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Bioengineering bacteriophages to enhance the sensitivity of phage amplification-based paper fluidic detection of bacteria

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ABSTRACT

Bacteriophage (phage) amplification is an attractive method for the detection of bacteria due to a narrow phage-host specificity, short amplification times, and the phages' ability to differentiate between viable and non-viable bacterial cells. The next step in phage-based bacteria detection is leveraging bioengineered phages to create low-cost, rapid, and easy-to-use detection platforms such as lateral flow assays. Our work establishes the proof-of-concept for the use of bioengineered T7 phage strains to increase the sensitivity of phage amplification-based lateral flow assays. We have demonstrated a greater than 10-fold increase in sensitivity using a phage-based protein reporter, maltose-binding protein, over the detection of replicated T7 phage viron itself, and a greater then 100-fold increase in sensitivity using a phage-based enzymatic reporter, alkaline phosphatase. This increase in sensitivity enabled us to detect 10^3 CFU/mL of *Escherichia coli* in broth after 7 h, and by adding a filter concentration step, the ability to detect a regulatory relevant *E. coli* concentration of 100 CFU/100 mL in inoculated river water after 9 h, where the current standard requires days for results. The combination of the paper fluidic format with phage-based detection provides a platform for the development of novel diagnostics that are sensitive, rapid, and easy to use.

1. Introduction

The application of bacteriophages (phages) as detection probes has many opportunities in rapid diagnostics. Phages are relatively easy to produce, they are host specific, can potential distinguish between viable and non-viable cells, and their use can allow for rapid signal amplification (Singh et al., 2012). The measurement of replicated phages in order to determine the presence of host bacteria is commonly referred to as phage amplification. In concept, a single phage adsorbs to and infects a single bacterial cell. During infection, the phage DNA is injected into the host resulting in a high-jacking of the metabolic machinery of the cell in order to produce replicated phages. At the appropriate point in the infection cycle, enzymes capable of degrading the bacteria cell wall are produced, rupturing the cell, and releasing tens to thousands of new phage into the environment. The new increased phage titer can be determined through traditional plating methods such as a plaque assay or other detections scheme such as ELISA. For a phage such as T7, which has broad range specificity for *Escherichia coli,* the time from infection to lysis occurs in as little as 25 min (Studier, 1972), after which over 100 infectious particles (i.e. new phages) are released (Sadowski, 1974). Therefore, in the time it would take an *E. coli* cell to double (Cooper and Helmstetter, 1968), there is a potential 100-fold increase in T7 phage, making it an attractive component in a detection scheme for *E. coli* host cells.

Advances in bioengineering have allowed the genetic modification of phages to co-express reporter peptides and enzymes during host cell infection, further enhancing the magnitude and speed of signal amplification (Alcaine et al., 2015a; Birmele et al., 2008; Hoang et al., 2014; Singh et al., 2012). Both modified and unmodified phage amplification schemes have been leveraged in bacteria detection methods (Smartt et al., 2012), but little research has attempted to combine phage amplification with a resource-limited amenable platform, such as lateral flow assays (LFA). Paper-fluidic (Koo et al., 2013; Wang and Nugen, 2013) and microfluidic (He et al., 2014; He and Nugen, 2014) based devices, like LFAs, are attractive testing platforms due to their ease-of-use, cost, and reliability. They have been shown to be effective platforms for the detection of analytes and pathogens in low-resource settings (Yager et al., 2006). The simplicity and ubiquity of the home pregnancy test highlights the attractiveness of the LFA, particularly when testing liquid matrices.

LFAs for the detection of pathogenic strains of *E. coli* have

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previously been investigated (Jung et al., 2005; Terao et al., 2013), and are commercially available for detecting some *E. coli* strains such as the DuPont™ Lateral Flow System for *E. coli* O157:H7 (DuPont, Wilmington, DE) and Neogen's Reveal 2.0 for *E. coli* O157:H7 (Neogen, Lansing, MI). The challenge with these immunobased lateral flow assays is the difficulty in developing a mixture of antibodies with a broad enough range to capture an entire species of bacteria, such as the generic *E. coli*, which is commonly used as indicator of water quality (Olstadt et al., 2007), and a marker of urinary tract infections (Wilson and Gaido, 2004). These LFAs also require a heat inactivation step, indicating that these assays in fact detect non-viable rather than viable cells. If the original sample has high levels of heat-killed cells, perhaps due to a validated pathogen reduction step, the test could result in a false positive. Furthermore, many of these LFAs require an enrichment of 8–24 h prior to testing. The use of phage-based assays can offer several advantages to these methods. Phage strain selection offers the ability to narrow or broaden the bacterial specificity of a test. Phages can only amplify within a viable host, thus reducing the potential of false positives cause by detecting non-viable bacteria. Additionally, the dynamics of phage amplification could conceivably reduce detection time.

