

Engineering bacteriophage for a pragmatic low-resource setting bacterial diagnostic platform

Joey N. Talbert, Samuel D. Alcaine & Sam R. Nugen

To cite this article: Joey N. Talbert, Samuel D. Alcaine & Sam R. Nugen (2016): Engineering bacteriophage for a pragmatic low-resource setting bacterial diagnostic platform, Bioengineered, DOI: [10.1080/21655979.2016.1184386](https://doi.org/10.1080/21655979.2016.1184386)

To link to this article: <http://dx.doi.org/10.1080/21655979.2016.1184386>



Accepted author version posted online: 31 May 2016.
Published online: 31 May 2016.



Submit your article to this journal [↗](#)



View related articles [↗](#)



View Crossmark data [↗](#)

Commentary

Engineering bacteriophage for a pragmatic low-resource setting bacterial diagnostic platform

Joey N. Talbert¹, Samuel D. Alcaine², Sam R. Nugen^{2,3}

¹Department of Food Science and Nutrition, Iowa State University, Ames, IA

²Department of Food Science, Cornell University, Ithaca, NY

³Department of Food Science, University of Massachusetts, Amherst, MA

Corresponding Author Email: snugen@cornell.edu

Abstract

Bacteriophages represent multifaceted building blocks that can be incorporated as substitutes for, or in unison with other detection methods, to create powerful new diagnostics for the detection of bacteria. The ease of phage manipulation, production, and detection speed clearly highlights that there yet remains unrealized opportunities to leverage these phage-based components in diagnostics amenable to resource-limited settings. The passage of regulations like the Food Safety Modernization act, and the ever increasing extent of global trade and travel, will create further demand for these types of diagnostics. While phage-based diagnostics have begun to entering the market place, further research is needed to ensure the potential benefits of phage-based technologies for public health are fully realized.

We are just beginning to explore the possibilities that phage-based detection can offer us in the future. The combination of engineered phages as well as engineered enzymes could result in ultrasensitive detection systems for low-resource settings. Because the reporter enzyme is synthesized *in vivo*, we need to consider the options outside of normal enzyme reporters. In this case, common enzyme issues such as purification and long-term stability are less important. Phage-based diagnostics were conceptualized from out-of-the box thinking and the evolution of these systems should be as well.

Keyword

bacteriophage, diagnostics, biosensor, low-resource setting, low-cost, synthetic biology

1 Introduction

Bacteria are an integral part of our world. They are present in the environment that surrounds us, the food and water we consume, and are vital inhabitants of our bodies. Of this vast bacterial population, a small percentage is pathogenic to humans, but this group represents a significant burden on public health. At both the national and global level, there has been increasing focus by public health agencies on emerging issues such as the dissemination of antibiotic resistance bacteria.¹ The added challenges in resource-limited settings, have driven research into the development of “lab-on-chip” technologies aimed at addressing issues with manufacturing, detection, automation, and power source to create diagnostics that are simple, cheap, and reliable and thus amenable to adoption in such settings.²

Paper-fluidic^{3,4} and microfluidic⁵ based devices have proven themselves to be effective platforms for the detection of chemicals and pathogenic organisms of interest in resource-limited settings⁶, and there is a large body of research applying them for detection of various bacterial, chemical, viral agents.⁷ Commercially available examples of these LFA based products for the detection of bacteria are the Lateral Flow System by DuPont (Wilmington, DE) and Neogen’s Reveal 2.0 product line, both of which have dipstick assays for the detection *E. coli* O157:H7, *Salmonella* spp., and *Listeria* (Neogen, Ann Harbor, MI).

2 Bacteriophage

Bacteriophages (phages) are a class of virus that infect prokaryotic bacteria. They were first discovered in 1915, and initially used as antimicrobial agents but were quickly supplanted by

antibiotics. With the increasing threat of multidrug resistant organisms, the study and application of phage therapy has become a growing field. Additionally, our increased understanding of phage diversity, and the creative applications of modern bioengineering tools, has led to renewed interest in bacteriophage. There are clear opportunities to leverage these advances and apply phage to enhance diagnostics, like paper-fluidic LFAs, geared to resource-limited settings.

