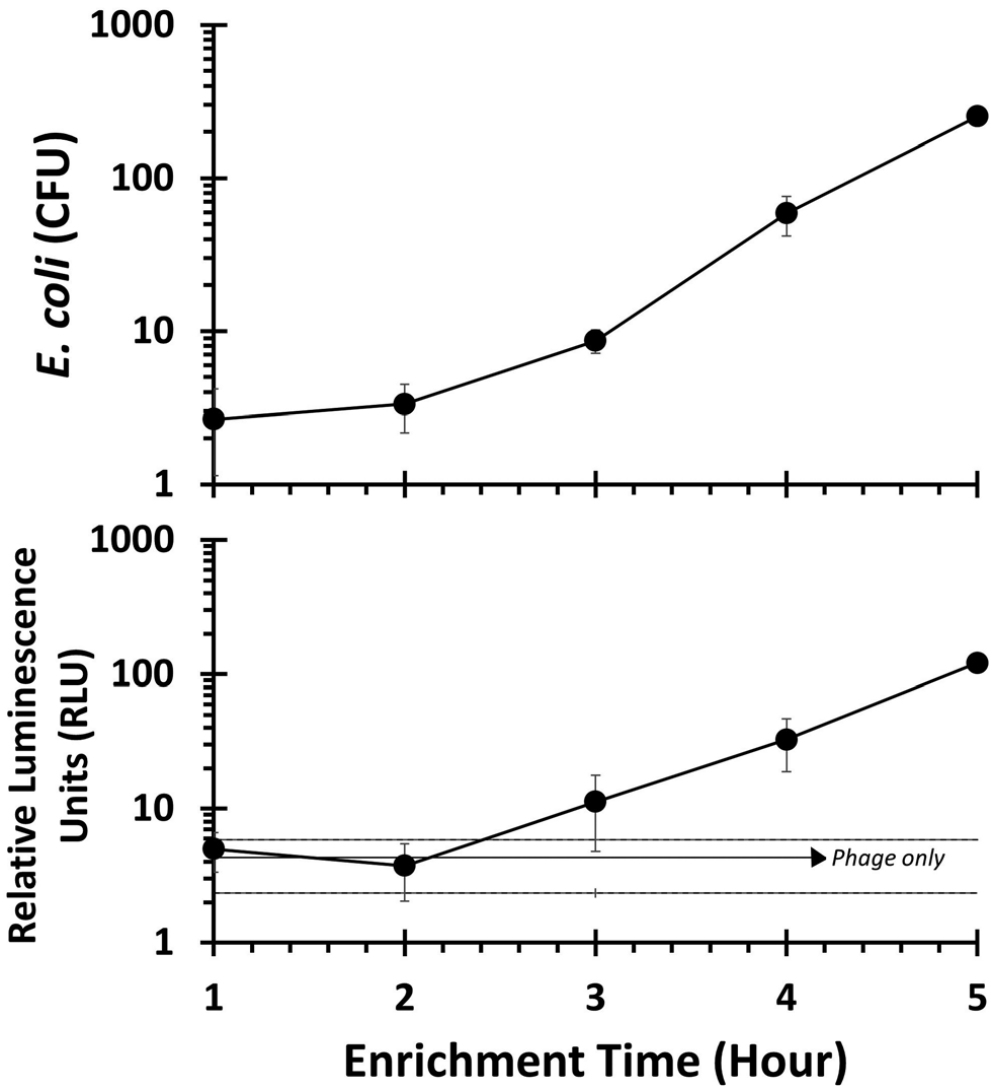

Figure 5: Stationary phase *E. coli* inoculated into lake water was enriched from 1 to 5 hours prior to being infected with $T7_{NLC}$. The results indicate that after 3 hours of enrichment, <10 CFU of *E. coli* are detectable from lake water. The "phage only" negative control (solid horizontal line) is represented as ±standard deviations (dashed lines). Data points represent the average of three replicates and error bars represent the standard deviation.

Phage-based assay conditions for the detection of *E. coli* in 100 mL water samples

The $T7_{NLC}$ phage was used to detect *E. coli* from 100 mL samples of water. Water samples were inoculated with varying concentrations of *E. coli* in order to determine the limit of detection for the assay. Prior to phage/cellulose addition, a 60 minute pre-enrichment step served to resuscitate any bacterial cells that were injured or had reached stationary growth (Fig. 2). While the time can be varied according to host growth rates, the pre-enrichment step is critical for a phage-based assay because phage require an actively growing host for successful infection.[63] Although two recombinant phages were generated in this work, only $T7_{NLC}$, which employed the cellulose affinity tag, was selected for the final detection assay. As seen in Fig. 6, the detectable NanoLuc signal was closely correlated with the bacterial cell population. The detection limit for $T7_{NLC}$ is <10 CFU mL^{-1} in a total assay time of 3 hours.

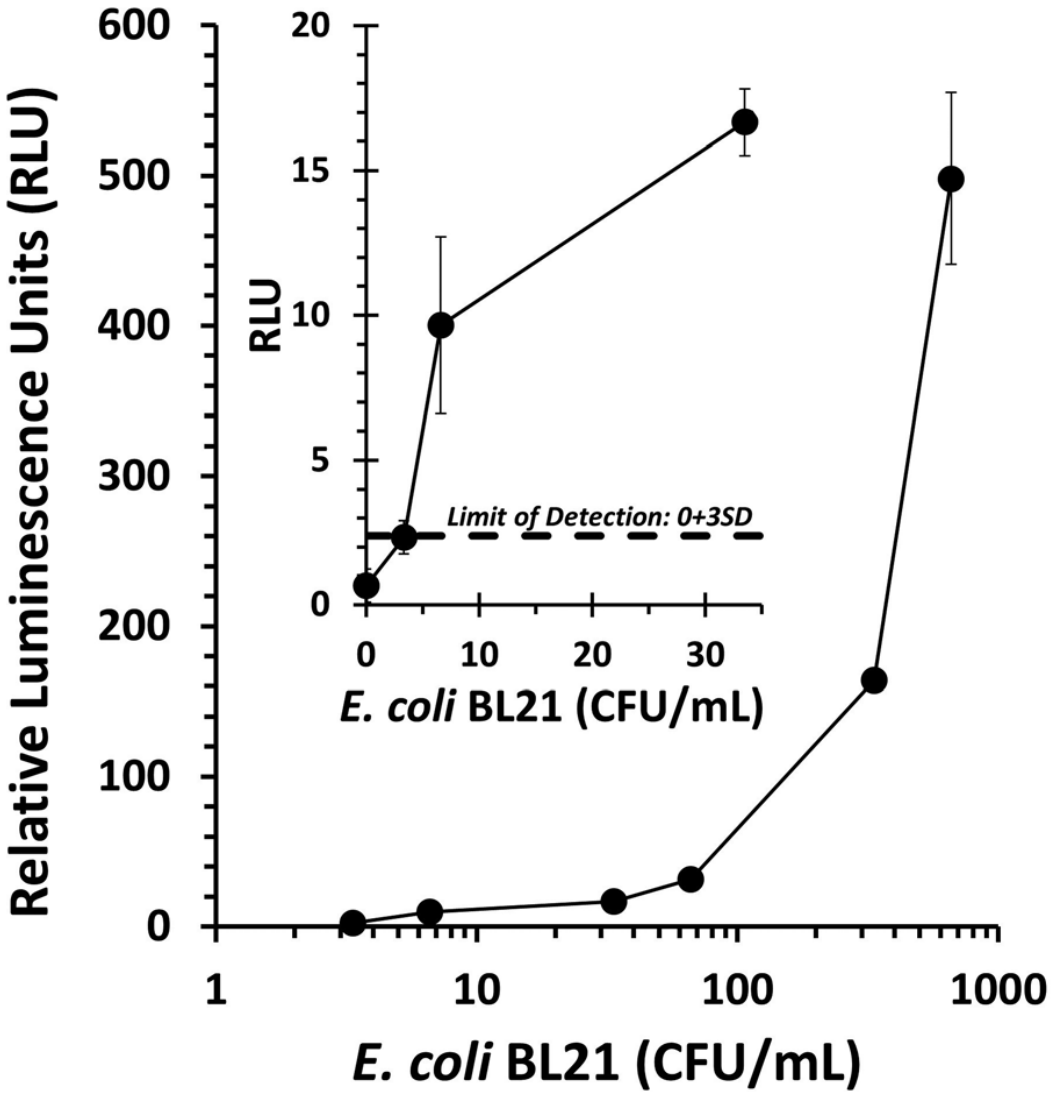

Figure 6: Drinking water (100 mL) containing *E. coli* BL21 was used to determine the dose response of the phage-based assay. Within the range tested, increasing *E. coli* concentrations correlated with stronger bioluminescent signal. The limit of detection was defined as the negative control (0 CFU mL^{-1}) plus three standard deviations. Data points represent the average of three replicates and error bars represent the standard deviation.

Furthermore, the results seen in Fig. 4 suggest a lower limit of detection is achievable and therefore it is likely that the demonstrated 100 mL water assay is: (i) not efficiently infecting the *E. coli* in the larger samples, or (ii) the free cellulose is not efficiently capturing the fusion reporter released from the resulting infections. Additionally, the effect of nonspecific binding must be taken into account as well. It is possible that some signal is lost due to the binding of the reporter enzyme to carbohydrates within the bacterial cell as well as nonspecific interactions with the polypropylene tubes used for the assay.[48]

CONCLUSION

Current methods for the detection of microorganisms in food samples are largely culture based, requiring trained laboratory personal multiple days before a definite result. Furthermore, many pathogenic strains of *E. coli* have such low infectious doses (<100 CFU) that the development of rapid and sensitive detection schemes is required to adequately mitigate potential risk to public health. The successful integration of a newly developed, highly active luciferase gene (*nluc*) into a phage genome, created a reporter phage capable

of rapidly detecting low concentrations of *E. coli*. The *nluc* gene was synthesized within an expression construct specifically engineered for T7 expression. Overexpression of the *nluc* gene was achieved by optimization of regulatory sequences as well as by insertion of the reporter into a highly expressed region of the phage genome. The expression construct employed the strongest native T7 promoter sequence, a custom ribosome binding site, a *pelB* leader sequence, and a codon optimized *nluc* gene. Finally, the most significant development of this work was the genetic fusion of a carbohydrate binding module (*cbm2a*) to NanoLuc within the previously described expression construct. As a result, the functionalized reporter was successfully concentrated through immobilization onto inexpensive, inert, insoluble microcrystalline cellulose. Microcrystalline cellulose dispersed into the bacteria-phage complex successfully captured the novel chimeric NanoLuc::CBM produced as a result of target cell infection. Following a full phage infection, the cellulose can be either actively pelleted by centrifugation or passively separated by gravity depending on access to resources. The insoluble cellulose pellet

