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RESEARCH PAPER

Colorimetric detection of *Escherichia coli* using engineered bacteriophage and an affinity reporter system

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Abstract

Reporter phage systems have emerged as a promising technology for the detection of bacteria in foods and water. However, the sensitivity of these assays is often limited by the concentration of the expressed reporter as well as matrix interferences associated with the sample. In this study, bacteriophage T7 was engineered to overexpress mutated alkaline phosphatase fused to a carbohydrate-binding module (ALP*-CBM) following infection of *E. coli* to enable colorimetric detection in a model system. Magnetic cellulose particles were employed to separate and concentrate the overexpressed ALP*-CBM in bacterial lysate. Infection of *E. coli* with the engineered phage resulted in a limit of quantitation of 1.2×10^5 CFU, equating to 1.2×10^3 CFU/mL in 3.5 h when using a colorimetric assay and 100 mL sample volume. When employing an enrichment step, $< 10^1$ CFU/mL could be visually detected from a 100 mL sample volume within 8 h. These results suggest that affinity tag modified enzymes coupled with a material support can provide a simple and effective means to improve signal sensitivity of phage-based assays.

Keywords Bacteriophage · Fusion · *E. coli* · Binding module · Cellulose · Magnetic

Introduction

Bacteriophage-based systems have emerged as a promising technology for the detection of bacteria in food matrices [1, 2]. Bacteriophages (or phages) can infect a targeted host cell with extraordinary specificity and rapidly propagate inside the host before lysing the cell. Reporter phage detection systems exploit this mechanism by introducing recombinant genes into the phage genome to enable expression by the host bacteria during infection. The reporter gene, when expressed, offers a unique method for detection of bacteria using standard colorimetric, fluorescent, or luminescence signal. Since bacteriophage are viruses, their genes can only express during the host infection, thereby confirming the presence of viable host bacteria. A variety of reporter genes have been engineered in phages including luciferase (*lux*, *luc*) [3], β-galactosidase (*lacZ*) [4, 5], green fluorescent protein (*gfp*) [6], and alkaline phosphatase (*alp*) [7].

While phage-based detection systems show promise, their utilization has been limited. Central to this challenge is the need to reliably detect bacteria at low concentrations from diverse and large volume samples. Although laboratory samples may not contain substances that interfere with the signal generated from reporter enzymes, real-world samples (e.g., environmental, food, and clinical samples) may contain compounds that quench the signal generated by the reporter enzymes, inhibit the enzyme, or restrict interpretation due to background noise (from compounds that can give a mistaken signal or from enzymes in the sample that compete for the substrate) [8–10]. In practical applications, dilution of the reporter is employed to reduce background/matrix interferences associated with inhibitors, pigments, and other compounds found in the food and lysed bacteria. Dilution, however, reduces the concentration of reporter available for detection. The signal potential of the reporter is also reduced when small volumes are used to identify its presence in a large volume sample. Often, only a small percentage of the expressed reporter is utilized for detection due to volume constraints. For example, in a 100 mL water sample, only 10–100 μL of the

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sample (containing the expressed enzyme) may be used for detection when using a plate reader or sensor. Given these limitations, it is critical that practical means to separate and concentrate the reporter be developed.

The coupling of separation and concentration to phage-based detection has been evaluated as a means to improve the sensitivity of detection. For water, filter methods have been used to separate and concentrate *E. coli* before phage-based detection. Using this approach, 50 CFU of *E. coli* K12 could be detected in 4 h using an M13KE phage with a β-galactosidase reporter and a fluorescent substrate [11]. While separation and concentration of bacteria before detection aids in reducing the limit of detection, the expressed reporter is still diluted (from addition of the phage), native enzyme from trapped bacteria can compete for the substrate, and the presence of inhibitors in the lysis can reduce activity. For samples where filtration is not possible due to fouling of the membrane, separation and concentration can be achieved by using immunomagnetic particles to capture bacteria before the introduction of phage. While immunomagnetic separation (IMS) has been used to reduce the detection limit of generic *E. coli* using phage-based systems [12], the high cost, batch-to-batch variation, and the need for specific environmental conditions (e.g., salinity, temperature, and pH) limit the use for food and agriculture applications [13]. Moreover, cells and nutrients present in the concentrated sample dilute the expressed reporter and may interfere with the resulting signal.