In previous work, our lab bioengineered two reporter T7 phage strains: i) $T7_{TEV}$ which carries a gene for tobacco etch virus (TEV) protease that is expressed upon infection of *E. coli* (Alcaine et al., 2015b); and ii) $T7_{ALP}$, which carries the *E. coli* alkaline phosphatase gene that is overexpressed during infection (Alcaine et al., 2015a). A co-product of $T7_{TEV}$ infection is maltose-binding protein (MBP), which acts improves solubility and proper folding of TEV protease prior to being cleaved from the construct (Kapust and Waugh, 1999). Approximately 2×10^3 molecules of TEV protease, and concurrently molecules of MBP, are produced per replicated phage during infection (Alcaine et al. 2015b). Given that T7 has a burst size of ~100 PFU per cell, there is a potential production of 2×10^5 MBP molecules per bacterial host. MBP is also used as a protein capture tag in purification schemes, so commercial antibodies are widely available, making the construction of an LFA for MBP quite pragmatic. Alkaline phosphate is commonly used as an enzymatic reporter in diagnostic assays (McComb et al., 1979). Antibodies against *E. coli* alkaline phosphatase are also readily available and relatively inexpensive. Furthermore, when paired with a colorimetric substrate like 5-bromo-4-chloro-3′-indolyphosphate and nitro-blue tetrazolium (BCIP/NBT), alkaline phosphatase requires no additional label, thus eliminating the need for a secondary reporter antibody and simplifying the standard sandwich LFA scheme. Both bioengineered T7 reporters: MBP due to its level of expression per cell; and alkaline phosphatase due to its additionally enzymatic activity; make attractive analytes for an LFA with the potential to improve sensitivity in a phage amplification-based detection scheme.

As part of this study, we developed and compared three LFA schemes targeting different reporter products resulting from a phage-host infection: i) the wild-type T7 phage virion itself; ii) a MBP reporter produced by the bioengineered $T7_{TEV}$ phage; and iii) an alkaline phosphatase reporter that is overexpressed by the bioengineered $T7_{ALP}$ phage. We then investigated whether an increase in LFA sensitivity translated into increased sensitivity for bacteria detection. To do this we tested a proof-of-principle application of our phage amplification-based lateral flow assay to detect low levels of *E. coli* in media and river water. We demonstrate the ability of bioengineered phage-based reporters to improve the sensitivity of phage amplification-based LFA detection schemes several orders of magnitude, thus enabling the rapid detection of a low bacterial concentrations in artificially contaminated broth and river water. Our research highlights the potential of bioengineered phage-based reporters in LFAs targeting bacteria, and we believe further research in this area can lead to the development and deployment of rapid, sensitive, and inexpensive diagnostics assays for bacteria detection.

2. Materials and methods

2.1. Bacteriophage strains working stock preparation

E. coli BL21 stock was purchased from EMD Millipore (Billerica, Massachusetts) and wild-type bacteriophage T7 stock was purchased from ATCC (BAA-1025-B2, Manassas, Virginia). *E. coli* BL21 culture was routinely grown overnight in 50 mL of Luria Broth (LB), pH 7.5, at 37 °C with 250 rpm shaking. An overnight culture (1 mL) was then added to 200 mL of fresh LB and incubated at 37 °C with shaking until an optical density (OD) of at least 0.6 at 600 nm was reached (approximately 3.5 h). Then 20 μL of T7 stock was added to the *E. coli* BL21 culture and allowed to incubate, with shaking, at 37 °C for 1.5 h. NaCl (5 g) was added to the 200 mL culture to prevent further phage adsorption to the cells. The culture was then divided into six 35 mL tubes and spun at 7500*g* on a Fiberlite F21-8x50y fixed angle rotor (ThermoFisher Scientific, Waltham, MA) for 10 min at room temperature. Supernatant was collected and filtered through 0.22 μm SCFA filter (Corning Life Science, Corning, NY). 40 mL of this filtered culture was divided into four 13.5 mL ultracentrifuge tubes and spun at $10^5 g$ for 2 h on a Fiberlite F65L-6X13.5 fixed angle rotor (ThermoFisher Scientific) at room temperature. The supernatant was removed and the pellets in each tube were re-suspended in 1 mL of 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, and then combined for a total of 4 mL of purified T7 working stock. Working stock of Phage $T7_{TEV}$, which causes *E. coli* to overexpress tobacco etch virus protease and maltose binding protein upon infection, and working stock of Phage $T7_{ALP}$ which causes *E. coli* to overexpress alkaline phosphatase upon infection, were prepared as previously described (Alcaine et al., 2015a, 2015b). For the sake of clarity, $T7_{TEV}$ will be referred to a $T7_{MBP}$, as we are interested in MBP production for this application. The titer of all T7 working stocks were determined following the double agar overlay plaque assay (Kutter and Sulakvelidze, 2005).