can be resuspended in enzyme substrate for immediate measurement or in a biological buffer for downstream applications. The use of cellulose to collect the reporter probe from the bulk sample solution served to reduce the overall sample volume by over 2 orders of magnitude. This was achieved by separating the 0.05 g cellulose added to the 125 mL sample and resuspending the cellulose-enzyme complex in less than 1 mL of substrate following phage infection. Future studies using cellulose filters to capture the reporter probes may demonstrate a significant improvement in the capture of the NanoLuc::CBM resulting in a lower limit of detection. The light emitted from NanoLuc is bright blue as evidenced by a sharp emission peak at 450 nm. When images of NanoLuc are separated in RGB channels, over 98% of signal emitted resides within the blue channel.[64] As a result, NanoLuc has significant potential to be incorporated into a multiplex recombinant phage biosensor (i.e. NanoLuc + Red and/or Green luciferases inserted into phages with different host ranges) that would be capable of rapid and simultaneous differentiation of specific analytical epitopes. Areas for further optimization include broadening phage host range,

increasing enzyme expression during phage infection, and minimizing the enzymatic loss of activity when genetically fusing affinity binding ligands. Genetic modifications to the tail fibers of T7 have been shown to successfully expand the range of permissive hosts.[24] Similar genetic engineering would be straightforward with $T7_{NLC}$ as the NanoLuc expression cassette bears no effect on the tail fibers. Increased enzyme expression will be achieved by further optimization of regulatory and signal sequences as well as the insert location within the phage genome. The optimal leader sequence must be proven empirically, as leader sequences other than *pelB* have been shown to significantly increase expression as well.[55] Furthermore, optimization of the linker sequence between NanoLuc and the CBM could function to fully retain enzymatic activity of our novel chimeric reporter as compared to the standard NanoLuc enzyme. Expression of a reporter enzyme with a CBM causes binding to cellulosic entities within the cell during expression as well as plastic species within standard laboratory tubing and glassware. Blocking of plastics with 1% BSA should lead to additional efficiencies and a lower limit of detection.[48] A current limitation to

phage-based assays remains the need for cocktails of phages in order to cover the desired host range. This is due to the high specificity of the phages which can often be limited to single strains. Ongoing work by the authors and other labs aims to customize the tail fibers of phages to allow tailored host ranges. Such work will allow a better utilization of phages as both detection and therapy tools.

CONCLUSION

Our results suggest that our novel recombinant phages are ideal for an ultrasensitive bacterial detection assay. Our proof-of-principle detection assay utilizes recombinant phages that express novel chimeric reporter enzymes to rapidly and sensitively detect less than 10 CFU mL^{-1} *E. coli*. Lower limits of detection can be reached by increasing the pre-enrichment times thereby allowing the bacteria to reach a higher concentration. As we demonstrated the successful genetic engineering of a phage to sensitively detect *E. coli*, similar efforts could provide low cost detection assays to other fields burdened with bacterial threats including food and water safety, medical diagnostics, animal health, and bio threat detection.

Conflicts of interest

The authors report no conflicts of interest.

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CHAPTER 4

A PHAGE-BASED ASSAY FOR THE RAPID, QUANTITATIVE, AND SINGLE CFU VISUALIZATION OF *E.COLI* (ECOR #13) IN DRINKING WATER*

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ABSTRACT

Drinking water standards in the United States mandate a zero tolerance of generic *E. coli* in 100 mL of water. The presence of *E. coli* in drinking water indicates that favorable environmental conditions exist that could have resulted in pathogen contamination. Therefore, the rapid and specific enumeration of *E. coli* in contaminated drinking water is critical to mitigate significant risks to public health. To meet this challenge, we developed a bacteriophage-based membrane filtration assay that employs novel fusion reporter enzymes to fully quantify *E. coli* in less than half the time required for traditional enrichment assays. A luciferase and an alkaline phosphatase, both specifically engineered for increased enzymatic activity, were selected as reporter probes due to their strong signal, small size, and low background. The genes for the reporter enzymes were fused to genes for carbohydrate binding modules specific to cellulose. These constructs were then inserted into the *E. coli*-specific phage T7 which were used to infect *E. coli* trapped on a cellulose filter. During the infection, the reporters were expressed and released from the bacterial cells following the lytic

infection cycle. The binding modules facilitated the immobilization of the reporter probes on the cellulose filter in proximity to the lysed cells. Following substrate addition, the location and quantification of *E. coli* cells could then be determined visually or using bioluminescence imaging for the alkaline phosphatase and luciferase reporters, respectively. As a result, a detection assay capable of quantitatively detecting *E. coli* in drinking water with similar results to established methods, but less than half the assay time was developed.

INTRODUCTION

Clean drinking water has been declared a fundamental human right, yet millions still lack access to consistently clean sources of potable water[1]. Pathogenic *Escherichia coli (E. coli),* a common drinking water contaminant, is a major cause of morbidity and mortality worldwide. While the WHO estimates approximately 63,000 annual deaths are due to *E. coli* infections, the added consequence of 5 million years of life lost (YLLS) and 5 million disability adjusted life years (DALYS) further compounds the suffering caused by this pathogenic contaminant[2]. In addition to GI tract infections, *E. coli* is responsible for 8.9% of sepsis cases, 29% of early onset neonatal sepsis cases and the majority of urinary tract infections[3,4]. Generic *E. coli* species which consist of both pathogens and non-pathogens, are ubiquitous in mammalian feces[5,6,7,8,9]. Because these organisms are naturally found in the feces of mammals in high concentrations, their presence is a biological indicator of fecal contamination and therefore possible pathogens in drinking water[10].

The United States EPA and FDA have set a limit of zero CFU generic *E. coli* in 100 mL for drinking water and postharvest produce rinse

water, respectively. Untreated agricultural water, such as that used for irrigation, has a maximum geometric mean (GM) of 126 CFU or less with a statistical threshold value (STV) of 410 or less of generic *E. coli* in a 100 mL sample[11]. These same requirements are used by the EPA for untreated recreational water. EPA Method 1603 is an approved drinking water assay that quantifies generic *E. coli* with an assay time of 24 hours. This drinking water assay contains a membrane (0.45 μm) filtration step to remove bacteria from the water sample prior to enrichment on selective and differential media. The bacteria CFUs are quantified directly on the filter following a lengthy enrichment[12].

An alternative assay used to detect generic *E. coli* in agricultural and produce rinse water is the most probable number (MPN) method. The MPN is determined by serial diluting the water sample in triplicate or pentaplicate followed by incubation in selective growth media. The tubes are then assessed for bacterial growth and the highest dilution numbers for each replicate are used to statistically estimate the MPN of the original sample.

While both EPA 1604 and MPN can detect a single *E. coli* CFU in 100 mL of water, the prolonged incubation periods necessary for visual identification make them less practical for time sensitive applications. For example, the days required to receive results for drinking water or recreational water may be too long to prevent individuals from becoming infected. Similarly, the results for *E. coli* counts in produce rinse water may not be available until after the produce has been sold and consumed. Due to rapid spoilage, many types of produce may be sold and consumed before microbial results from traditional methods are available. Therefore, there is a significant need to rapidly detect *E. coli* in water samples while maintaining high sensitivity and quantification.

While the identification of indicators and/or pathogens often involves culturing of serological, food, or environmental samples, new technologies aim to significantly decrease assay times. Although some advanced technologies (e.g. optical nanostructures, surface enhanced Raman spectroscopy, flow cytometry, etc.) have shown promise as sensitive detection methods, they typically process only small, relatively clean samples. The true bottleneck to rapid

detection methods remains the separation of a target analyte from a large complex matrix.

Currently, a pragmatic system that integrates sample preparation and rapid pathogen detection from large volume samples remains elusive. While the vast majority of research focuses on improving sensitivity of pathogen detection rather than separation/concentration, an ideal detection platform would incorporate all these components into one rapid, sensitive and specific detection assay. Additionally, while some regulations may be satisfied with binary presence/absence results, others, such as those for irrigation water and untreated recreational water require quantitative results. Therefore, there is a need to develop a rapid detection assay with the ability to detect 1 CFU/100 mL of viable target bacteria, while also providing fully quantitative results. These attributes mimic those of the current EPA 1603 method which is widely used for the testing of generic *E. coli* from water samples.

A new generation of rapid tests to detect bacteria utilize engineered bacteriophages[13,14]. Bacteriophage (phages) are viruses that infect bacteria in a strain-specific manner. Phages exhibit specific host

ranges due to the complex interactions involved in phage attachment to the bacterial cell surface. Following adsorption, the phage injects its genome into the bacterial host and initiates infection. Then bacterial DNA transcription and translation systems begin the process of replicating many more infectious phage particles along with the lytic enzymes responsible for eventual bacterial lysis. These viral predators contain robust biorecognition elements commonly employed in sensitive bacterial detection assays[13,14,15,16,17,18,19,20,21,22]. We took advantage of a naturally evolved viral infection process that dramatically alters bacterial protein expression to overexpress engineered reporter enzymes.

While, previous studies have reported the integration of phage-based testing and filtration for water testing, the migration of reporter probes prohibited full quantification and single CFU visualization[23]. We hypothesized that the fusion of an affinity binding module to the reporter probe could effectively immobilize the probe in vicinity to the lysed bacterial host, allowing improved quantification. Therefore, a phage-based detection assay for generic *E. coli*, modeled after the widely used membrane filtration assay, EPA Method 1603, for

drinking water was developed. First, highly active reporter enzymes was genetically fused to a carbohydrate binding module (CBM) with specificity for cellulose. The reporter enzymes were then genetically engineered into a phage specific to *E. coli*. Following infection, the bacteria trapped on a cellulose filter lysed releasing the reporter probes, which then immobilized locally. The addition of bioluminescent or colorimetric substrates allowed a visual quantification of the host *E. coli* colonies on the filter. Here we integrate several technologies to construct a phage-based detection platform that is quantitative and can allow the visualization of individual CFU's from a 100 mL water sample. The power of our approach is that it integrates a custom phage that produces a highly active fusion enzyme which can be immobilized to allow improved quantification.

MATERIALS & METHODS

Reagents and materials

The luminescent substrate, NanoGlo, was purchased from Promega (Madison, WI, USA) and prepared immediately before use according to the manufacturer's specifications. All other reagents were

purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. The colorimetric phosphatase substrate, 5-bromo-chloro-3-indolyl-phosphate p-toluidine salt (BCIP), was prepared as a stock solution (20 mg/mL) in N,N-Dimethylformamide (DMF) and stored at –20 °C. A BCIP working solution (2 mg/mL) was prepared in 1 M Diethanolamine (DEA) buffer immediately prior to use. Bioluminescent images were captured using long exposures (30 s) with a DSLR camera (Rebel T6, Canon, Melville NY, USA) in a dark box (LTE-13, Newport Corporation, Irvine, CA, USA). Nalgene™ disposable analytical test filter funnels (145-0045) for water testing were used to house the regenerated cellulose filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany). All filters were 47 mm diameter with a 0.22 μm pore size.