An alternative approach utilizing separation and concentration for phage-based detection has been demonstrated by Martelet et al. [1]. Rather than separating the bacteria, this study relied on the separation and detection of progeny phage produced during phage infection of *E. coli*. The resulting progeny phage was separated and concentrated using immunomagnetic particles specific to the progeny phage, then detected using MALDI MS. This method was determined to be more sensitive than immunomagnetic separation of bacteria for the detection of *E. coli* and allowed for detection down to 1 CFU/mL. While capable of low detection limits, this method is not cost-effective for low-resource settings. Additionally, as reporter proteins are typically expressed in much higher concentration than progeny phage, it is expected that the separation and concentration of reporter proteins (specifically, enzymes) can improve the time to detection.

Several proteins and peptides have been identified that have demonstrated specific affinity to material supports such as carbohydrates, metals, silica, and synthetic polymers [14–21]. These material-binding proteins or peptides can be introduced as fusion tags by inserting the gene for the affinity tag adjacent to the gene coding for the protein of interest. When the protein is expressed, the fusion tag is also expressed at one or both terminus ends of the protein as an attached moiety to the desired enzyme [22]. Carbohydrate-binding modules (CBMs) represent one class of material-binding proteins, and CBMs with cellulosic binding capabilities can be found naturally as part of cellulases and cellobiohydrolases [23–25]. The binding affinity of these proteins towards cellulose can be so high that release must be performed under denaturing conditions [26]. As a fusion tag, CBMs have been expressed with T4 lysozyme [27], *cis*-epoxysuccinic acid hydrolase [28], k- and λ-carrageenases [29], and phytase [30]. The objective of this study was to engineer bacteriophage T7 to overexpress a CBM-tagged alkaline phosphatase reporter upon infection of *E. coli* in a model system, and to determine the subsequent colorimetric detection capability of the system when paired with magnetic cellulose-based particles to enable separation and concentration of the reporter.

Methods and materials

Materials

p-Nitrophenyl phosphate (p-NPP) was purchased from Thermo Fisher Scientific. T7 Express Competent *E. coli* (high efficiency) was purchased from New England Biolabs. Magnetic macroporous bead cellulose (types MG) Iontosorb MG100 was purchased from Iontosorb.

Bacterial strains and culture media

In this study, T7 Express cells (New England Biolabs) were used for propagation of phages and as generic bacteria for detection studies. *E. coli* T7 Express cell was grown overnight in 5 mL of Luria−Bertani broth (10.0 g of tryptone, 5.0 g of yeast extract, 10.0 g of sodium chloride in 1 L of distilled water). The next day, cells were serially diluted in LB media and plated to determine the count.

Construction of ALP* and ALP*:Cex phages

The reporter was produced as a double mutant (D135G/D330N) of *E. coli* alkaline phosphatase (GenBank accession no. M29664.1) and designated as ALP*. The CBM gene of exoglucanase (Cex) from *Cellulomonas fimi* (GenBank accession no. M15824.1) was fused at the C-terminus of ALP* to produce ALP*:Cex [31]. The T7-based reporter phage was engineered as previously described by Hinkley et al. [32]. Briefly, the T7Select genome (Millipore Sigma, Burlington, MA, USA) was digested with *Hind*III-HF, resulting in a cut at the 3′ of the capsid gene. DNA inserts complementing the capsid gene with a stop codon followed by the reporter genes were inserted between the digested DNA using in vitro DNA assembly (New England Biolabs, Ipswich, MA). The assembled genomes were then transformed into *E. coli* (enhanced BL21 derivative-T7 Express) to initiate infection and replication of the engineered phages. Genomes of the engineered phages,

NRGp1, which contained the gene for ALP*, and NRGp2, which contained the gene for ALP*:Cex, were submitted for whole genome sequencing to determine correct assembly.