2.1 Bacteriophage as Detection Agents

Phages are becoming increasingly popular as probes in bacterial detection schemes.⁸ These viruses have several potential advantages over other biorecognition elements, such as antibodies and DNA. First, phages are relatively easy to produce as they are readily propagated in the presence of their host and can be subsequently purified for use. A second advantage is that phage are host specific, and a single or multiple phage types can be used to target a specific bacterial strain, species, or group of closely related bacterial species. The successful use of reporter-tagged phage for bacterial detection has been demonstrated using both fluorescent⁹ and enzymatic¹⁰ tags. The VIDAS® UP by Biomérieux (St. Louis, MO) is a commercially available detection platform that utilizes reporter-tagged phage components as detector probes in an ELISA format for the detection of *Salmonella*, *E. coli* O157:H7, and *Listeria* with reported superior performance to PCR-based methods.¹¹ Phage-display schemes have also allowed for the development of novel phage-based probes by enabling the bioengineering of phage capsids to incorporate proteins with affinities to chemicals and molecule unrelated to bacterial detection.¹²

2.2 Bacteriophage as Agents for Signal Amplification

The use of phages as a detection probes also allows for signal amplification, commonly referred to as phage amplification. In concept, a single phage adsorbs to and infects a single bacterial cell. The phage DNA is injected into the host, and through a series of mechanisms, it commandeers the metabolic machinery of the cell into the production of new phage. At the end of the infection cycle holin and a lysozyme-like enzyme are produced, rupturing the cell wall, and releasing tens to hundreds of new phage into the environment. The *E. coli* phage T7, for example, has an infection-to-lysis time of 25 minutes under ideal conditions, and over 100 new phage released at the end of a cycle. For comparison, *E. coli* cells take approximately the same amount of time to replicate under optimal growth conditions, so a traditional bacterial enrichment for amplification results in only 2-fold increase using versus the potential 100-fold increase in T7 phage in the same amount of time. There other phages with burst sizes greater than a 1000-fold¹³, making their incorporation into bacterial detection schemes quite attractive. Furthermore, since phage replication requires healthy, growing bacterial cells, detection of phage amplification can be used to specifically identify the presence of only viable cells in a sample. This is another advantage phage-based detection schemes have over immunological and PCR-based schemes, which may detect extraneous antigens or DNA from non-viable cells resulting in higher false positive rates.

Advances in molecular techniques, have enabled the combination of both phage and enzyme based amplification for bacterial detection. Phages have been genetically modified to express reporter enzymes with high turnover rates, enabling more rapid and sensitive detection.

Luciferase has been successfully incorporated into several phage strains targeting bacterial pathogens like *E. coli* O157:H7[70], *Yersinia pestis*¹⁴, and *Bacillus anthracis*¹⁵. Commercially,

Sample6 (Cambridge, MA) has developed a phage-based bioluminescent enzymatic reporter platform for the detection of *Listeria* in environmental samples. In the literature luciferase appears to be a popular choice as an enzymatic reporter, but recent research has also shown the successful incorporation of enzymes such as peroxidases¹⁶, proteases¹⁷, and alkaline phosphatase^{18, 19} as phage-mediated reporters.

3 Reporter Enzymes in Genetically Engineered Phage

The use of genetically engineered phage to selectively infect target bacteria and, subsequently, express a reporter enzyme has been well described.²⁰ However, the current approaches have been limited by signal production and signal/noise ratio. In this regard, the full potential of genetically engineered phages for bacteria detection has not been realized. Biology has provided a number of diverse tools to allow for improved analysis. The use of these tools, in tandem with proper instrumentation and appropriate substrates, creates a significant opportunity to enhance the capabilities of genetically engineered phage for bacteria detection. In this section, we describe opportunities to improve upon the current state of the art using biological approaches as well as instrumentation and analytical methodology. It is important to note that these approaches to optimization are interdependent, and must be considered alongside the application to provide the best system for the intended use.

3.1 Enzyme Optimization

The selection of a suitable enzyme to be expressed following phage infection of bacteria is paramount in designing an improved detection system. Ideally, the enzyme should have a high

substrate turnover, be distinguishable from native enzymes in the bacteria, and be readily expressed.

Turnover number (k_{cat}) for enzymes represents the number of molecular conversions that can be achieved per unit time for a single active site. A higher turnover number indicates faster velocity at a set enzyme concentration. While turnover numbers can be as high as 10^7 sec^{-1} , enzymes used in genetically engineered phage have turnover numbers of ca $10^0 - 10^2 \text{ sec}^{-1}$. Improving the turnover number can be achieved by selecting an enzyme with a higher turnover, mutating the enzyme, or adding cofactors that enhance activity. Enzymes with inherently high turnover, such as catalase and carbonic anhydrase, may be limited by the availability of proper instrumentation to measure low concentrations of product. However, other enzymes such as acid phosphatase or glucose-6-phosphate dehydrogenase have high turnover (ca $10^5 - 10^6 \text{ sec}^{-1}$) and conversion can be analyzed using common instrumentation.^{21, 22} Contrary to the use of enzymes in other diagnostic applications, enzymes expressed by phages only need to be viable within the duration of the assay. This requirement enables a large variety of high turnover enzymes to be considered in the design of the genetically engineered phage.