2.2. Lateral flow assay construction

Lateral flow assay test strips were constructed as follows. A 10 cm by 2.5 cm strip of nitrocellulose membrane (AE 98, Whatman International, Kent, England) was backed with a 10 cm by 4 cm strip of PET plastic using 3 M 465 adhesive transfer tape. For the test line, a solution of the appropriate antibody: (i) T7 tail fiber monoclonal antibody (EMD Millipore); (ii) MBP polyclonal antibody PA1-989 (ThermoFisher Scientific); or (iii) *E. coli* alkaline phosphatase polyclonal antibody ab7321 (Abcam, Cambridge, MA); was printed across the backed membrane in a 95 mm line to achieve a concentration of ~0.2 μg of antibody per mm using a Linomat IV (Camag Scientific, Inc., Willmington, NC). The T7 tail fiber antibodies were dialyzed overnight against PBS buffer pH 7.2. The membranes were placed in a vacuum oven at 37 °C for 1 h to dry. The membranes were then blocked in 1% non-fat dry milk powder, (Omniblok™, American Bioanalytical, Inc., Natick, MA) in PBS buffer, pH 7.2 with shaking for 30 min. Membranes were then removed from the blocking solution, blotted dry, and then placed in PBS buffer, pH 7.2, for 5 min with shaking to remove excess blocking agents. Membranes were removed from the wash solution, blotted dry, and further dried in a vacuum oven at 37 °C for 1 h. The membranes were removed, and an absorbent pad (CF5; Whatman International) was adhered to 1.5 cm PET overhang using 3 M 465 adhesive transfer tape. The membrane was then cut

into 4 mm wide test strips.

2.3. Lateral flow assay procedure

The T7 LFA was performed as follows: 50 μL of sample and 5 μL of 5% non-fat dry milk in PBS were placed in a 10 mm glass test tube, and the end of a test strip was then placed into the solution. Following absorption of the sample solution, the test strip was transferred to a fresh tube containing 30 μL of PBS buffer, pH 7.2, resulting in a wash step. Once the buffer was absorbed, the test strip was placed on a heat block (50 °C) for 20 min. The heating step was found to be necessary in order to expose the target peptide sequence of the reporter antibody. A 10 μL solution containing the biotinylated T7 monoclonal capsid antibody (EMD Millipore) diluted 1:200 in PBS, pH 7.2, was then run up the strip, followed by 30 μL of PBS, pH 7.2, as a wash. Lastly, 5 μL of a 1:200 dilution in PBS of streptavidin conjugated QDot 605® (ThermoFisher Scientfic), was then run up the strip followed by 30 μL of PBS, pH 7.2, as a final wash.

The MBP LFA was performed as follows: 50 μL of sample was incubated with 10 μL of biotinylated polyclonal anti-MBP antibody diluted 1:200 in PBS, pH 7.2 for 10 min. The biotinylated polyclonal anti-MBP antibody was prepared using the EZ-Link™ Sulfo-NHS-Biotin kit (ThermoFisher Scientific) and is the same polyclonal anti-MBP antibody used for the strip test line. After the incubation step, the sample was combined with 5 μL of PBS buffer, pH 7.2, containing 2.5% non-fat dry milk 0.25% Tween 20 (ThermoFisher Scientific) in a small glass test tube, and the end of a test strip was placed in the solution. Following absorption of the sample solution, the test strip was transferred to a fresh tube containing 30 μL of PBS buffer, pH 7.2, as a wash. Next, 5 μL of a 1:200 dilution in PBS of streptavidin conjugated QDot 605® (ThermoFisher Scientific), was run up the strip followed by 30 μL of PBS, pH 7.2, as a final wash.