Bacteria, phages and culture media

E. coli BL21 was obtained from ATCC (Manassas, VA USA) and *E. coli* (ECOR #13), a strain isolated from a healthy human, was obtained from the Thomas S. Whittam STEC Center (East Lansing, MI, USA). Bacterial cultures were initially stored at –80 °C in 25% glycerol prior to use and were grown in Luria Bertani (LB) broth and

plated on LB agar. Overnight cultures of *E. coli* were prepared in 10 mL of LB inoculated with a single bacterial colony and incubated (37 °C, 200 rpm, 18 hr). Serial dilutions were performed in sterile phosphate buffer saline (PBS). Bacteria and phages were enumerated using standard plate counts and double overlay plaque assays, respectively. The lytic coliphage T7 (T7Select 415-1) used in this work was designed as a cloning vector for routine phage display applications. The phage DNA used in this study was purified from propagations of T7Select 415-1 DNA in *E. coli* BL21, purchased from EMDMillipore (Burlington, MA, USA).

Phage Stock Solutions

Exponentially growing host cells (200 mL) were infected with phage at an MOI of 0.1 until cellular lysis caused a significant decrease in OD600 (1.5–2 h). Low speed centrifugation was used to clear cellular debris (3,200 × g, 10 min, 4 °C) before sterile filtration (0.22 μm). Polyethylene glycol 6000 (PEG6000; 4%) and sodium chloride (NaCl; 0.4 M) were added and incubated overnight at 4 °C to precipitate phage particles. Phage were pelleted by ultracentrifugation (35,000 × g, 120 min, 4 °C), resuspended in

phosphate buffered saline (PBS, pH 7.4), enumerated, and stored at 4 °C. All phage used in detection assays were diluted to 1 × 109 PFU/mL in LB, sterile filtered (0.22 μm) and stored at 4 °C.

Phage DNA Isolation

Phage lysates of sufficient concentration (>10^{11} PFU/mL) were used for genome extraction and purification. The phage stock solution was treated with sodium dodecyl sulfate (SDS; 2%) for 20 min. at 70 °C to disrupt the capsid release phage genomic DNA. After cooling on ice, DNA was precipitated with sodium acetate (0.3 M) and ethanol (70%). The sample was centrifuged (10,000 × g, 10 min, 4 °C) and the supernatant was passed through the Qiagen Genomic Tip 100/G according to the manufacturer's recommendations.

Reporter Enzyme Expression Constructs

A phosphatase and luciferase were chosen as reporter enzymes to transduce colorimetric and luminescent signals, respectively. Muller et al. performed random mutagenesis on bacterial alkaline phosphatase and demonstrated that two amino acid substitutions (D153G/D330N) increased enzymatic activity by more than two orders of magnitude26. Similarly, Hall et al. engineered a

bioluminescent enzyme and substrate system (NanoLuc & NanoGlo, respectively) capable of generating a much stronger signal compared to other commonly employed luciferases[24]. An affinity binding motif with irreversible binding to crystalline cellulose (CBM2a) was identified in the xylanase 10A gene from Cellulomonas fimi[33], and employed as a genetic fusion to specifically immobilize the chosen reporter enzymes34. The CBM gene was genetically fused to the C-terminus of the reporter enzyme while a strong T7 promoter and ribosome binding site were inserted upstream to force high levels of expression. Finally, the expression cassette was flanked by regions homologous to the phage multicloning site and synthesized as a double stranded DNA fragment by IDT (Coralville, Iowa, USA).

Construction & Isolation of Reporter Phages

Phage genomic DNA was isolated and purified from propagations of T7 Select 415-1 as described previously. Purified phage DNA was digested with HindIII to prepare the vector for reporter gene insertion. The reporter gene, containing homology to each vector arm, was added to the phage genomic vector at a 2:1 molar ratio

and was assembled using NEBuilder® Hifi DNA Assembly Master Mix (NEB, Ipswitch, MA). Transformations were performed in electrocompetent *E. coli* DH10B (MegaX, ThermoFisher) in 1-mm cuvettes under standard conditions. Recovery was performed in SOB with shaking until visible signs of lysis occurred. Serial dilutions were performed until double overlay plaque assays revealed individual plaques. Correct clones were identified with application of enzymatic substrate and imaging as described previously. Positive plaques were further evaluated using PCR to verify insert size and full genome sequencing. The constructs for the inserted genes are shown in Fig. 1.

Enzyme Substrates & Imaging

Alkaline Phosphatase

The phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphatase, 4-toluidine salt (BCIP) was dissolved in DMF (20 mg/mL) and stored at –20 °C. Immediately before use, BCIP stock was diluted tenfold in diethanolamine buffer (1 M DEA, pH 10.1) and applied directly to the filter. A short incubation (37 °C, 10 min.) generated sufficient color development for imaging. All

images were captured with a DSLR camera on an LED light box (AGPTek, Brooklyn, NY, USA).

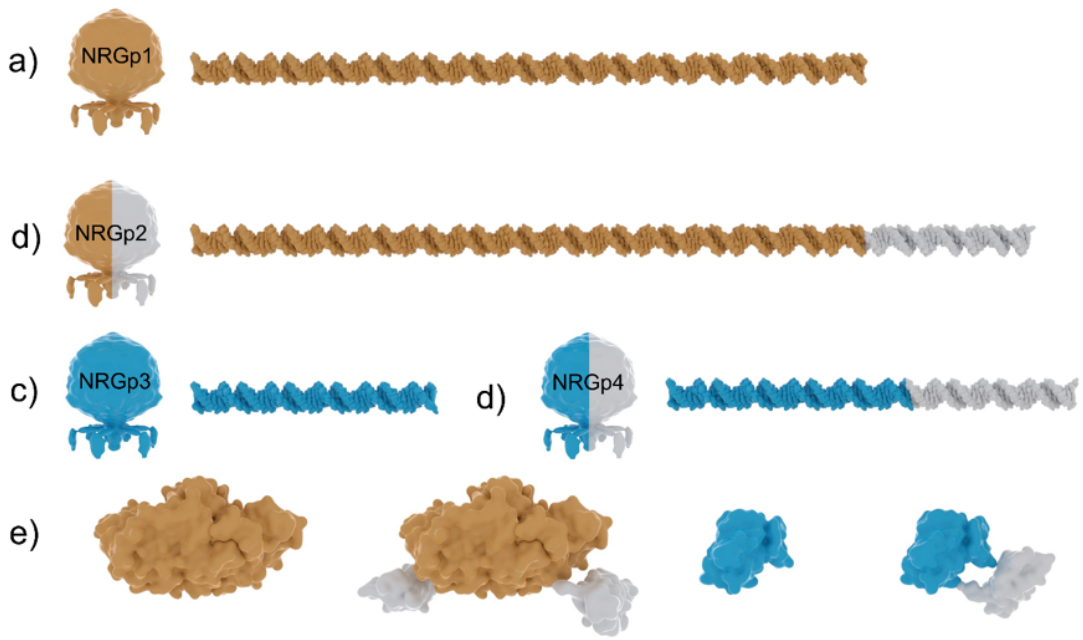

Figure 1: Reporter enzyme fusion design for the engineered bacteriophages. Reporter genes were synthesized within expression constructs as described previously. The inserted nucleic acid lengths for a) alkaline phosphatase, b) alkaline phosphatase + CBM, c) NanoLuc and d) NanoLuc + CBM are shown in relative scale. The resulting proteins (e) are represented in scale using pdb files 5ibo, 1kh7, 1exg for NanoLuc, Alkaline Phosphatase and CBM2a, respectively.

NanoLuc

NanoGlo buffer was prepared according to the manufacturer's recommendations immediately before use. The filter membranes were fully saturated in substrate (~300 μL) prior to imaging. Long exposure bioluminescent images were captured with a DSLR camera in a dark box using 30 second exposure times.

Assay procedure

The phage-based procedure used the initial filtration steps of EPA method 1603 followed by phage infection and imaging (Fig. 5). Drinking water samples (100 mL) were obtained from a local municipal water source (Ithaca, New York, USA) and autoclaved. The sterile drinking water samples were inoculated with varying concentrations of stationary phase *E. coli* (ECOR #13) and filtered according to the procedure outlined in EPA Method 1603. Following filtration, the filter membrane was removed and placed onto an absorbent pad saturated with LB broth. The filters were incubated (37 °C, 8–12 hours) to allow for colony growth. Following the initial enrichment, a phage solution (2 mL, 109 PFU/mL in LB) was applied to the filter and incubated (37 °C, 90 min) to initiate phage infection

and reporter probe expression. After brief drying on a sterile absorbent pad, the enzymatic substrates were applied and imaged as described previously.

In order to characterize the performance of the phage-based method, the concentration of *E. coli* in the water samples was determined in parallel using the EPA method 1603[12].

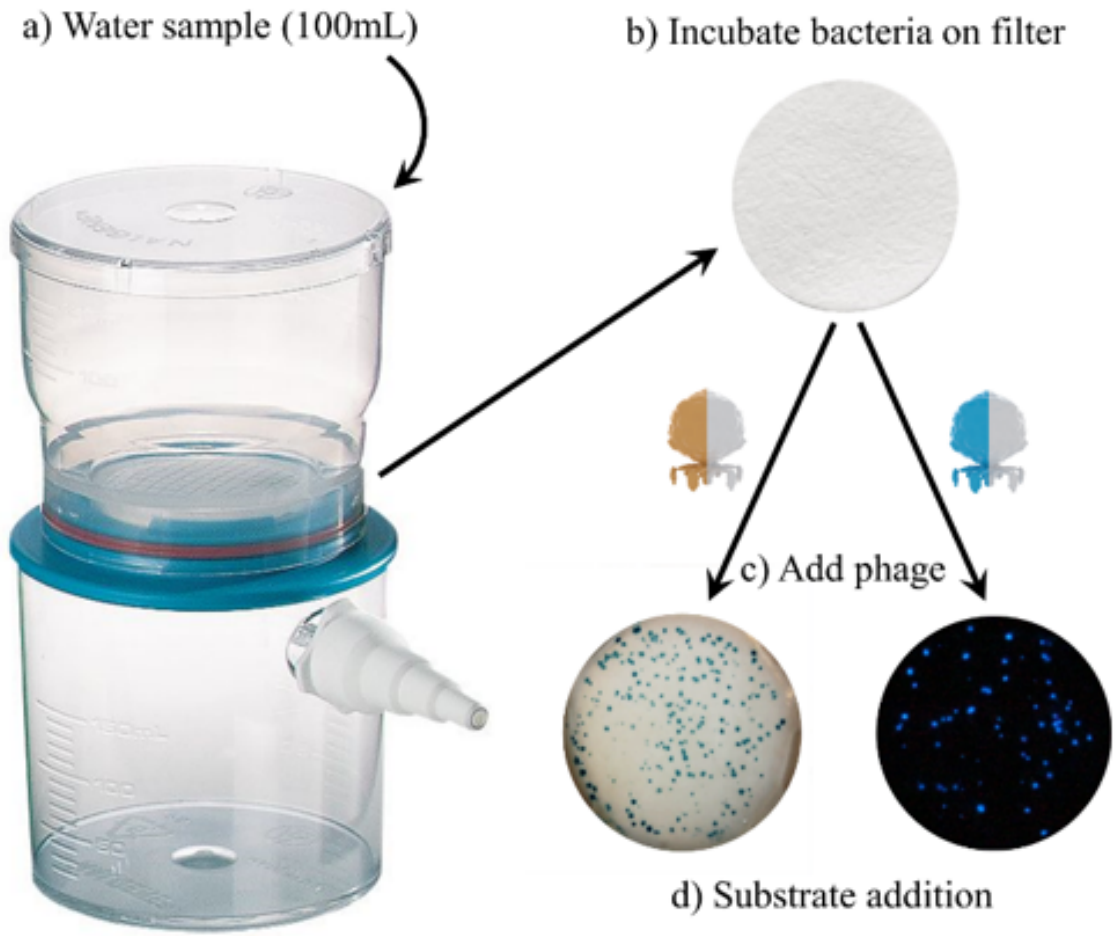

Figure 5: Schematic diagram of the phage-based detection assay. a) The 100 mL water sample passes through the filter, retaining any bacterial contaminants. b) The filter is placed on media and incubated to allow for colony formation. c) Recombinant phages are added to force expression of the desired reporter. d) Substrate addition provides a colorimetric signal for NRGp2 and a bioluminescent signal for NRGp4.