Mutation in the structure of a native enzyme can provide an alternative means to increase turnover. Alkaline phosphatase, for instance, has been mutated to increase k_{cat} >40 fold---enabling improved substrate conversion and signal production.²³ Moreover, if the product of an enzyme conversion is not amenable to a specific detection method, co-reactants may be utilized. These reactants enable improved signal output or sensitivity. For example, tris(2-carboxyethyl)phosphine (TCEP) has been employed to improve signal production from alkaline

phosphatase, and $\text{Ru}(\text{bpy})_3^{2+}$ has been utilized as a co-reactant to facilitate alcohol dehydrogenase-catalyzed ethanol detection via electrochemiluminescence.^{24, 25}

In addition to the above approaches, reduced inhibition of the native enzyme can also be used to enhance signal production. This approach is illustrated by the incorporation of Coenzyme A to react with a luciferase inhibitor (dehydroluciferyl-adenylate) that is formed as a side-product during signal production. Removal of the inhibitor, in this manner, allows the enzyme to generate an enhanced signal.

Proper selection of the reporter enzyme can enhance not only signal production, but also the signal/noise ratio. Native enzymes present in the bacteria may contribute to background noise by reacting with the enzyme substrate. If a problematic native enzyme is expressed during analysis, alternative enzymes may be required. The interference may be reduced by employing a non-native enzyme as the reporter enzyme. Additionally, a thermostable reporter enzyme may be expressed.²⁶ In this approach, the reaction mixture, following cell lysis, can be heated to denature or reduce the activity of any native lysate enzymes that are not compatible with the higher temperature, thus limiting their contribution to substrate conversion.

Optimization of the concentration and quality of enzymes expressed per cell during phage infection provides another means of enhancing signal production. Ribosomal binding site sequence can be altered to increase the translation of the desired enzyme. However, if the translation rate is too high, the enzyme may be folded incorrectly. Therefore, it is valuable to use design calculators to help maximize efficacy of translation.²⁷ Likewise, codon optimization has proven to be a valuable tool for protein expression and should be applied in the initial design of

the gene sequence used for the engineered phage. If the enzyme is not soluble following lysis (i.e. inclusion bodies are formed), the application of a fusion tag, such as maltose binding protein, glutathione S-transferase, or SUMO, to the gene coding for enzyme expression should be considered.

3.2 Optimization of Instrumentation and Analytical Methodology

Along with biological approaches, instrumental methods and substrate selection are essential for optimization of genetically engineered phage systems. Both substrate and instrumentation should be accessible and deliver the necessary sensitivity, precision, and accuracy within the required timeframe for the intended application.

Optical, spectrophotometric, fluorescence, luminescence, electrochemical, and electroluminescence approaches have been employed to measure signal production. While these techniques have been well-described, it's worth highlighting some advantages, disadvantages, and opportunities. Optical detection, utilizing a visible substrate, is advantageous when a yes/no answer is desired. Though not as sensitive as other assays, optical detection by aggregation and precipitation of a substrate on a material surface can be a useful and simple detection method.²⁸

Spectrophotometric assays allow for inexpensive quantification and tend to provide a stable signal, but are prone to background interference and low sensitivity. Bioluminescence and chemiluminescence are extremely sensitive, being able to detect as low as 10^{-20} moles.²⁹

Moreover, these systems are not as prone to background interference as other systems, and portable devices are also being developed, which will aid with point-of-care applications.

However, a limited number of substrates are available for luminescence, and the signal is quickly

produced and relatively short lived (seconds -- minutes) after adding the substrate. These substrates prevent the enzyme from being able to build up signal with time, as they do with other assays. Fluorescent methods are sensitive and allow for signal build-up, however, the instrumentation is more expensive and not as well suited for point-of-care applications. Electrochemical reactions utilize either an oxidoreductase or a redox product from an enzymatic reaction. These systems are sensitive and reactions can be analyzed using inexpensive instrumentation. Moreover, electrochemical recycling of the enzyme product enables signal amplification. Background interference, however, can occur from common molecules in the detection matrix and may influence the signal/noise ratio. Electrochemiluminescence is a hybrid approach that improves on the sensitivity of electrochemistry while enabling greater assay versatility and substrate recycling when compared to chemiluminescence.³⁰ This approach is expected to be utilized to a greater extent in future applications.