Propagation and purification of the engineered phage

For propagating the engineered phage, 50 μL of overnight *E. coli* culture was inoculated in 10 mL of LB media in a 50-mL tube and grown for 3–4 h at 37 °C/150 rpm. Subsequently, 10 μL of stock engineered phage was inoculated in the culture and incubated for 3–4 h until the culture became clear. The lysate was then centrifuged at 3000×*g* for 20 min at room temperature (ca. 21 °C) to remove cellular debris.

Phage expressing ALP*:Cex was purified by mixing the lysate with 20 mg microcrystalline cellulose (50 μM) in 5 mL of total volume at room temperature (ca. 21 °C) under end-to-end rotation using a mini-tube rotator for 1 h. The solution was centrifuged at 3000×*g* at room temperate for 5 min to separate the protein-bound cellulose particles from the solution. Phage expressing ALP* was purified using Ni-NTA by mixing 0.5 mL Ni-NTA resin with 5 mL of the lysate at room temperature (ca. 21 °C) under end-to-end rotation using a mini-tube rotator for 1 h. The solution was then centrifuged at 3000×*g* at room temperature for 5 min to separate the resin-bound protein from the phage. In both cases, 20 μL of cell lysate was assayed using p-NPP before and after cellulose addition to evaluate the background signal in pure phage lysate. The phage lysate in LB media was stored at 4 °C until further use. The phage titers (PFU/mL) were determined by a plaque assay using a double agar overlay. Briefly, the serially diluted phages (100 μL) were added into the melted top LB agar (2 mL) containing the overnight *E. coli* culture (20 μL). The contents were then poured over the underlay plate. After overnight incubation at 37 °C, plaques were counted.

Separation and concentration of the reporter

An overnight culture of *E. coli* was serially diluted in LB media to achieve 1–10^6 CFU/mL in 1, 10, and 100 mL of LB media. Cells were infected with 10 μL of engineered phage (diluted in LB media) expressing ALP* or ALP*:Cex so that the final phage concentration was at least 10 times higher than the initial concentration of bacteria. The mixture was then incubated at 37 °C with shaking at 175 rpm for 2 h. Hydrated magnetic cellulose 10, 20, and 40 mg was added to 1, 10, and 100 mL of the samples and shaken at 175 rpm at room temperature for another 1 h. Following immobilization, the suspension was subjected to magnetic separation for 10 min to separate protein-bound magnetic cellulose from the lysate. Separated particles were washed two times with 100 mM DEA, pH 10.0 containing 100 mM NaCl, and 10 mM $MgCl_2$ before activity detection. For enrichment studies, the above protocol was followed using 100 mL of bacterial culture that was incubated in LB media for 4 h at 37 °C before the incorporation of the phage.

Detection of enzyme activity

To determine the activity of the soluble enzyme, a volume of supernatant (10 μL) from a 1 mL solution containing10^6–10^7 CFU/mL *E. coli* and 10^8 PFU/mL of engineered phage expressing ALP* or ALP*:Cex (incubated for 2 h at 37 °C and 175 rpm) was added to 50 μL p-NPP to give a final substrate concentration of 25 mM. To determine the activity of the immobilized enzyme, 25 mM p-NPP was added to tubes containing magnetically separated particles to give a final volume of 60 μL. The tubes were incubated at 37 °C for 30 min. Following incubation, tubes were placed on a magnetic rack, and 30 μL of the resultant supernatant was transferred to a 384-well microplate and read at 405 nm using a BioTek Synergy H1. All colorimetric assays were performed under optimum condition for ALP*/ALP*:Cex (1 M DEA buffer, pH 10.0 with 10 μM of $ZnCl_2$, and 10 mM $MgCl_2$ at 37 °C) and under saturating substrate conditions (25 mM of p-NPP) based on the K_m of ALP*:Cex (4.9 ± 1.1 mM). Percent relative activity of the soluble and immobilized enzymes was determined from the activities obtained from 1 mL solutions of phage and *E. coli*. Activities were standardized on a colony-forming unit (CFU) basis and expressed as a percentage relative to the mean activity of soluble ALP*. Unit activities were determined from pre-saturation absorbance values (≤ 1.5) using a molar extinction coefficient of 16,200. One unit was capable of producing 1 μmol of product per minute under the conditions of the assay. Unmodified T7 phage (which does not express a reporter enzyme following infection) and *E. coli* cells without the addition of phage were assayed as controls.