Following filtration, the filter was placed onto modified mTEC agar and incubated according to the methods specifications. Colonies were counted after the required 24-hour incubation period.

Image analysis

Images of the bioluminesnce on the capture filters were analyzed using ImageJ. The relatively low background of the bioluminescent images allowed the pixel intensities to be multiplied by three resulting in an improved visualization of the spots. The spots were counted both visually as well as using the imageJ software to allow a determination of the accuracy of the image analysis. Spot sizes and distribution were determined using ImageJ particle size distribution.

RESULTS

Phage Construction, Isolation & Characterization

The *alp, alp::cbm and luc, nluc::cbm* reporter genes were successfully inserted into coliphage T7 to generate the reporter phages NRGp1 (MH651795), NRGp2 (MH651796), NRGp3 (MH651797), & NRGp4 (MH651798), respectively. Initial screening was performed using PCR and Sanger sequencing was used for

validation. Full genome sequencing revealed no significant mutations in the remainder of the genome. No significant differences in plaque morphology, burst size and/or lysis times were observed between the recombinant and wild type phages (data not shown).

Reporter probe characterization

NanoLuc, a luciferase engineered from deep sea shrimp, was selected as a luminescent reporter due to its strong signal, small size and low background[24]. NanoLuc is an ideal reporter enzyme candidate for this work as it has previously been employed in phage-based detection assays[13] and also as a genetic fusion partner[25]. The alkaline phosphatase double mutant was selected as a colorimetric reporter due to its high enzymatic activity, low background and the wide variety of substrates available[26].

We used the lytic phage T7, originally designed as a phage display platform (T7 Select), which contains a multiple cloning site directly downstream of the capsid gene[27,28]. The expression cassette contained a stop codon upstream of the reporter in order to highly express the standalone reporter enzyme as opposed to the capsid fusion commonly employed in phage display. This insertion location

has previously been used in both phage display[29] and reporter phage applications[15,30]. Furthermore, the strong phi10 T7 promoter[31] and a custom ribosome binding site were used to promote enzyme expression[32].

The enzymatic activity of the two reporters used, alkaline phosphatase and NanoLuc luciferase, have been engineered to be over two orders of magnitude greater than their respective counterparts[24,26]. In this work, these two highly active enzymes were functionalized with an affinity binding motif through the genetic fusion of a carbohydrate binding module (CBM) from Cellulomonas fimi with specificity to cellulose (CBM2a). As seen in Fig. 1, four total recombinant phages were generated (two of which carried celulose binding functionality) and were used in detection assays.

To evaluate the binding affinity of the CBM fusions, phage lysates containing the respective reporter enzyme were slowly spotted onto the center of a cellulose filter allowing for passive diffusion to completely saturate the membrane. Following drying in ambient air, the respective substrates were applied and images of the filters were captured. As seen in Fig. 2, T7 with a NanoLuc+CBM gene

(NRGp4) and T7 with an alkaline phosphatase + CBM gene (NRGp2) displayed limited diffusion and a stronger, concentrated signal whereas T7 with a NanoLuc gene (NRGp3) and T7 with an alkaline phosphatase gene (NRGp1) both exhibit significant diffusion and signal dilution. Similar relative binding affinities were displayed when the filters were washed with a biological buffer prior to substrate addition. To a lesser extent, the same phenomenon was observed when the respective phages were used in the detection assay.

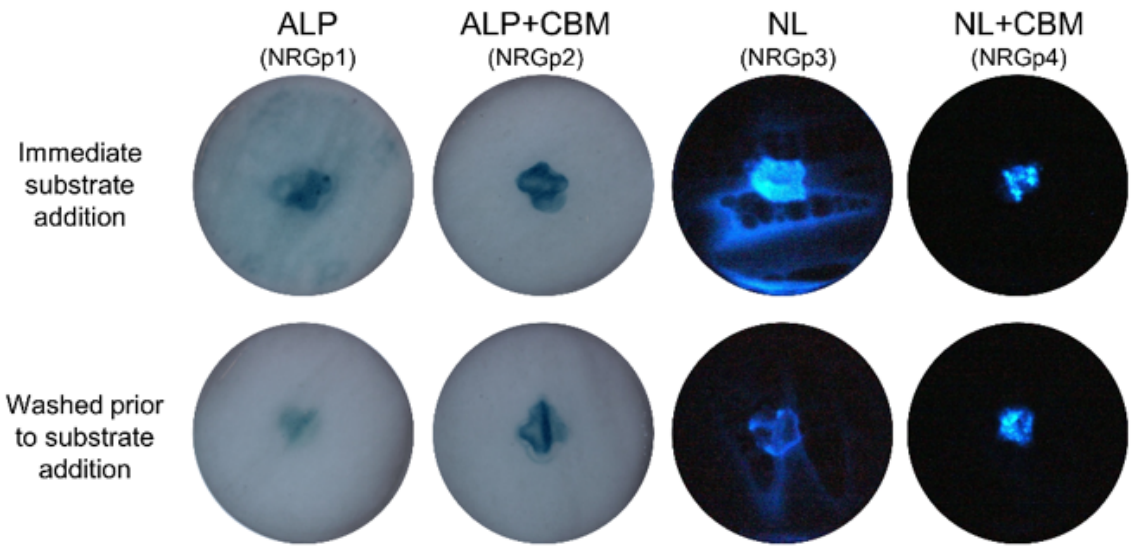

Figure 2: Reporter probes fused with a carbohydrate binding module significantly limits diffusion across. The reporter probes were spotted (1 mL) onto the center of the filter until the entire filter was saturated by diffusion. The effect of the carbohydrate binding module (CBM) fusion to the reporter enzymes was visualized by the degree of diffusion from the center of the filter (top row). The binding strength was further evaluated by washing the filter with PBS before substrate addition (bottom row).

Detection of *E. coli* colony forming units

Determination of assay time

Detection assays employing membrane filtration require pre-enrichment to allow the bacteria on the filter to grow to a sufficient density cells at which a detectable signal can be generated. The amount of pre-enrichment needed for a bacterial colony to grow to a detectable size depends on several factors (generation time, growth conditions, reporter enzyme kinetics, etc.) and was investigated for the proposed assay. While the first visible indication of colony formation occurred well before 8 hours of pre-enrichment for ECOR #13, 8 hours was selected as the ideal pre-enrichment time to minimize false negative results by ensuring that smaller colonies grew large enough to produce a measurable signal. With only 90 minutes required for phage infection and 15 minutes for substrate addition and imaging, our phage based detection assay provided quantifiable results in less than half the time of traditional detection assays.

Visualization of colonies

The NanoLuc enzyme, when complexed with its substrate NanoGlo, exhibits a blue luminescent signal with a peak emission at 460 nm. To mitigate signal decay, a camera was placed in close proximety to the cellulose filters. Although alkaline phosphatase has a wide range of available substrates, the colorimetric substrate 5-bromo-chloro-3-indolyl-phosphate p-toluidine salt (BCIP) was used to visualize bacterial colonies infected with either NRGp1 or NRGp2. The insoluble blue precipitate which formed as a reaction product between alkaline phosphatase and BCIP was easily visualized by the naked eye, with no requirements for specialized equipment (Fig. 3).

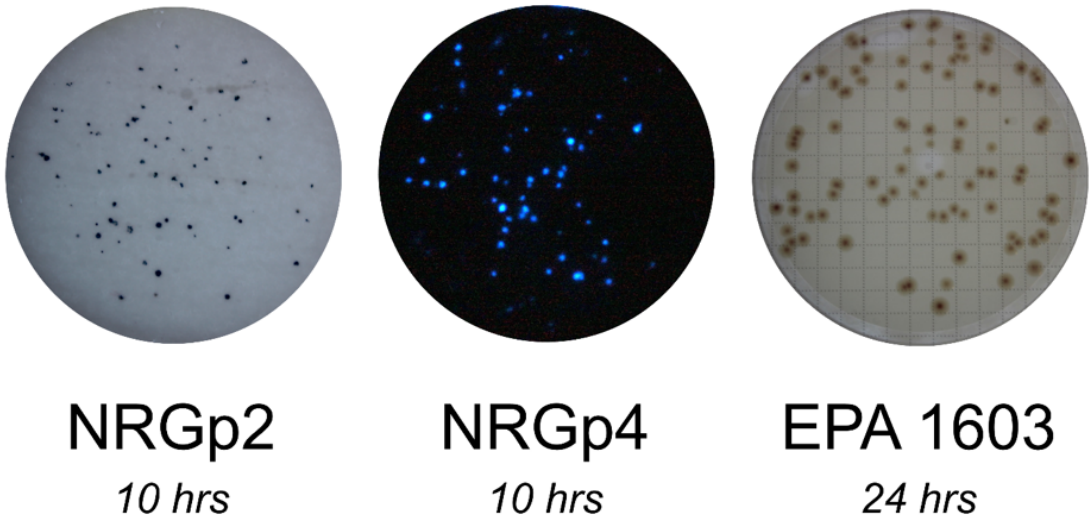

Figure 3: Visual comparison of the EPA method 1603 with the phage-based methods. While the EPA method requires a significantly longer time for results, the use of phage-based reporters allows a more rapid determination. The times listed are the total assay times including incubation. All filters diameters are 47 mm.

Phage Based Detection vs. EPA Method 1603

EPA Method 1603 requires pre-enrichment on selective and differential media (modified mTEC) to enumerate total coliforms for water quality testing. Drinking water samples were spiked with varying concentrations of *E. coli* (ECOR #13) and evaluated using EPA Method 1603, the NRGp4 phage method, and the NRGp2 phage method.

The phage-based methods used 8 hours of pre-enrichment, followed by a 90-minute phage infection period. From initial filtration to final results, the total assay time for the phage-based methods was approximately 10 hours, whereas the EPA method requires 24 hours before accurate counts could be observed. Images of representative filters are shown in Fig. 3.