The ALP LFA was performed as follows: 50 μL of sample was added to a small glass test tube, and the end of a test strip was placed in the solution. Following absorption of the sample solution, the test strip was transferred to a fresh tube containing 30 μL of PBS buffer, pH 7.2, as a wash. Finally, the test strip was transferred to a third tube containing, 1 mL of 1-Step™ NBT/BCIP Substrate Solution (ThermoFisher Scientific) pre-warmed to 37 °C, and incubated for two hours at 37 °C before reading.

2.4. Bacterial limit of detection using $T7_{MBP}$ and $T7_{ALP}$

Three separate overnight cultures of *E. coli* BL21 were serially diluted in LB to achieve 10^3, 10^2, and 10^1 CFU/mL. A 900 μL aliquot of each dilution and a negative control of LB, containing no bacterial cells, were placed in wells of a 96-well DeepWell™ plate, and mixed with 100 μL of 10^3 PFU/mL of $T7_{MBP}$ or 10^4 PFU/mL $T7_{ALP}$ in LB. The samples were incubated at 37 °C with 200 rpm shaking for either 5 or 7 h for 7_{ALP}, and 7 h for $T7_{MBP}$. Samples were then passed through a 4 mm 0.45 μm Millex®-HV filter, stored at 4 °C until use. The assays were performed as previously described using either the MBP or ALP lateral flow test trips, with exception that 100 μL of sample, rather than 50 μL, was run up the strip. The overnight cultures were serially diluted and plated on LB agar to confirm bacterial concentration.

2.5. Bacteria detection in river water using $T7_{ALP}$

River water (2 L) was collected from Fort River in Amherst, MA and autoclaved to deactivate any naturally occurring bacteria. Three separate overnight cultures of *E. coli* BL21 were diluted and used to inoculate three separate 300 mL aliquots of water at a target concentration of 100 CFU/100 mL. Each river water aliquot was divided into three separate 100 mL subsamples and then passed through a 0.22 μm SCFA filter to separate and concentrate the *E. coli* cells. The cells were then back-flushed off the filter with 1 mL of LB to re-suspend the captured cells in an appropriate growth media. Each sample was added to a 15 × 100 mm glass test tube, resulting in three 1 mL tubes corresponding to each of the initial 300 mL river water aliquots. Then for each triplicate, 100 μL of $T7_{ALP}$ (10^5 PFU/mL) in LB was added to one tube, and 100 μL of LB to the second, the contents of the third tube were plated to confirm level of *E. coli* recovery. The samples were incubated at 37 °C with 200 rpm shaking for 7 h. Samples were then passed through a 4 mm, 0.45 μm Millex®-HV filter, stored at 4 °C, and tested as previously described using the ALP lateral flow test trips with exception that 100 μL of sample, rather than 50 μL, was run up the strip. The overnight cultures were serially diluted and plated on LB agar to confirm bacterial concentration.

2.6. Image and statistical analysis

Digital images of test strips were then processed using a digital camera, or in the case of fluorescent reporters, a CCD imaging station (Kodak, Rochester, NY, USA). Signal analysis (signal integration) for the fluorescent reporters was performed using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). The $\log_{10}$ of all test line signals was taken and then evaluated for statistically significance in Origin Pro version 9.0.0 (Northampton, MA). All test line signals were normalized to the test line signal of the negative control for the run, and then analyzed for significance using a single-sample, one-sided *t*-test, mean $<=1$, with an alpha level of 0.05. In all figures error bars represent one standard deviation (SD) $\pm$ from the sample mean and a star (*) indicates a significance ($p < 0.05$).

