

# Bacteriophage Effectiveness for Biocontrol of Foodborne Pathogens Evaluated via High-Throughput Settings

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## Abstract

Bacterial pathogens continually challenge food safety systems worldwide. With increasing concerns about the emergence of heat- and sanitizer-resistant bacteria, novel antibacterial agents, are urgently needed. A bacteriophage-based biocontrol strategy is the therapeutic use of phages to control bacterial pathogens in agricultural settings. Phage biocontrol is increasingly accepted as a sustainable technology, effective at decontaminating foodborne pathogens. To ensure effective biocontrol outcomes, systematic screening of phage combinations against targeted bacteria under required environmental conditions is crucial. Antibacterial efficacy of phage cocktails may be affected by phage genera and combination, targeted bacterial strains, the multiplicity of infection, temperature, and time. To formulate a phage cocktail with superior efficacy, the proposed method was to systematically evaluate the effectiveness of individual phages and phage cocktails in killing foodborne bacterial pathogens under targeted conditions. Bacterial killing efficacy was monitored by measuring optical density at desired temperatures and durations. Superior phage efficacy was determined by complete inhibition of bacterial growth. The proposed method is a robust, evidence-based approach to facilitate formulating phage cocktails with superior antibacterial efficacy.

## Introduction

Bacteriophages (phages) are viruses that naturally invade bacterial cells, disrupting bacterial metabolism and causing lysis of the bacterium. In contrast to conventional antimicrobials (e.g., antibiotics), phage host spectrums are relatively narrow, only capable of infecting a targeted set of bacterial species or strains and thus should minimize collateral effects on microbiota that benefit animal and human

health. With the rise of antimicrobial resistance (AMR), phages and their derivatives lead to alternative antimicrobials to control bacterial infectious diseases, including AMR bacterial infections in humans and animals<sup>1,2</sup>. Phages have confirmed therapeutic potential against >20 bacterial pathogens that cause superficial infections and infections

of the upper respiratory system and gastrointestinal tract of humans<sup>3</sup>.

In agricultural settings, a phage-based biocontrol strategy is the therapeutic use of phages to control bacterial pathogens. Phage biocontrol is well accepted as a green technology, effective at decontaminating foodborne pathogens (e.g., Shiga-toxin producing *Escherichia coli* (STEC), *Salmonella*, and *Listeria*) in various foods<sup>4,5</sup>. In addition, phages can be used as sanitizers to disinfect food processing surfaces and animal hides, which can be integrated into conventional antimicrobial systems (e.g., chemicals, steam, and hot water pasteurization) to enhance desired outcomes and reduce environmental impacts. The use of phages to reduce zoonotic bacteria in animals is also promising<sup>1</sup>. However, there is a need to address the technical challenges to improve outcomes from the phage biocontrol approach to be popularly applied in diverse food production systems. The main challenge is the impaired effectiveness of phages due to the development of bacteria-resistant mutants<sup>5</sup> and changes in bacterial physiology due to exposure to environmental stressors<sup>6</sup>.

For minimizing the risk of phage resistance, phage cocktails (i.e., a combination of multiple phages) are proposed and have improved biocontrol potency in agriculture and aquaculture settings<sup>7</sup>. However, from several studies, it has been proved that phage cocktails did not always offer better effectiveness than the administration of a single phage. For instance, a cocktail of 3 T4-like phages had a narrower host range against *E. coli* strains<sup>8</sup>. Furthermore, AKFV33, a member of *Tequintavirus*, had greater efficacy than a cocktail of four phages in removing *E. coli* O157 from beef, despite the incubation temperatures applied<sup>4</sup>. Recently, it has been reported that the effectiveness of individual

phages does not predict the efficacy of phage cocktails for control of O157<sup>9</sup>, as interactions among multiple phages can alter efficacy. Most importantly, numerous factors, such as phage genera and combinations, targeted strains and MOIs, and incubation temperatures and times, may impact interactions between phages. Therefore, carefully screening combinations of phages against specific bacteria to assess phage synergy or facilitation, or at least to ensure minimal phage antagonism under specific environmental conditions, is vitally important for optimal outcomes. Here, a method is described to systematically assess the efficacy of various phage combinations against foodborne pathogens under a range of environmental conditions. The benefit of this approach is to enable screening of all possible biotic and abiotic factors predicted to affect the antibacterial efficacy of phages in natural settings. In the protocol, STEC O157 and their infecting phages are employed as an example.