Each dilution of *E. coli* was run in triplicate for each of the three methods. A negative control containing no inoculated *E. coli* was also for all methods. The CFU counts for the methods were compared to determine agreement. For each dilution, EPA Method 1603 was compared to the NRGp4 method resulting in a linear relationship with a slope of 0.89 ($R^2 = 0.99$). Similarly, EPA Method

1603 was compared to the NRGp2 method resulting in a linear relationship with a slope of 1.02 (R^2 = 1.00). As seen in Fig. 4, the variations observed between the phage-based methods falls within the variation of the EPA test. Two-factor ANOVA with replication was used to determine the difference between EPA Method 1603 and the two phage-based methods at the several bacteria concentrations reported. The results indicate there was no significant difference at the $p<0.01$ level, between EPA Method 1603 and the NRGp2 method [$F(1,3)=1.105$, $p=0.306$] and EPA Method 1603 and the NRGp4 method [$F(1,3)=1.667$, $p=0.689$]. We can conclude that both the phage-based tests fall within the natural variation of the accepted EPA Method 1603 at low bacterial concentrations, and therefore provide comparable results in less than half the time.

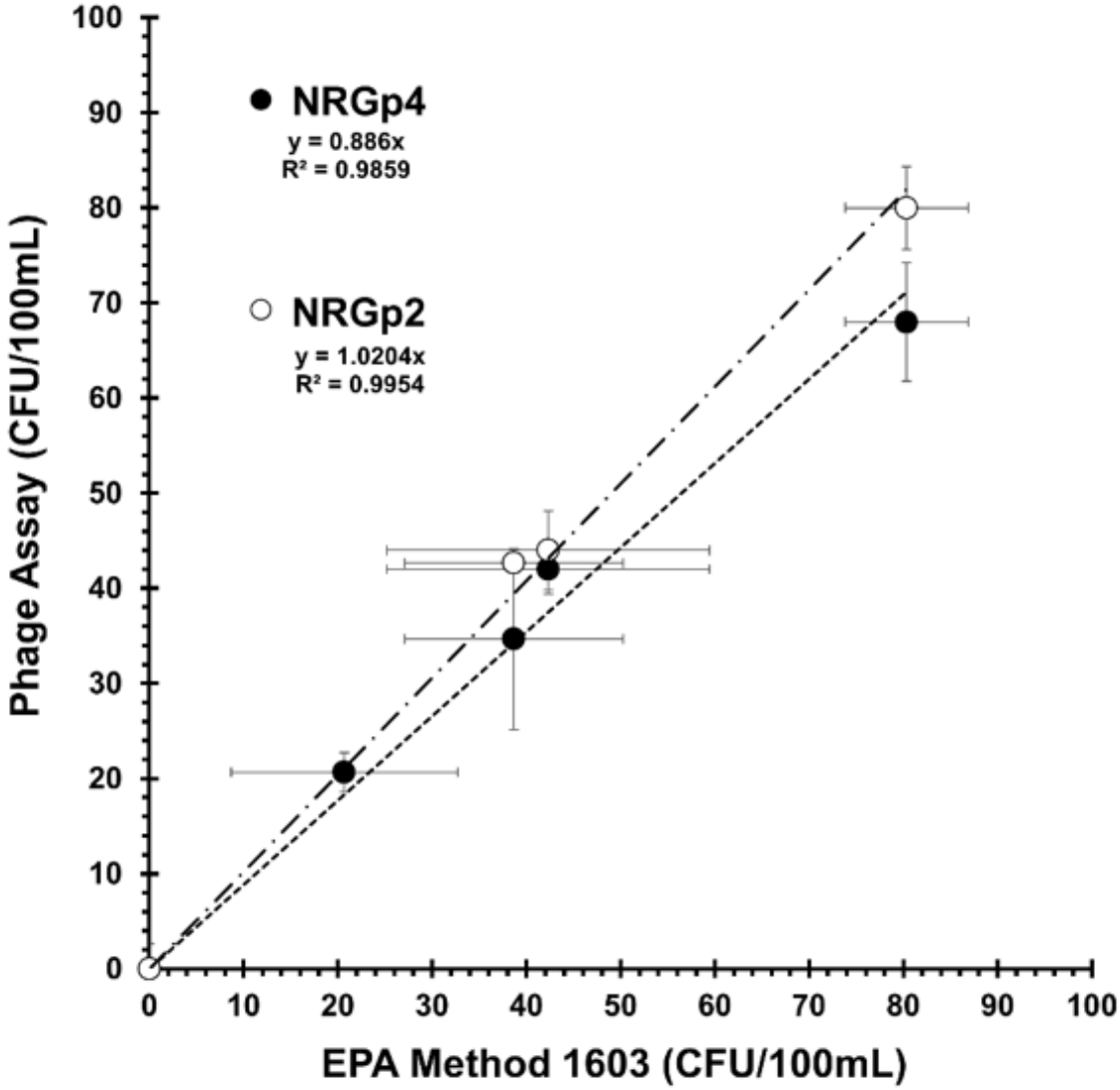

Figure 4: Performance comparison of the phage-based methods against the approved EPA Method 1603. Dilutions of *E. coli* in 100 mL of water were tested in triplicate using all three methods. Both NRGp2 (ALP+CBM) and NRGp4 (NanoLuc+CBM) methods showed similar CFU counts over the different concentrations. Error bars represent the standard deviation of three replicates.

DISCUSSION & CONCLUSION

Detection of bacterial pathogens or their indicators in larger samples of water is critical to ensuring safe drinking water in developing countries. Given the zero tolerance regulatory requirements for drinking water of 0 CFU/100 mL, sample sizes below 100 mL risk the possibility of false negative results. Additionally, concentration methods which result in low capture efficiencies could also contribute to false negatives. We have developed a phage-based detection assay that utilizes the cellulosic filter as a functional surface with a high capture efficiency. We have also demonstrated the effectiveness of the method to deliver quantitative results which are statistically similar to the established method within the required range. Two phage-based filtration assays were compared to the current 24-hour EPA method and yielded similar results in only 10 hours. Given that phages exist for almost all bacteria, this method can be modified for other pathogens and indicators in other liquid samples such as urine, beverages, and environmental waters.

The use of reporter probes with fusion tags, which enable them to bind the filter, allowed the labeling of the individual CFU locations,

and prevented the bleeding and overlapping of the enzyme product. The discrete localization of the enzyme product in proximity to the lysed bacterial cells allowed the counting of the individual CFUs and thus enabling quantification. As with all methods, higher bacterial concentrations resulted in overlapping colonies that leads to natural variations in counts. While the 47 mm filters used in the assay are recommended for < 100 CFU in order to reduce the frequency of overlapping colonies, larger filters could be used to allow for a larger dynamic range.

Given the practical limitations of isolating a single viable CFU for the validation of single CFU detection ability of an assay, we performed a direct comparison between our proposed methods and the established EPA method using the same inoculated water samples. From these results, similar variability within all assay formats was observed.

By using the engineered phages and reporter probes with cellulose-affinity, we have incorporated the filter material into the signal generation and readout. This resulted in a lab-on-a-filter that could be used to identify discrete CFUs from large samples in a

significantly shorter time than the current standard. As will all bacterial detection assays, a high degree of specificity is critical. In order to ensure the proper host range, phage-based assays and therapeutics commonly use cocktails of phages with differing specificities. In the future, the ability to use genetic engineering to engineer the host range of phages will have a significant impact on their utility. In the assay presented in this report, we selected a single *E. coli* isolate to demonstrate the detection platform. The isolate, ECOR #13, was isolated from a human and therefore represents an indicator of fecal contamination. While a field-ready version of this assay would most likely require a cocktail of engineered phages for the appropriate host range, we have demonstrated a proof of principle for an assay format which could enable faster field testing than those currently used in the field which require up a minimum of 24 hours to achieve quantitative results.

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CHAPTER 5

A SYRINGE-BASED BIOSENSOR TO RAPIDLY DETECT LOW LEVELS OF *ESCHERICHIA COLI* IN DRINKING WATER USING ENGINEERED BACTERIOPHAGES*

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ABSTRACT

A sanitized drinking water supply is an unconditional requirement for public health and the overall prosperity of the human race. Potential microbial and chemical contaminants of drinking water have been identified by a joint effort between the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) who together establish guidelines that define, in part, that the presence of *Escherichia coli* in drinking water is an indication of inadequate sanitation and a significant health risk.

As *Escherichia coli* is a nearly ubiquitous resident of mammalian GI tracts, no detectable counts of such an organism in 100 mL drinking water is the standard used worldwide as an indicator of sanitation.

One important threshold requires that 100 mL of drinking water contain no detectable *Escherichia coli* (*E. coli*). In response, we developed a rapid bacteriophage-based detection assay with detection limit capabilities comparable to traditional methods in less than a quarter of the time.

We coupled membrane filtration with selective enrichment using genetically engineered bacteriophage (phage) to identify less than 20 CFU *E. coli* in 100 mL drinking water in 5 hours.

The combination of membrane filtration with phage infection produced a novel assay that demonstrated the rapid, selective and sensitive detection of an indicator organism in large volumes of drinking water as recommended by the leading world philanthropic organizations.

INTRODUCTION

Every person on the planet has a fundamental human right to water and sanitation.[1] Unfortunately, millions still lack access to potable water sources within reasonable walking distance from their homes.[2] Not only does microbial contamination of drinking water contribute significantly to morbidity and mortality worldwide, it disproportionally affects members of low income countries.[3] Improper sanitation of drinking water sources is strongly correlated with the presence of coliforms (a widely variable group of gram-negative rod shaped bacteria that possess a range of biochemical attributes[4]). *Escherichia coli* (*E. coli*) is a member of the coliform family, is a near ubiquitous resident of mammalian GI tracts[5] and thus has been determined an appropriate indicator of fecal pollution from warm-blooded animals. In 2015, even though over 5 billion people were lucky enough to utilize water sources free from contamination, almost 850 million people still lacked access to a basic drinking water source.[6] Of those 850 million people, children typically bear the brunt of the disease burden as their developing immune systems cannot effectively eradicate ingested coliforms in improperly

sanitized drinking water.[7] The resulting diarrheal disease typically results in severe dehydration, a condition that necessitates a clean drinking water source for improvement in well-being. More specifically, *E. coli* is responsible for nearly a third of neonatal sepsis cases[8] and the majority of urinary tract infections worldwide.[9]

Even though the detection any indicator species will have inherent limitations and biases,[10] the detection of *E. coli* as an indication of poor sanitation has been widely successful in the improvement of water supplies worldwide.[2, 11-14] While standard culture-based techniques are reliable, results require anywhere from 24 to 72 hours,[15, 16] a relative eternity for a community water supply in require of remediation.[17]

The rapid detection of bacteria remains a significant challenge and many research approaches have been developed to overcome that challenge.[18] One promising area of rapid microbial detection assays are bacteriophage-based diagnostics.[19] Bacteriophages (phages) are obligate bacteria-infecting viruses that have co-evolved with bacteria for most (if not all) of the more 3 billion years that bacteria have existed on the planet.[20] Reporter phages have been modified

to include an exogeneous reporter gene in the phage genome. The newly created recombinant phage expresses the exogenous cellular biomarker upon phage infection in addition to new phage progeny.[21] Measurement of the reporter enzyme activity allows for correlation between signal output (absorbance, fluorescence, luminescence, etc.) and initial bacterial populations.