Statistical methods

All experiments were conducted using three independent tests. One-way ANOVA using Tukey's pairwise comparison was applied to determine significant differences ($p < 0.05$). In the event of unequal variance (as determined by the Brown–Forsythe test), the Steel–Dwass test was applied for multiple comparisons. The quantitation limit was defined as ten times the standard deviation of the blank divided by the regression slope.

Results and discussion

Detection system

The premise of the assay system is described in Fig. 1. Following phage infection of the host bacteria, a reporter enzyme containing a carbohydrate-binding module (CBM) is

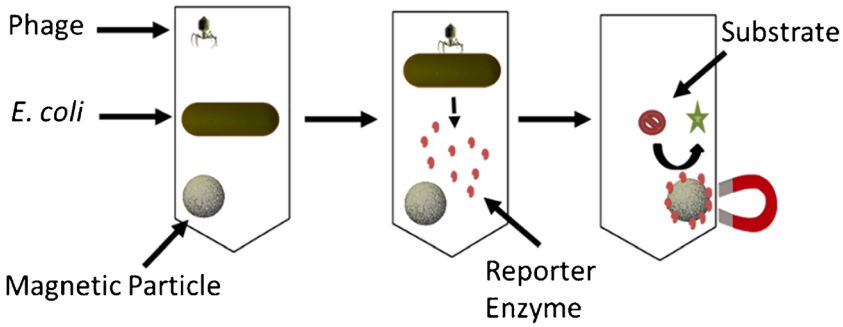

Fig. 1 Schematic of the reporter phage detection system

expressed. The CBM fusion tag enables immobilization of the reporter to magnetic cellulose particles. Particles with the immobilized enzyme are separated and concentrated. After the addition of the substrate, the resulting color is evaluated using a spectrophotometer or visual observation. The assay design utilizes inexpensive components (e.g., magnetic cellulose, phage, and substrate) as well as colorimetric detection to promote adoption in resource-limited settings that require minimal equipment.

Construction of ALP* and ALP*:Cex phages

The successful insertion of the reporter genes was observed in the genome sequences of the engineered phages. The sequences for NRGp1 (MH651795) and NRGp2 (MH651796) have been submitted to the NCBI.

Reporter expression, activity, and immobilization

As seen in Fig. 2, both native ALP* as well as ALP*:Cex could be expressed from T7 phage in the model *E. coli*. However, modification of the enzyme resulted in a total activity that was ca. 42% lower than that of native ALP* on a per CFU basis. This result corroborates our in vitro studies that found the expression level and the k_{cat} for ALP* to be greater than that of ALP*:Cex, and suggests that the increase in size and/or changes in the tertiary structure of alkaline phosphatase following modification may reduce the amount of total activity of the enzyme following expression [31]. When cellulose was introduced to the system containing expressed ALP*, very little bound activity (< 1% relative to the soluble ALP*) could be recovered following particle separation and washing (Fig. 2). Conversely, ca. 75% of the soluble activity of ALP*:Cex could be recovered in the immobilized form (44% activity per CFU relative to soluble ALP*) following the addition of cellulose particles. These results suggest that the addition of the Cex fusion tag enables direct binding of catalytically active alkaline phosphatase to cellulose following phage infection and reporter expression.