## Protocol

### 1. Preparation of buffer and reagents

1. Make 500 mL of tryptic soy broth (TSB) (15 g of TSB powder and 500 mL of ultrapure water) and autoclave.  
**NOTE:** This can be stored at room temperature for up to 3 months or at 4 °C for up to 6 months.
2. Make 500 mL of tryptic soy agar (TSA) (20 g of TSA powder and 500 mL of ultrapure water) and autoclave.  
**NOTE:** This can be stored at 4 °C for up to 3 months.
3. Make 500 mL of Phosphate Buffered Saline (PBS; 4 g of NaCl, 0.1 g of KCl, 0.77 g of Na<sub>2</sub>HPO<sub>4</sub>, and 0.12 g of KH<sub>2</sub>PO<sub>4</sub>, 500 mL of ultrapure water), measure and adjust the pH to 7.2 ± 0.2 at 25 °C and autoclave.  
**NOTE:** This can be store at 4 °C for up to 6 months.

4. Make 200 mL of 1 M MgSO<sub>4</sub> (49.294 g and 200 mL of ultrapure water) and sterilize via 0.22 μm polyethersulfone filter.
5. Make 500 mL of TSB with 10 mM MgSO<sub>4</sub> (mTSB; 15 g of TSB powder, 500 mL of ultrapure water, and 5 mL of 1 M MgSO<sub>4</sub>) and autoclave; let the autoclaved media cool to room temperature. Apply the aseptic technique. Using a serological pipette, carefully transfer 5.0 mL of 1 M MgSO<sub>4</sub>; swirl gently to mix.

## 2. Preparation of bacterial culture

1. To prepare Bacterial Research Laboratory Culture (BRLC), remove glycerol stock vial(s) from the freezer inventory and transfer to the laboratory.
2. Use a disposable inoculation loop or equivalent, dip into the vial, remove a scraping of inoculum (frozen slush), and streak onto a TSA plate or equivalent within a level II biological safety cabinet. Incubate the plate at 37 ± 2 °C for 15–18 h.
3. Bag the prepared BRLC plates and store at 4 °C.  
**NOTE:** General expiry period for BRLC: 14 days at 4 °C after generation.
4. Inoculate a single colony of each *E. coli* O157 strain from the BRLC plates to be tested in 10 mL of TSB and statically incubate at 37 °C for 18 h to reach 9 log<sub>10</sub> CFU/mL.
5. Prepare a serial dilution of the overnight cultures (the following morning), each *E. coli* O157 strain using mTSB containing 10 mM of MgSO<sub>4</sub> to achieve 4–5 log<sub>10</sub> CFU/ml (or other desired inoculum level).

6. Mix an equal volume of an overnight culture of each strain to achieve 4–5 log<sub>10</sub> CFU/mL in total (or as desired) to prepare the bacterial mixture.
7. Immediately place the diluted bacterial culture at 4 °C for pending use.
8. Dilute the inoculum culture 10 or 100-fold and plate 0.1 mL aliquots of these dilutions on the TSA plates to obtain the isolated colonies.

## 3. Preparation of phage working solutions

1. Propagate high-titer working stocks for each phage to be screened (≥10<sup>8</sup> PFU/mL) by following the standard methods<sup>4</sup>.  
**NOTE:** The general expiry period for the phage stocks is 3 months in a plastic bottle at 4 °C after generation.
2. To achieve the desired titer of, e.g., ~10<sup>8</sup> PFU/mL for lysis kinetics, dilute individual phage preparations with mTSB containing 10 mM of MgSO<sub>4</sub>. Prepare phage cocktails by mixing equal volumes of each working stock with the same titer in all the possible combinations.

## 4. Preparation of *in-vitro* lysis kinetics for individual phage and phage cocktails

1. Prepare serial 10-fold dilutions of each phages in Columns 1–8 in sterile 96-well microplates to set up the microplate assay.  
**NOTE:** Four phages against one bacterial strain can be tested in duplicate, in adjacent columns, on each plate. The remaining four columns are for controls, which are without phage, as well as mTSB blank (**Figure 1**).
2. Place 180 μL of mTSB to wells from Columns 1 to 12 of the 96-well microplate.