3. Results and discussion

3.1. Lateral flow assay limit of detection

We compared the limits of detection of three schemes for phage-amplification based lateral flow assays (Fig. 1). The first scheme (Scheme 1) targeted the wild type T7 phage viron itself, and used a fluorescent quantum dot reporter. The second scheme (Scheme 2) targeted MBP, a protein co-produced during $T7_{MBP}$ amplification, also using a fluorescent quantum dot reporter using an engineered bacteriophage. The third scheme (Scheme 3) targeted alkaline phosphatase, an enzyme that was also overproduced during $T7_{ALP}$ amplification, and whose activity was detected using the substrate BCIP/NBT resulting in a dark blue precipitate. Dilution series of stock T7, $T7_{MBP}$, and $T7_{ALP}$ were prepared in triplicate and tested using the corresponding lateral flow assay. The reporter signal produced at the test line was proportional to the corresponding phage titer of the sample and therefore used to determine the minimum level of phage amplification necessary to produce a signal. The test line signal of each dilution series sample were analyzed using ImageJ and compared to the signal at the test line of the negative control strip for the series (Fig. 2a). A representative set of strips can be found in Fig. 2b, these images were adjusted for brightness and contrast in order to better visualize the test line. Scheme 1 resulted in a visually distinguishable test line in samples containing at least $10^{6.8}$ PFU of wild type T7 (Fig. 2b), however statistically significant fluorescent signals were only observed at the test line in samples containing at least $10^{7.8}$ PFU of wild type T7 (Fig. 2a). In scheme 2, we were able to visually distinguish and detect a statistically significant test line signal in samples containing at least $10^{6.2}$ PFU of $T7_{MBP}$ (Fig. 2). At the highest concentration tested, $10^{8.2}$ PFU, a statistically

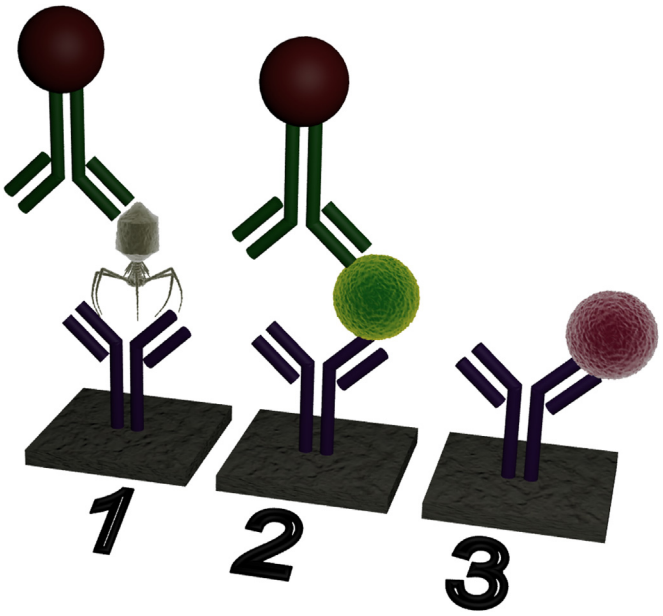

Fig. 1. Lateral flow assay (LFA) Schematic. The LFA consists of a nitrocellulose membrane with a test line containing capture antibodies (purple) against a product of the phage-bacteria infection. Scheme 1: The increase in bacteriophage titer is quantified using an anti-T7 tail fiber capture antibody (purple) and a quantum dot modified with anti-T7 capsid antibodies (green). Scheme 2: Maltose binding protein (MBP) from the infection of an engineered bacteriophage is quantified with anti-MBP polyclonal capture (purple) and reporter (green) antibodies. Similar to Scheme 1, a quantum dot is used for transduction. Scheme 3: The enzyme alkaline phosphatase (ALP) resulting from the use of an engineered phage infection is captured on the test line with anti-ALP polyclonal antibodies (purple). The precipitating dye BCIP/NBT, is then used to form a dark blue precipitate line through the enzymatic reaction with ALP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significantly signal was not detected ($p=0.0502$). With scheme 3 we were able to visually distinguish and detect a statistically significant test line signal in samples containing at least $10^{5.5}$ PFU of $T7_{ALP}$ (Fig. 2). Based on a sample volume of 50 μL, the final phage amplification levels required for detection were calculated to be: $10^{9.1}$ PFU/mL of wild-type T7; $10^{7.5}$ PFU/mL of $T7_{MBP}$, and $10^{6.8}$ PFU/mL of $T7_{ALP}$. Thus using a protein reporter produced by a bioengineered T7, we lowered the required phage amplification levels for a detectable signal by 1.6 $\log_{10}$, a great than 10-fold increase in sensitivity. Using the alkaline phosphatase reporter further reduced necessary phage amplification levels by a total of 2.3 $\log_{10}$, greater than 100-fold improvement in sensitivity. These results highlight the potential of targeted bioengineering of phage to produced reporter molecules that can enhance the performance of phage amplification-based LFAs.