Separation, concentration, and detection

A range of concentrations of *E. coli* at a set phage concentration and varying sample volumes (1 mL, 10 mL, and 100 mL) was evaluated to determine the scaling capability of the designed system to separate and concentrate the reporter. As the sample volume increased, higher absorbance values at lower bacteria concentrations were observed (Supplementary Figure 1). At a sample volume of 1 mL, concentrations of 10^5 CFU/mL could be routinely distinguished through visual observation, while 10^4 CFU/mL and 10^3 CFU/mL could be routinely distinguished at sample volumes of 10 mL and 100 mL, respectively (Fig. 3). While a significant increase ($p < 0.05$) in total recovered activity was observed with

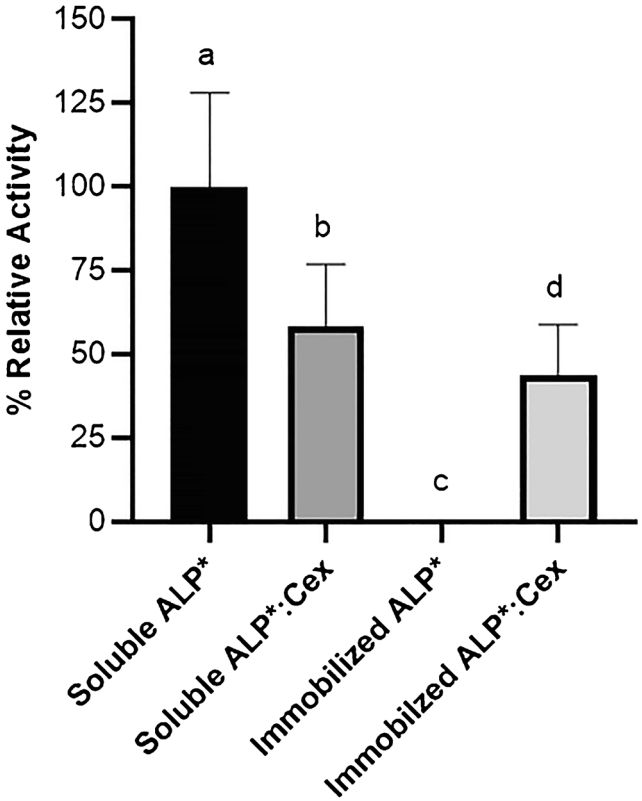

Fig. 2 Percent relative activity of soluble ALP* and ALP*:Cex in the supernatant prior to the addition of cellulose particles and immobilized ALP* and ALP*:Cex following the addition of cellulose particles