3. Add 20  $\mu\text{L}$  of diluted individual phages or phage cocktails ( $\sim 10^8$  PFU/mL) to wells 1-8 of the top row of the microplate (Row A).
  4. For the phage-free and Blank control, add 20  $\mu\text{L}$  of mTSB to the top well of Columns 9, 10, and 11.
  5. With a 12-channel pipette, dilute down the plate. Transfer 20  $\mu\text{L}$  from row to row. Mix the well contents by gentle, repeated aspiration, ejection (at least five times), and changing tips between dilutions. Remove 20  $\mu\text{L}$  from the last row (Row H).
 

**NOTE:** Using tips with filters is recommended to prevent cross-contamination.
  6. Set up a reservoir for each strain, use 1 mL pipette to transfer 2-3 mL of the diluted culture into the reservoir. For columns 1-10, use a multichannel pipette to add 20  $\mu\text{L}$  of the diluted bacterial culture to each well. Change tips between each addition.
  7. Cover and incubate the microplate at desired conditions (e.g., 37 °C for 10 h or 22 °C for 22 h).
  8. At 2 h or other desired intervals, remove the microplates from the incubator.
4. From the **Select Plate Type** window, choose **96 Well Plate** from the drop-down list (**Supplementary Figure 2**).
  5. Select **Absorbance** for detection method, the **Endpoint/Kinetic** option for read type, and **Monochromators** for optics type. Click on OK (**Supplementary Figure 3**).
  6. For Read Step, enter **600 nm** for wavelengths and select **Normal** for read speed. Click on **OK** (**Supplementary Figure 4**).
  7. To set the temperature for the assay, click on the **Incubate** checkbox and select **Incubator On**. Enter the desired temperature in the **Temperature** box. Click on **OK**. To prevent condensation on the plate lid during incubation, set a temperature gradient by entering a value (**1-3**) in the **Gradient** box. Click on **OK** (**Supplementary Figure 5a**).
 

**NOTE:** Click on the **Preheat Before Continuing With the Next Step** checkbox for the 37 °C incubation temperature. This step is not required for the test condition at 22 °C (RT).
  8. Next, click on the **Kinetic** checkbox to open the Kinetic Setup. Set the run time for 10 h for 37 °C incubation or 22 h for RT. Enter 2 h for reading interval. Click on **OK** (**Supplementary Figure 5b**).
  9. Click on the **Shake** checkbox in the **Procedure** window to set up the shake condition, if needed, for each kinetic read (**Supplementary Figure 6a**).
  10. Select **Linear** for Shake Modes and change Duration to 30 s. Set Linear Frequency value to 731 cpm (2 mm), and then click on **OK** (**Supplementary Figure 6b**).

## 5. Determination of optical density

1. Examine for optical density at 600 nm ( $\text{OD}_{600\text{nm}}$ ) using a Microplate Reader.
 

**NOTE:** It is advised to set up and save the assay protocol in the program before test plate preparation.
2. Turn on the microplate reader and open program.
3. Select the **Simple** mode in the **Startup Options** window and select **Create a New Protocol** in **Task Manager** (**Supplementary Figure 1a,b**).

11. In the **Protocol Summary Dialogue** window, click on **Plate Layout** and select **Blanks, Assay Controls, and Samples**. Click on **Next (Supplementary Figure 7)**.

**NOTE:** Enter **1** in **Number of Different Control Types** for negative control.

12. After defining the settings for each well type, click on **Finish**.
13. Select a **Well ID** in the left-hand side interface, and then assign it to the matrix shown in the plate layout window. Click on **OK (Supplementary Figure 8)**.
14. Click on the **Read Plate** button and save the protocol as a .prt file and click on **Save (Supplementary Figure 9)**.
15. Insert the plate and click on **OK**.
16. Once the experiment is completed, save the experiment as a .xpt file and click on **Save (Supplementary Figure 10)**.
17. Export data to Excel by clicking on the **Yes** button in the message window box for further analysis (**Supplementary Figure 11**).
18. Click on the **Save Yes/No** selection and the **Don't Ask Again** checkbox to save preference.

## 6. Data analyses

1. Repeat at least two independent experiments as described above. Compile results from all the independent trials. Calculate the average and standard deviation of the OD<sub>600</sub> from each phage-treated and -free culture.
2. Perform square root of the OD values at 600 nm and analyze them using an appropriate statistical model for each bacterial strain and temperature.

**NOTE:** For SAS software, the MIXED model and the least-squares to differentiate means ( $P < 0.05$ ) are selected. For each strain, assign panels A–G to each phage treatment of which overall anti-O157 efficacy across time and MOIs differed ( $P < 0.05$ ).