The field of bioluminescence has been advancing for 70 years, from McElroy's pioneering work [22] to the development and widespread implementation of the NanoLuc luciferase.[23-33]

Luminescent reporter phages have been developed for the sensitive detection of common foodborne pathogens such as *Escherichia coli*, [24, 34, 35] *Listeria monocytogenes,*[36] *Salmonella* Typhimurium,[37] *Mycobacterium tuberculosis*,[38] *Vibrio parahaemolyticus*,[39] among others.[40-45] The NanoLuc reporter is uniquely suited for sensitive detection assays as it conferse a low background signal coupled with high dynamic range.[33]

In addition, the orthogonality of the NanoGlo chemical substrate system[33] offers consistently low background levels in a wide range of sample conditions, a characteristic pointing its use towards low

cost implementation. As sensitivity increases, distinguishing signal from noise becomes a major challenge in the establishment of the limit of detection.

The selective nature of phages is well documented,[46] and therefore our phage-based detection assay is well suited in situations where indicators and/or pathogen detection is pertinent.[47] To create the recombinant phages used in our novel detection platform, we inserted an enzyme expression cassette into a wild type phage genome to force expression of a heterologous reporter enzyme in addition to new phage progeny upon phage infection. The NanoLuc enzyme was selected as our reporter enzyme as it is more than 100x more active than its luminescent counterparts,[33] and this highly active reporter enzyme is already widely deployed in a variety of detection assays[25, 26, 31, 32] including bacteriophage-based schemes.[24, 48, 49] The NanoLuc enzyme was further functionalized by genetically fusing a cellulose binding module (CBM) to the C-terminus of the NanoLuc reporter gene. The CBM selected for this work (CBM2a) has previously been fused to a dimeric reporter enzyme (Alkaline Phosphatase) that allows for successful

immobilization on cellulose while still retaining enzymatic activity.[50] In comparison, NanoLuc is a much smaller, monomeric reporter that is frequently utilized as a genetic fusion tag.

Upon phage infection, the expression levels of the reporter enzyme have a direct effect upon the limit of detection as fewer cells are required to produce enough reporter for the signal to rise above the detection limit. As a result, we modified the upstream regulatory regions of the reporter enzyme cassette to determine the optimal sequences that permit the detection of the fewest concentration of cells.

Herein we propose a rapid (5 h) bacteriophage-based approach for the sensitive detection of the indicator bacteria *Escherichia coli*, the target of many regulatory requirements.[51] The novel detection assay coupled membrane filtration with bacteriophage infection to generate a luminescent signal if viable *Escherichia coli* were present. The combination of sample concentration with heterologous reporter enzyme expression upon bacteriophage infection produced a detection limit of less than 20 CFU in 100 mL of drinking water.

MATERIALS & METHODS

Materials & Reagents

NanoGlo (luminescent substrate) was purchased from Promega (Madison, WI, USA) and prepared immediately prior to use. Regenerated cellulose filters (diameter 13 mm, pore size 0.2 μm) were fitted within polycarbonate reusable syringe filter housings (Sartorious Stedim Biotech GmbH, Goettingen, Germany) and autoclaved prior to use in detection assays. Assembled, autoclaved filters were fitted to sterile single use syringes (100 mL, Wilburn Medical, Kernersville, NC, USA) to perform filtration. All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Bacterial Strains & Growth Conditions

Escherichia coli BL21 was obtained from ATCC (Manassas, VA, USA) and *E. coli* ECOR13, a reference strain of *E. coli* isolated from a healthy human, was obtained from the Thomas. S. Whittam STEC Center (East Lansing, MI, USA). Bacterial stocks were stored at -80 °C in 25% glycerol prior to use and cultured in Luria Bertani high salt (LB) broth and plated on LB agar. Overnight cultures of *E. coli* BL21 and *E. coli* ECOR13 were cultivated in Luria-Bertani medium (12-16 hours, 37 °C, 250 rpm). The concentration of *E. coli* ECOR13 used

in detection assays was determined by standard plate counts on LB agar (24 hours, 37 °C).

Bacteriophage T7 was propagated on *E. coli* BL21 using standard protocols. Briefly, an overnight culture of *E. coli* BL21 was subcultured in LB (200 mL, 37 °C, 250 rpm, ~2 hours) and grown to mid exponential phase (OD_{600} = 0.6). Phages were added to the bacterial culture at a multiplicity of infection (MOI) of 0.1, and incubated (37 °C, 250 rpm) until lysis was observed (~2 hours). Cellular debris was removed via centrifugation (3,200 x *g,* 10 min, 4 °C) before sterile filtration (0.22 μm). Phage particles were precipitated by overnight incubation (4 °C, 18 hours) with polyethylene glycol 6000 (PEG6000, 4%) and sodium chloride (NaCl; 0.4 M) before ultracentrifugation (35,000 x *g*, 120 min, 4 °C). Phage were resuspended in phosphate buffered saline (1x PBS, pH 7.4) and stored at 4 °C. Standard double agar overlay assays were used to enumerate phage samples.[52] Phage stock solutions used in detection assays were diluted to 10^9 PFU/mL in LB, sterile filtered (0.22 μm) and stored at 4 °C.

DNA Isolation

DNA was prepared in accordance with standard procedures.[53] Briefly, concentrated phage stocks (5 mL, $>10^{11}$ PFU/mL) were treated with sodium dodecyl sulfate (5 mL; 4%) at 70 °C for 20 minutes before cooling on ice. Potassium acetate (5 mL, 2.55 M, pH 4.8) was added, the samples were centrifuged (10 min, 10,000 x *g*, 4 °C) and the supernatant was applied to an anion exchange resin (Qiagen Genomic Tip 100/G) in accordance with the manufacturer's specifications.

Recombinant Phage Construction

The optimized NanoLuc-CBM fusion reporter gene was inserted into the genome of T7Select to create reporter bacteriophage NRGp5. Integration of the gene was performed using homologous recombination as previously described.[48] Briefly, the T7Select genome was prepared as a cloning vector by propagation of the phage in *E. coli* BL21, followed by purification and restriction digestion of the genome to generate two vector arms. Homology to the upstream and downstream regions of the insertion site were included at the N and C terminal ends of the reporter gene fragment,

respectively. The three DNA fragments consisting of the reporter gene and two vector arms were assembled using in vitro DNA assembly (New England Biolabs, Ipswich, MA, USA). The assembled DNA was then transformed into *E. coli* (MegaX DH10B, Thermofisher), and incubated for 120 minutes until visible lysis occurred. The mutant T7 (NRGp5) was identified using a plaque assay followed by application of NanoGlo substrate (Promega, Madison WI, USA) on individual plaques. The pate was then imaged with a 30 second exposure time (Rebel T6, Canon, Melville NY, USA) in a dark box (LTE-13, Newport Corporation, Irvine, CA, USA) to identify luminescent plaques. The mutants were isolated and propagated on *E. coli* BL21 in accordance with standard techniques.[52]

Phage characterization

The genomes from the NRGp5 phages were isolated and submitted for sequencing. Characterization of the phage infection was compared to the original T7Select. The phage host *E. coli* ECOR13 was grown from stationary phase in LB media (3 h, 37 °C, 250 rpm) using 24 well microplates (Greiner Bio-One North America Inc.,

Monroe, NC, USA). The optical density at 600 nm (OD_{600}) was determined at periodic time intervals to observe the growth of the bacterial cells. Phage addition (negative control, 10^7 PFU/mL T7Select, or 10^7 PFU/mL NRGp5) took place at 180 minutes and the optical density (OD_{600}) was monitored for 300 minutes using a Synergy Neo2 microplate reader (Biotek Instruments, Winooski, VT, USA).

The luminescence during the phage infections was monitored for a negative control, T7Select, NRGp5, as well as a previously developed NRGp4.[48] Luminescent signals were measured using the same microplate reader (Biotek Instruments, Winooski, VT, USA) in sterile 24 well suspension culture plates (Cellstar, Greiner Bio-One, Monroe, NC, USA) with a 0.1 s integration time. To approximate growth conditions in the detection assay, the host *E. coli* ECOR13 was grown from stationary phase in LB supplemented with NanoGlo (3 h, 37 °C, 250 rpm). Phage addition took place at 180 minutes and luminescence was measured at 30 minute intervals for 300 minutes.

Dose response

The sensitivity of phage NRGp5 to differentiate concentrations of bacteria in growth media was evaluated. Phage NRGp5 (10^7 PFU/mL) was added to serial dilutions of mid-exponential phase *E. coli* ECOR13 (0 to 10^5 CFU/mL) and incubated (1.5 h, 37 °C) for to allow the phage infection to proceed. Aliquots were mixed 1:1 with NanoGlo substrate and luminescence was measured on a plate reader. Bacterial concentrations were confirmed using standard plate counts.

Infection of non-viable cells

The ability of phage NRGp5 to differentiate between viable and nonviable bacterial cells was evaluated by treating identical cultures with either alcohol (inactivation) or a biological buffer (control) before phage infection. Briefly, *E. coli* ECOR13 was harvested at mid-exponential phase (OD_{600} = 0.5), separated into identical aliquots, and centrifuged (3,000 x g, 5 min). The cell pellet was resuspended in either ethanol (70%) or phosphate buffered saline (PBS; 1x) and incubated at room temperature for 10 minutes. The cells were pelleted again (3,000 x g, 5 min), resuspended in sterile autoclaved

drinking water (100 mL, 20 °C) and used as analytical samples for the phage-based diagnostic assay.

Phage-Based Syringe Filter Detection Assay

An overall detection scheme can be seen in Figure 1. Drinking water was autoclaved to account for any natural flora before aliquots (100 mL) were deliberately spiked with known concentrations of *E. coli* ECOR13. Samples were then passed through 0.22 μm regenerated cellulose filters (Sartorious Stedim Biotech GmbH, Goettingen, Germany) to separate the bacteria. The filters were removed and incubated on LB media (3 h, 37 °C) in order to resuscitate the bacteria. Phage NRGp5 (10^7 PFU/mL) was applied to the enriched bacteria on the filter and incubated (1.5 h, 37 °C) for expression of the luminescent reporter. The filters were fully submerged in NanoGlo substrate and luminescence was measured every 12 seconds for 5 minutes in a spectrophotometer to capture peak signal generation. The variability of the blank was used to calculate the limit of detection using the standard method of adding three times the standard deviation of the blank to the mean blank value.