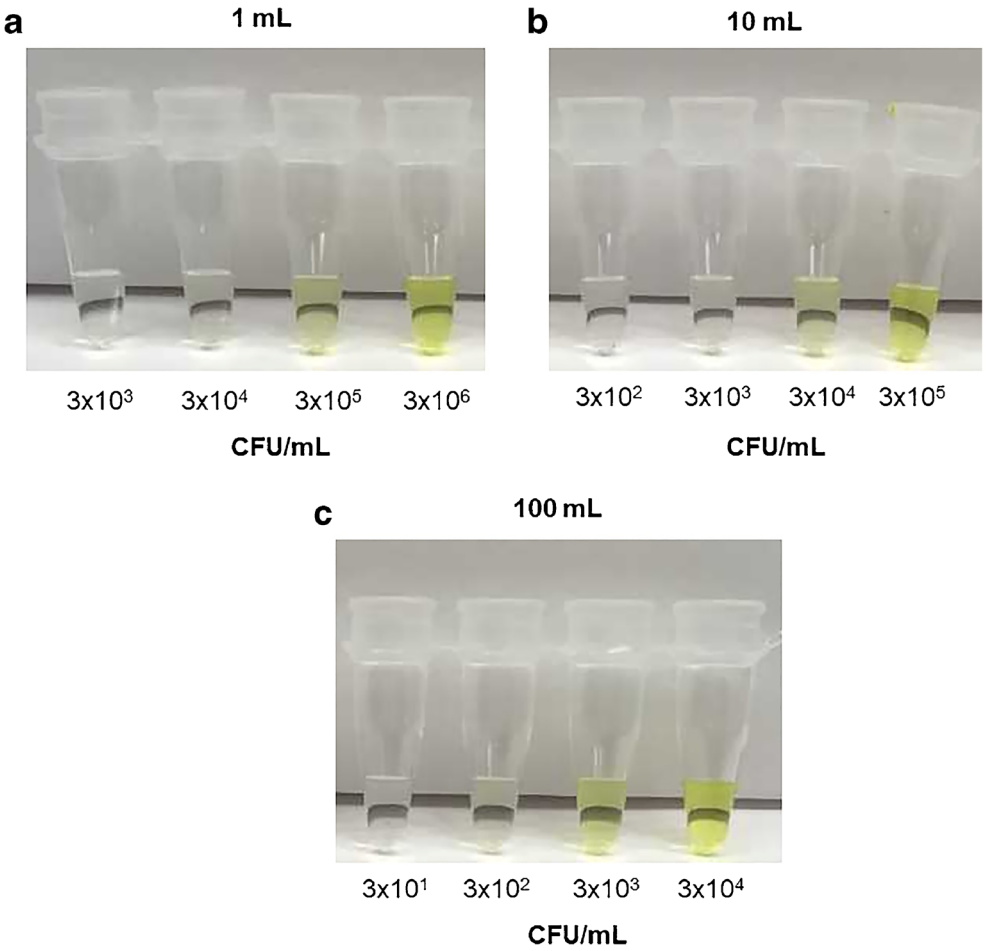

Fig. 3 Visual signal of immobilized ALP*:Cex expressed from phage-infected *E. coli* in **a** 1 mL, **b** 10 mL, and **c** 100 mL sample volumes

increasing sample volume (Fig. 4a), no significant difference was seen when the activity was adjusted for the total number of cells in the sample volume (Fig. 4b). These results suggest that the output per cell is independent of sample volume and that the ability to detect lower concentrations of bacteria with increasing sample volume is due to separation and concentration of the reporter. When the signal was evaluated independent of volume, the quantitation limit was determined to be 1.2×10^5 CFU (Fig. 5).

To determine the feasibility of the assay to enable rapid presence/absence detection (≤ 8 h total assay) of concentrations of *E. coli* relevant to food and water applications (≤ 100 CFU/mL), the designed system was coupled with a bacteria enrichment step. An enrichment step enables the initial concentration of bacteria to reach higher concentrations before initiation of the assay. As demonstrated in Fig. 6, visual detection of less than 10 CFU/mL could be achieved when using a 100 mL sample and a 4-h enrichment time (8-h total assay time). These results indicate that coupling enrichment with the designed reporter system offers the possibility of visual presence/absence detection of practical concentrations of *E. coli* within a standard 8-h work shift. Detection at these lower initial concentrations is achievable provided that there is enough time for the initial concentration of bacteria to reach