3.2. Detection of *E. coli* in broth using $T7_{MBP}$ and $T7_{ALP}$ amplification-based lateral flow strips

We developed a phage amplification matrix, with varying initial *E. coli* cell and phage concentrations, to provide guidance on the maximum level of phage amplification we could reasonably expect over time (Supplemental materials). This information allowed us to understand whether the sensitivity our three LFA schemes would be sufficient to detect phage-amplification from low levels of *E. coli.* Based on the phage amplification matrix (Fig. S1) and the sensitivities of our LFA schemes (Fig. 2) we choose an initial phage inoculum level of 10^2 PFU/mL for further $T7_{MBP}$ testing and an initial phage inoculum level of 10^4 PFU/mL for further $T7_{ALP}$ testing. The sensitivity of the T7 viron targeted LFA was not adequate for successful detection of *E. coli* cells concentrations at or below 1000 CFU/mL, and so this scheme was not used for further testing.

We incubated several concentrations of *E. coli* BL21, from 10^1 to 10^3 CFU/mL, and a negative control consisting of LB, at 37 °C with an inoculum of either $T7_{MBP}$ or $T7_{ALP,}$ for 7 h for the former, and 5 and 7 h for the later. At the end of incubation, we filtered the samples to remove cellular debris, and ran a 100 μL aliquot up the appropriate lateral flow strip as previously described. Note, an added benefit of increasing the volume of the sample aliquot to 100 μL, is that it lowers the amount of phage amplification needed to be reached in the sample by 0.3 $\log_{10}$, i.e. for scheme 3, based on $T7_{ALP}$, the necessary level went from $10^{6.8}$ PFU/mL to $10^{6.5}$ PFU/mL and from $10^{7.5}$ to $10^{7.2}$ for scheme 2, based on $T7_{MBP}$. A statistically significant signal from phage amplification could be detected at the test line from samples with an initial *E. coli* concentration of 10^3 CFU/mL for scheme 2 after 7 h and for scheme 3 after 5 h (Fig. 3a). We were able to detect a statistically significant signal from a starting concentration of 10^2 and 10^3 CFU/mL of *E. coli* (Fig. 3a) with LFA scheme 3 after a 7-h incubation. Based on the phage amplification matrix (Fig. S1), we had expected scheme 3 to be able to detect a signal 10 CFU/mL of *E. coli*, but did not. It is possible that the 1-h decrease in incubation was sufficient to prevent amplification levels from reaching those observed within