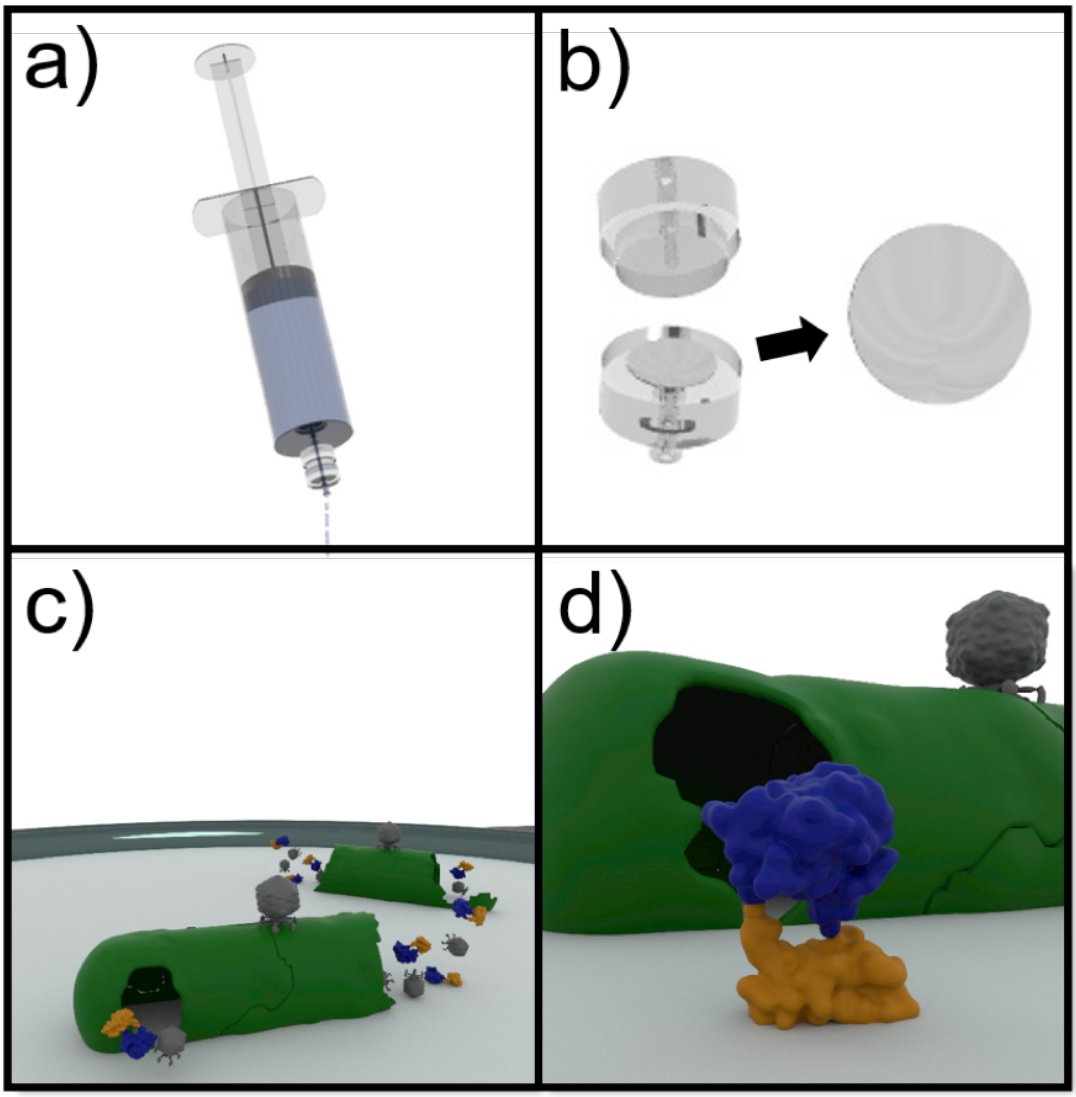

Figure 1: Scheme for the detection of E. coli in drinking water. a) The water is filtered through a 0.22 μm cellulose filter in order to separate the bacteria. b) The filter is then removed from the housing and placed on LB media in order to resuscitate the trapped bacteria. c) Following the application of the engineered phages (grey), an infection cycle results in the expression and release of a reporter enzyme consisting of NanoLuc (blue) and a carbohydrate binding module (orange) with specificity to cellulose. d) The fusion enzyme binds to the cellulose filter and the luminescent activity can then be determined.

RESULTS & DISCUSSION

Recombinant phage construction

The newly developed luciferase, NanoLuc, was selected as a preferred reporter because it is a small, highly active, monomeric enzyme.[33] The 19 kDa luciferase was genetically fused to a carbohydrate binding module to create a 31 kDa fusion enzyme. This novel fusion reporter has demonstrated the simultaneous capability to generate a luminescent signal while specifically bound to a cellulosic substrate. The whole genome sequencing revealed no mutations to the insertion cassette as well as no insertions, deletions or significant mutations outside of the cloning site.

Phage Characterization

The successful insertion of the reporter gene was determined by visualizing the luminescence of phage plaques. Reporter gene expression was initially confirmed via long exposure photography of double overlay plaque assays where NanoGlo substrate was directly applied to well isolated plaques. Luminescent plaques were isolated and propagated for detection assays and submitted for whole genome sequencing. The ability of phage NRGp5 to infect and lyse

indicator bacteria was directly compared to the original T7Select in order to evaluate if the addition of the reporter enzyme gene had a deleterious effect on the apparent fitness of the phage. As seen in Figure 2, there was no statistical difference between the magnitudes, or rate of optical density decrease, resulting from addition of the two phage types. Insertion of the NanoLuc-CBM reporter was performed in an intergenic region immediately downstream of the capsid without any native gene knockouts, as confirmed by whole genome sequencing. The genetic insertion served to increase the genome size of T7Select by less than 3% (37.3 kb vs. 38.3 kb) leaving it with a genome still smaller (~38kb) than that of its 39.7kb wild type counterpart.[54]

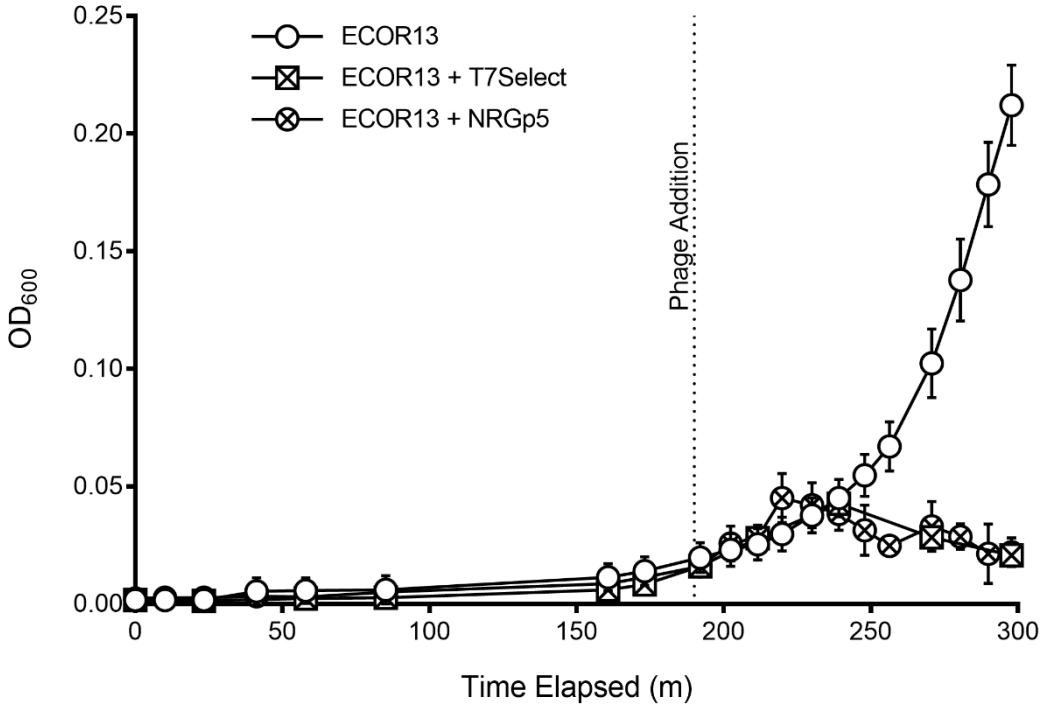

Figure 2: Phages (NRGp5 or T7Select) were added to individual cultures of *E. coli* (ECOR13) after 3 hours of enrichment. The *E. coli* in samples without phages added continued to exponential phase while the application of phage resulted in a decline in optical density after about two hours. There was no statistical differences in the infection characteristics between the two phages.

During phage infection, T7 DNA is replicated in concatemers and successful phage maturation relies on the specific recognition and cleavage of *cos* sites located at the termini of each genomic copy. If the genetic insertion is too large then progeny phages will be unable to fit the genome into the limited space within the capsid.

Packing of the modified genome into the capsid was not expected to cause a significant loss in fitness as larger reporters have previously been inserted into T7 with no apparent lack of fitness.[55, 56] Figure 3 suggests that only cultures infected with phage NRGp4 or NRGp5 produced luminescent signals above the limit of detection. While changes to the promoter and ribosome binding sites upstream of the reporter only served to decrease luminescence signal during the infection of identical cultures (data not shown), removal of the N-terminal secretion signal used in phage NRGp4 [48] produced nearly a half log increase in signal intensity (Figure 3) when identical cultures were infected.

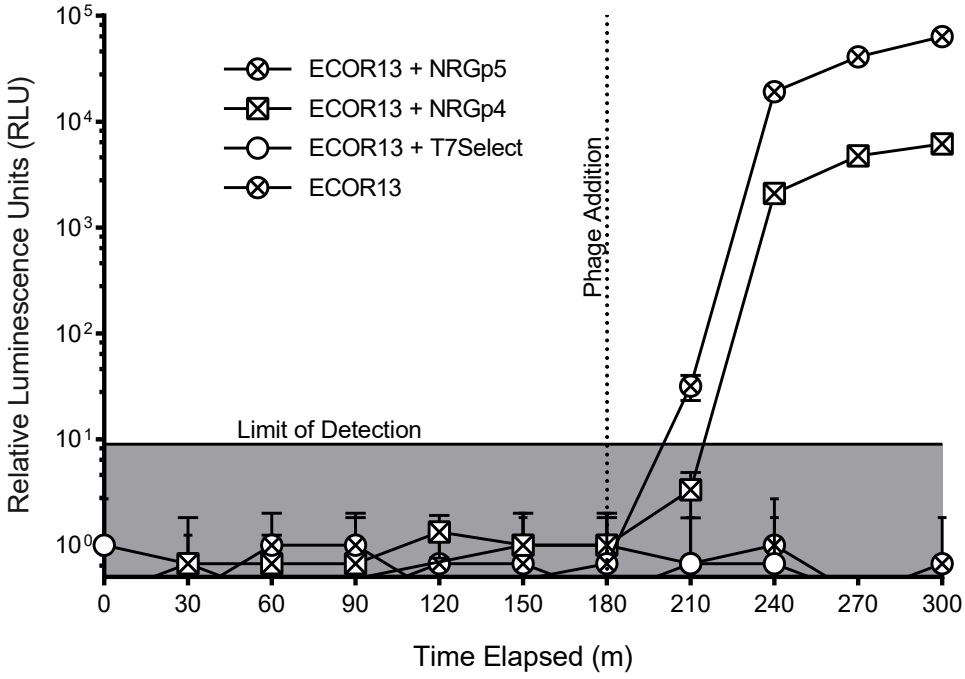

Figure 3: Luminescence was measured from *E. coli* samples with and without the phages T7Select, which did not contain a gene for a reporter enzyme, NRGp4, which contained a gene for NanoLuc-CBM, and NRGp5, which contained an optimized NanoLuc-CBM gene. It can be seen that the reporter genes resulted in luminescence with the optimized gene providing a higher signal. The lowest positive signal was calculated as the negative control +3x the standard deviation using a minimum of three replicates.