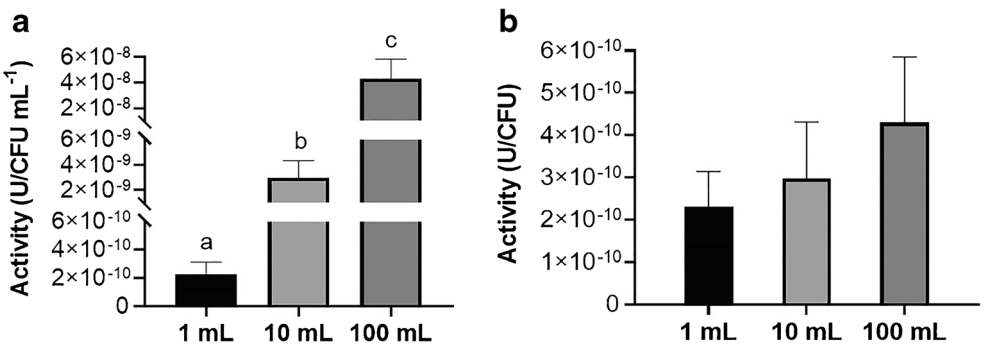

Fig. 4 Activity of immobilized ALP*:Cex expressed from phage-infected *E. coli* as a function of sample volume on **a** a cell concentration basis and **b** a per cell basis

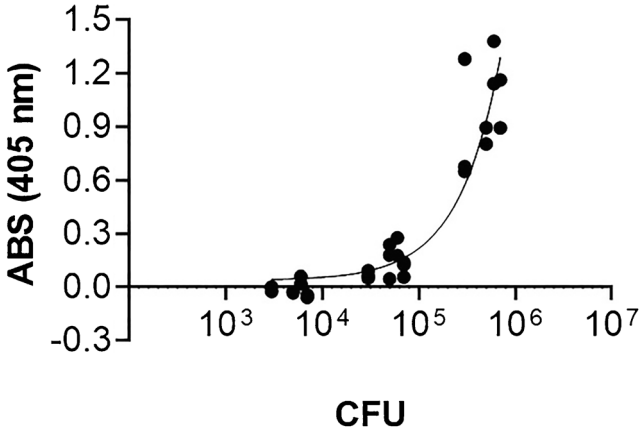

Fig. 5 Regression of absorbance values at 405 nm as a function of the initial number of *E. coli* cells in a sample

the limit of quantitation before the addition of phage and that the sample volume is sufficiently large enough to yield the necessary amount of the reporter.

Conclusion

In this study, we have engineered T7 phage to overexpress a fusion reporter enzyme following infection of *E. coli* in a model system. When expressed in phage, modification of an alkaline phosphatase reporter with a Cex fusion tag results in a reduction of activity of the soluble enzyme. However, incorporation of the fusion tag to the reporter allows for facile immobilization of the reporter on magnetic cellulose particles following phage infection of the targeted host. This technology enables the separation and concentration of the reporter from varying sample volumes when used in a model solution and provides a means to enhance bacteria detection with increasing sample volume. When paired with a colorimetric readout and cell enrichment, the developed phage-based system demonstrates the potential for rapid (≤ 8 h total assay) and user-friendly detection of bacteria in sample volumes relevant to food and water analysis.

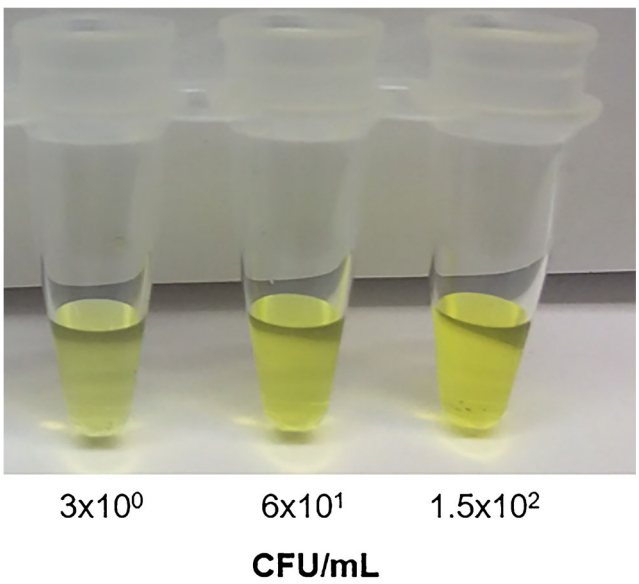

Fig. 6 Visual signal of immobilized ALP*:Cex expressed from phage-infected *E. coli* in a 100 mL sample following a 4-h enrichment at 37 °C

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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