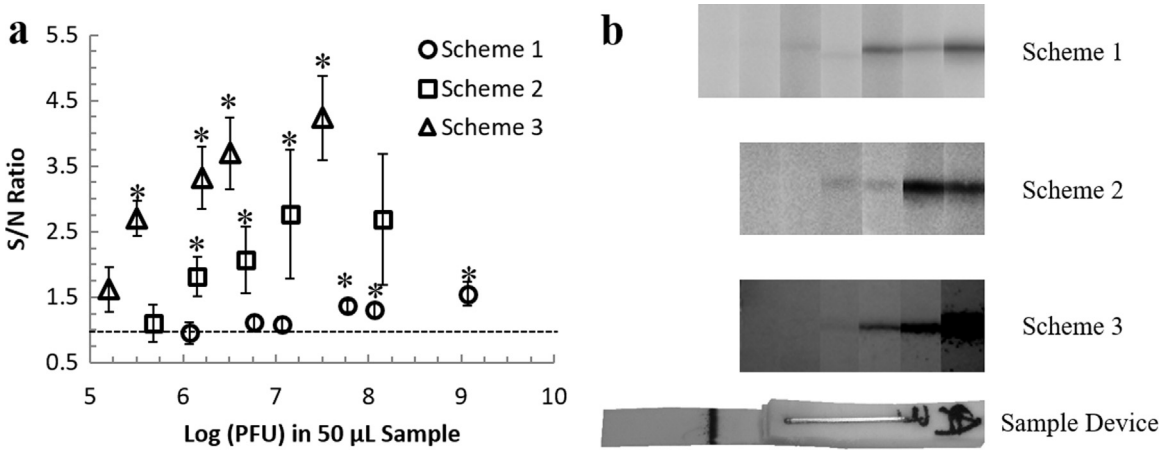

Fig. 2. LFA Limit of Detection. (a) 50 μL samples of a dilution series of wild-type T7, $T7_{MBP}$, and $T7_{ALP}$ phage stock were tested using the respective LFA scheme: Scheme 1 for the T7 viron, Scheme 2 for maltose-binding protein, and Scheme 3 for alkaline phosphatase. Sample test line signals were normalized to the negative control test line signal (S/N) for Schemes 1 and 2. The dotted line denotes an S/N ratio =1. Error bars represent one standard SD ± from the sample mean and a star (*) indicates a significance ($p < 0.05$). (b) Representative strips of all three LFA scheme trials. The left most strip corresponds to the negative control, and each subsequent strip is increasing phage titer corresponding to the respective data points in part a. The sample device includes the nitrocellulose test strip as well as the affixed absorbent pad.

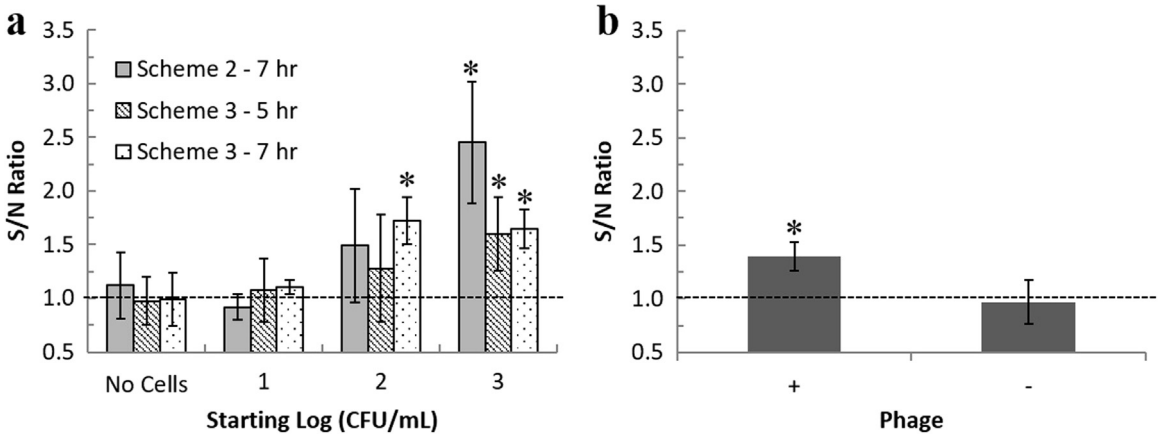

Fig. 3. Bacteria Detection. (a) Samples of each *E. coli* concentration were tested with the corresponding LFA scheme following either a 5 or 7 h incubation at 37 °C with $T7_{ALP}$ or $T7_{MBP}$; (b) River water containing 100 CFU/100 mL of *E. coli* was filter concentrated and cells re-suspended in LB with (+) or without (-) $T7_{ALP}$ and incubated for 7 h at 37 °C. 100 μL of each samples were tested with the LFA scheme 3. In both graphs sample test line signals were normalized to the negative control test line signal (S/N). The dotted line denotes an S/N ratio=1. Error bars represent one SD ± from the sample mean and a star (*) indicates a significance ($p < 0.05$).

8 h in our earlier matrix. It is also possible that the insertion of alkaline phosphatase into the T7 genome, and its subsequent overexpression during infection could lower the amplification efficiency of the bioengineered $T7_{ALP}$ in comparison to the wild type used for the matrix, and thus not achieve the same level of amplification. The scheme 3 strips were allowed to develop in BCIP/NBT overnight, and a visually detectable line did form at the test line for 10 CFU/mL *E. coli* samples (data not show).

3.3. Proof-of-principle detection of E. coli in river water using $T7_{ALP}$ amplification-based lateral flow strips

E. coli is commonly used as an indicator of drinking and recreational water quality (Edberg, 2000), and the rules for produce safety proposed by the FDA as part of the Food Safety Modernization Act (FSMA) will also establish microbiological quality criteria for agricultural water. These produce safety rules will require regular water monitoring for generic *E. coli* with a rolling 5-sample mean limit of 126 CFU/100 mL and a statistical threshold value of 410 CFU/100 mL (FDA, 2013). While there are many commercially available tests to detect *E. coli* in water, the majority of these tests take anywhere from 24 to 48 h to provide results (Olstadt et al., 2007). This time delay presents significant challenges for farmers in regards to water use management and appropriate corrective action response to the discovery of non-complaint water.