The results suggest that the newly developed reporter phage NRGp5 is a viable candidate for use as a more sensitive biosensor element in the phage-based detection assay.

Dose Response

The phage NRGp5 was also used to infect *E. coli* ECOR13 at varying bacterial concentrations (Figure 4). The results suggested a relatively linear response between 10^0 – 10^5 CFU/mL. This broad dynamic range in a detection scheme is common for the NanoLuc reporter enzyme that has shown a dynamic range over 8 orders of magnitude.[33] A limit of detection (LOD) was determined using a lowest positive luminescence of the average negative control background plus three times its standard deviation (0+3SD). As shown in Figure 4 an LOD between 10 and 100 CFU/mL can be achieved using NRGp5 and ECOR13 in bulk media without any filtration or enrichment.

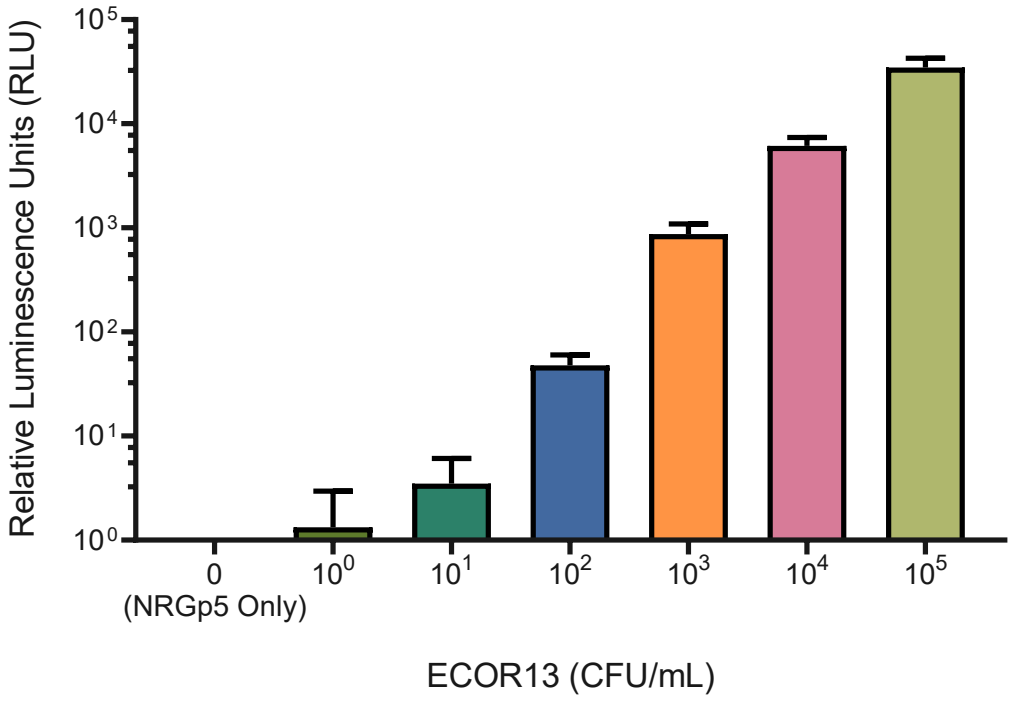

Figure 4: NRGp5 was added at a high MOI to varying concentrations of *E. coli* ECOR13. The luminescence of the lysate resulting from the infections was determined following the addition of substrate. The increase in signal was relatively linear to the initial log concentration of bacteria suggesting a broad dynamic range. From these experiments, a limit of detection between 10 and 100 CFU/mL was determined. The lowest positive signal was calculated as the negative control + 3x the standard deviation using a minimum of three replicates.

Infection of non-viable cells

Because drinking water often undergoes treatment steps in order to kill potentially harmful pathogens, the ability to distinguish between viable and non-viable bacterial is critical for reliable results. Given that phages utilize the genetic machinery of the host bacteria for successful replication, it was not expected that NRGp5 could replicate in non-viable cells. In order to determine if the assay is able to distinguish between viable and non-viable bacterial cells, *E. coli* ECOR13 cells were treated with 70% ethanol and washed prior to analysis. As demonstrated in Figure 5, only phages added to non-ethanol treated cells were able to produce luminescence following substrate addition while bacterial cells treated with ethanol did not produce luminescence following phage addition.

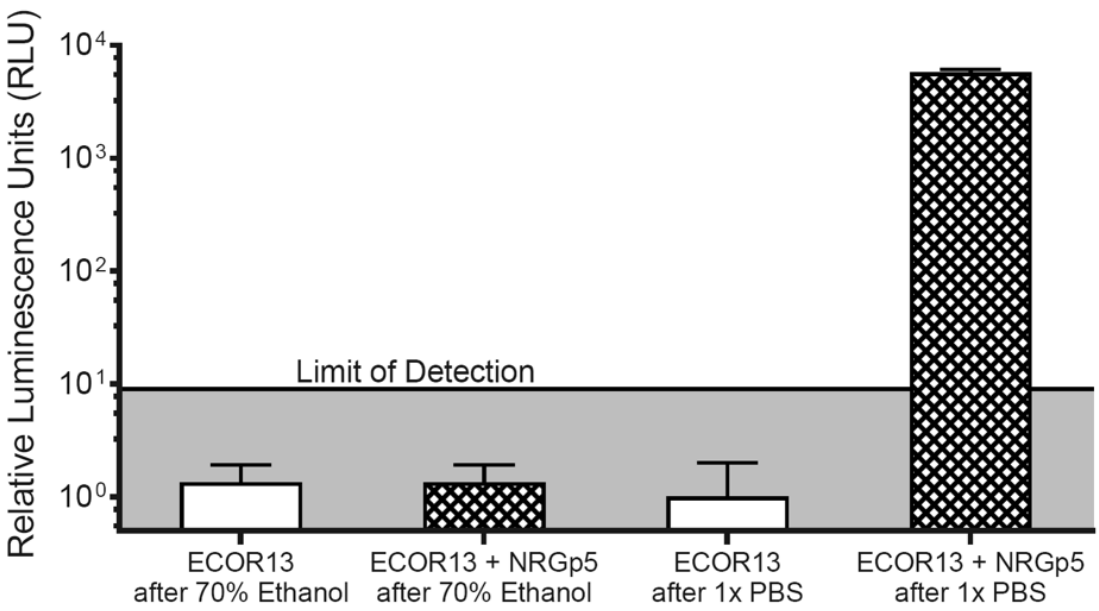

Figure 5: In order to demonstrate the ability of the phage-based assay to distinguish between viable and non-viable

E. coli **ECOR13 cells, the bacterial cells were exposed to either 70% ethanol or PBS, and washed. The cells were then either infected with NRGp5 or incubated without phage. The only variant to display luminescence following incubation and substrate addition was the non-ethanol treated** ***E. coli*** **ECOR13 with the NRGp5 phages.**

Phage-Based Syringe Filter Detection Assay

We improved the limits of detection achieved in bulk media without enrichment by adding filtration and enrichment steps prior to phage infection. The limit of detection improved by several orders of magnitude from ~100 CFU per mL (Figure 4) to 10 to 20 CFU per 100 mL (Figure 6). While positive signals were detected for fewer bacterial cells, the variability of those results did not render the reproducibility required for an acceptable proportion of false negative outcomes. Nevertheless, our novel detection assay realized multiple improvements in limits of detection over previously developed technology and approaches the limits of detection of standard culture based techniques in much less time.

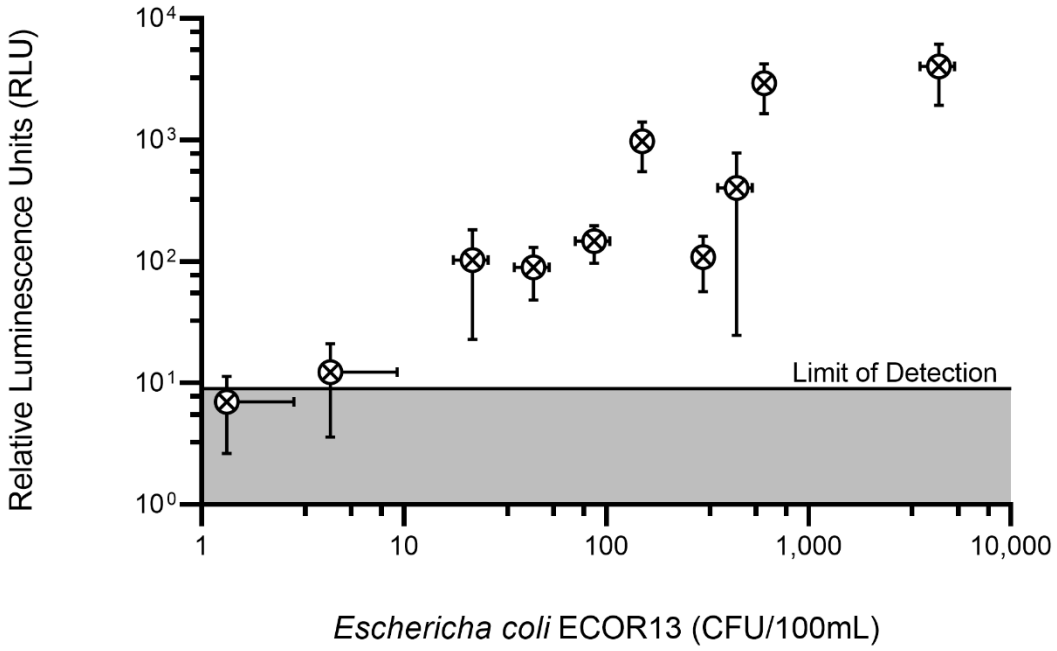

Figure 6. Performance of the phage-based syringe filter detection assay. Water (100mL) was processed through a syringe filter (0.22um, regenerated cellulose) where bacteria (*E. coli* ECOR13) were enriched prior to the addition of phage NRGp5. Reporter enzymes were expressed, immobilized onto the filter, and luminescence was measured.

Further improvements to the limit of detection at these bacterial concentrations is increasingly challenging due to the inherent nature of bacterial cells in large sample volumes. Unlike fully miscible solutes, bacterial cells are discreet units incapable of nearly infinite dilution and this effect is illustrated in Figure 6 as the horizontal error bars increase in size as cell populations decrease.

CONCLUSION

The presented research displays a specific, rapid and effective detection assay for indicator *E. coli* in large drinking water samples based on heterologous enzyme expression via bacteriophage infection. The specific advantages of phages make them excellent candidates as biorecognition elements. Not only are phage incredibly species specific, they are also capable of differentiating between live and nonviable cells.

We have demonstrated the successful insertion of a reporter enzyme cassette into a bacteriophage genome to create the reporter phage NRGp5. This recombinant phage forced the overexpressed of a highly active bifunctional reporter enzyme that was immobilized onto a regenerated cellulose membrane filter and facilitated the

rapid detection of low numbers of indicator bacterial cells. Improvements in enzymatic activity and expression as well as phage host range will serve to create a robust and sensitive detection assay with potential to improve the lives of people around the world.

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