As noted, previously, the nature of the substrate can influence phage detection capabilities. Depending upon the application, the substrate should have a suitable limit of detection and detection range. For all applications, the reaction kinetics for the expressed enzyme should be well-described prior to implementation. Both k_{cat} and K_m are dependent on the substrate used, in addition to the assay environment (e.g. pH, temperature, ion concentration). Therefore, optimization of genetically engineered phage system should include kinetic screening experiments. For subsequent assays, detection can be enhanced by conducting analysis under optimized conditions, with substrate concentrations maintained at greater than twice the K_m throughout the duration of the reaction in order to assure the enzyme is operating at maximum velocity.

4 Conclusion

While the use of bacteriophages for the detection of bacterial pathogens has been evolving for several years, advances in synthetic biology, enzyme engineering and specific techniques such as CRISPR-CAS 9 will allow these phage-based systems to reach far beyond current capabilities. The phage-based detection model can be a pragmatic solution to low-resource settings because phages are relatively easy to transport and store. In typical sandwich assay type systems, the reporter probe such as an enzyme-tagged antibody is commonly the self-life limiting component. The phage-based system can allow the use of enzymatic amplification steps without the need for storage concerns as the reporter is not synthesized until the phage encounters a host cell. The unique format of the bacteriophage detection schemes will allow future bacterial diagnostics to be performed relatively quickly and at low-cost.

5 References

1. Laxminarayan R, Duse A, Wattal A. Antibiotic resistance-the need for global solutions (vol 13,pg 1057, 2013). *Lancet Infectious Diseases* 2014; 14:675-.
2. Mark D, Haeberle S, Roth G, von Stetten F, Zengerle R. Microfluidic lab-on-a-chip platforms: requirements, characteristics and applications. *Chemical Society Reviews* 2010; 39:1153-82.
3. Koo CKW, He F, Nugen SR. An inkjet-printed electrowetting valve for paper-fluidic sensors. - *The Royal Society of Chemistry*, 2013:10.1039/C3AN01114C.
4. Wang Y, Nugen SR. Development of fluorescent nanoparticle-labeled lateral flow assay for the detection of nucleic acids. *Biomedical Microdevices* 2013; 10.1007/s10544-013-9760-1.
5. He F, Grimes J, Alcaine SD, Nugen SR. A hybrid paper and microfluidic chip with electrowetting valves and colorimetric detection. *Analyst* 2014; 139:3002-8.
6. Yager P, Edwards T, Fu E, Helton K, Nelson K, Tam MR, Weigl BH. Microfluidic diagnostic technologies for global public health. *Nature* 2006; 442:412-8.
7. Ngom B, Guo YC, Wang XL, Bi DR. Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review. *Analytical and Bioanalytical Chemistry* 2010; 397:1113-35.
8. Hagens S, Loessner MJ. Application of bacteriophages for detection and control of foodborne pathogens. *Applied Microbiology and Biotechnology* 2007; 76:513-9.

9. Goodridge L, Chen JR, Griffiths M. Development and characterization of a fluorescent-bacteriophage assay for detection of *Escherichia coli* O157 : H7. *Applied and Environmental Microbiology* 1999; 65:1397-404.
10. Willford JD, Bisha B, Bolenbaugh KE, Goodridge LD. Luminescence based enzyme-labeled phage (Phazyme) assays for rapid detection of Shiga toxin producing *Escherichia coli* serogroups. *Bacteriophage* 2011; 1:101-10.
11. Lu JZ, Tang MJ, Liu HL, Huang LH, Wan ZG, Zhang H, Zhao F. Comparative Evaluation of a Phage Protein Ligand Assay with VIDAS and BAX Methodology for Detection of *Escherichia coli* O157:H7 Using a Standard Nonproprietary Enrichment Broth. *Journal of Aoac International* 2012; 95:1669-71.
12. Petrenko VA, Vodyanoy VJ. Phage display for detection of biological threat agents. *Journal of Microbiological Methods* 2003; 53:253-62.
13. Fan HH, Huang Y, Mi ZQ, Yin XY, Wang L, Fan H, Zhang ZY, An XP, Chen JK, Tong YG. Complete Genome Sequence of IME13, a *Stenotrophomonas maltophilia* Bacteriophage with Large Burst Size and Unique Plaque Polymorphism. *Journal of Virology* 2012; 86:11392-3.
14. Vandamm JP, Rajanna C, Sharp NJ, Molineux IJ, Schofield DA. Rapid Detection and Simultaneous Antibiotic Susceptibility Analysis of *Yersinia pestis* Directly from Clinical Specimens by Use of Reporter Phage. *Journal of Clinical Microbiology* 2014; 52:2998-3003.