Our bacterial limit of detection using scheme 3 was 100 CFU/mL *E. coli*, essentially two orders of magnitude higher that the FSMA agricultural water limit. Filtration of 100 mL samples is a commonly used method for bacterial concentration when performing *E. coli* testing of water. To explore whether this concentration step would help us to reach the essential 1 CFU/mL FSMA limit, we took 100 mL samples of sterilized river water and spiked them with ~100 CFU of *E. coli* strain BL21, passed them through a 0.45 μm filter to capture the *E. coli* cells. The filter was then back-flushed with 1 mL of LB to re-suspend the cells and inoculated with $T7_{ALP}$. To further demonstrate that any positive signal was due to alkaline phosphatase production from $T7_{ALP}$ amplification and not due to alkaline phosphatase endemic to *E. coli*, our negative controls were samples spiked with *E. coli* but not inoculated with phage. Samples were incubated for 7 h at 37 °C. An aliquot (100 μL) of each of these samples was tested using LFA scheme 3, and the strips developed in BCIP/NBT for 2 h. Pictures were taken and analyzed for signal. We were able to detect a statistically significant signal at the test line in the samples inoculated with $T7_{ALP}$, and detected no signal in negative controls (Fig. 3b). This proof-of-principal demonstrates the potential for phage amplification-based LFAs to rapidly detect regulatory relevant levels of bacteria, though further work is needed to explore the impact of other microbial organisms that would be co-concentrated in the sample.

The assay benefits from the long term storage stability of a lateral flow assay (Posthuma-Trumpie et al., 2009) and the use of bacteriophages. Unlike the enzymes the bacteriophage may carry the gene for, the virus can be stabilized for storage at room temperature by lyophilization (Dai, et al. 2014). At 4 °C, the phages can be stored up to 37 months (Merabishvili et al., 2013).

To understand the reproducibility of our LFA schemes we used the LOD runs to calculate the intra-assay coefficient of variation. For each data point in Fig. 2a we calculated the average and standard deviation of the log10 (signal at test line) across the three independent runs. We divided the standard deviation by the average signal to provide a coefficient of variation (CV) for each point, and then took the average of the CVs for all the data points for a scheme to give us the intra-assay coefficient of variation for the respective scheme. Our intra-assay CVs were 6.8%, 9.9%, and 6.2% for the T7, MBP, and ALP LFAs respectively. Our LFAs are handmade devices to demonstrate the potential improvements in sensitivity that bioengineered phage could provide phage amplification-based LFAs, and we would expect the CVs to decrease further if these schemes were translated to a commercial manufacturing setting.

This proof-of-principal demonstrates the potential for phage amplification-based LFAs to improve detection time of regulatory relevant levels of bacteria. The gold standard for detection of generic *E. coli* in water are culture-based enzymatic tests, but they typically require 18–48 h to provide a result (Olstadt et al., 2007; Rompré et al., 2002). DNA based and immunological based methods have been used for detection of generic *E. coli*, however their adoption has been limited due to issues of specificity, false positive detection of non-viable cells, and test complexity (Ahmed et al., 2015, Frahm and Obst, 2003, Rompré et al., 2002). Purely enzymatic tests have shown the potential to provide results within two hours, but suffer from potential false positives from non-target bacteria (Ahmed et al. 2015, Rompré et al. 2002, Wildeboer et al. 2010).

4. Conclusion

Our goal was to investigate the use of bioengineered phage to

improve the sensitivity of phage-amplification based lateral flow assays for the detection of bacteria. We demonstrated that by bioengineering phage to overexpress protein and enzymatic reporters, that we could improve the sensitivity of phage-amplification based LFA by over 100-fold. This increase in sensitivity enabled the detection 10^3 CFU/mL of *E. coli* after 7 h, and a regulatory limit of 100 CFU/100 mL of *E. coli* when combined with a filter concentration step in 9 h. The LFA format is ideal for use in low-resource settings, such as some clinics, farms, and food production facilities; and modern molecular techniques allow for the replication of our alkaline phosphatase reporter scheme in other phage to ensure broad species coverage and allows for the targeting of other bacterial species of significance. Further research into LFA-amenable phage-delivered reporter peptides and enzymes will unlock the power and sensitivity of phage-based diagnostics, allowing for the development of novel and versatile bacteria detection assays.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.03.047.

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