15. Sharp NJ, Vandamm JP, Molineux IJ, Schofield DA. Rapid Detection of *Bacillus anthracis* in Complex Food Matrices Using Phage-Mediated Bioluminescence. *Journal of Food Protection* 2015; 78:963-8.
16. Hoang HA, Abe M, Nakasaki K. A novel colorimetric method for the detection of *Escherichia coli* using cytochrome c peroxidase-encoding bacteriophage. *FEMS microbiology letters* 2014; 352:97-103.
17. Alcaine SD, Tilton L, Serrano MAC, Wang M, Vachet RW, Nugen SR. Phage-protease-peptide: a novel tripecta enabling multiplex detection of viable bacterial pathogens. *Applied Microbiology and Biotechnology* 2015; 99:8177-85.
18. Alcaine SD, Pacitto D, Sela DA, Nugen SR. Phage & phosphatase: a novel phage-based probe for rapid, multi-platform detection of bacteria. - The Royal Society of Chemistry, 2015:7629 - 36.
19. Alcaine SD, Law K, Ho S, Kinchla AJ, Sela DA, Nugen SR. Bioengineering bacteriophages to enhance the sensitivity of phage amplification-based paper fluidic detection of bacteria. *Biosensors and Bioelectronics* 2016; 82:14-9.
20. Schmelcher M, Loessner MJ. Application of bacteriophages for detection of foodborne pathogens. *Bacteriophages* 2014; 4:e28137.
21. Hansen T, Schlichting B, Schonheit P. Glucose-6-phosphate dehydrogenase from the hyperthermophilic bacterium *Thermotoga maritima*: expression of the *g6pd* gene and

characterization of an extremely thermophilic enzyme. *Fems Microbiology Letters* 2002; 216:249-53.

22. Tham SJ, Chang CD, Huang HJ, Lee YF, Huang TS, Chang CC. Biochemical Characterization of an Acid Phosphatase from *Thermus thermophilus*. *Bioscience Biotechnology and Biochemistry* 2010; 74:727-35.

23. Muller BH, Lamoure C, Le Du MH, Cattolico L, Lajeunesse E, Lemaitre F, Pearson A, Ducancel F, Menez A, Boulain JC. Improving *Escherichia coli* alkaline phosphatase efficacy by additional mutations inside and outside the catalytic pocket. *Chembiochem* 2001; 2:517-23.

24. Wang D, Kinchla AJ, Nugen SR. Rapid detection of *Salmonella* using a redox cycling-based electrochemical method. submitted 2015.

25. Zhang L, Xu Z, Sun X, Dong S. A novel alcohol dehydrogenase biosensor based on solid-state electrogenerated chemiluminescence by assembling dehydrogenase to Ru(bpy)₃²⁺-Au nanoparticles aggregates. *Biosensors and Bioelectronics* 2007; 22:1097-100.

26. Hagens S, Wouters Td, Vollenweider P, Loessner MJ. Reporter bacteriophage A511:: celB transduces a hyperthermostable glycosidase from *Pyrococcus furiosus* for rapid and simple detection of viable *Listeria* cells. *Bacteriophage* 2011; 1:143-51.

27. Salis HM. THE RIBOSOME BINDING SITE CALCULATOR. In: Voigt C, ed. *Synthetic Biology, Pt B: Computer Aided Design and DNA Assembly*. San Diego: Elsevier Academic Press Inc, 2011:19-42.

28. Cao R, Guan L, Li M, Tian J, Shen W. A zero-step functionalization on paper-based biosensing platform for covalent biomolecule immobilization. *Sensing and Bio-Sensing Research* 2015; 6:13-8.
29. Bronstein I, Edwards B, Voyta JC. 1,2-DIOXETANES - NOVEL CHEMI-LUMINESCENT ENZYME SUBSTRATES - APPLICATIONS TO IMMUNOASSAYS. *Journal of Bioluminescence and Chemiluminescence* 1989; 4:99-111.
30. Chen XM, Su BY, Song XH, Chen QA, Chen X, Wang XR. Recent advances in electrochemiluminescent enzyme biosensors. *Trac-Trends in Analytical Chemistry* 2011; 30:665-76.