3. Define superior phage efficacy based on OD<sub>600nm</sub> value  $\leq 0.01$  corresponding to no detectable bacterial growth (limit of detection:300 CFU/mL).
  1. Analyze effects of time, incubation temperature, *E. coli* O157 strains, MOIs, and phage types on phage efficacy using an appropriate statistical model.
 

**NOTE:** For SAS software, use GLIMMIX with random measures.
  2. Calculate Odds Ratios to compare superior efficacy for different environmental and biological factors of interest.

## Representative Results

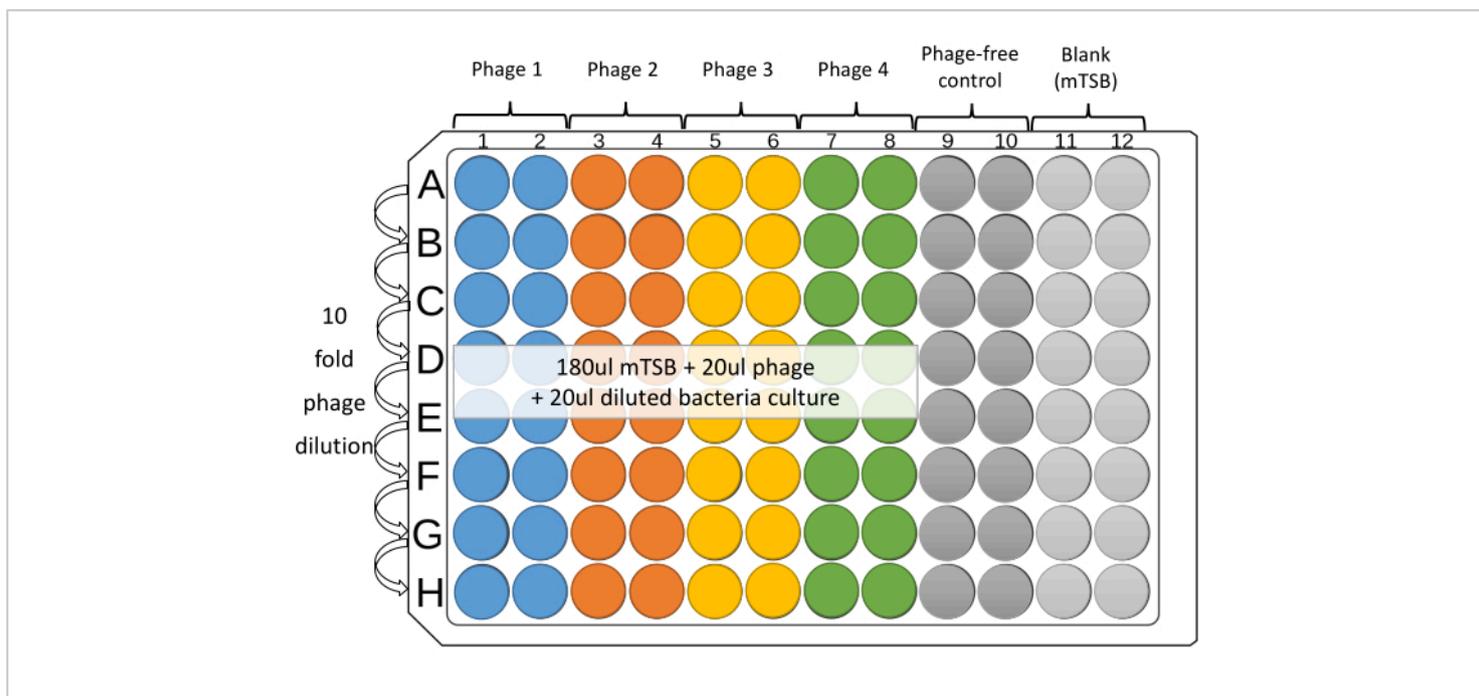
Following the protocol, a comparison of the anti-O157 phage efficacy with various phage combinations, temperatures, times, and MOIs was performed. Impacts of incubation temperature and times, MOIs, phages, and bacterial strains used on enhancing anti-*E. coli* O157 efficacy is presented in **Table 1** (odds ratio table)<sup>9</sup>. The percentage (%) of optical turbidity measurement  $\leq 0.01$  yielded was analyzed by each phage preparation under each experimental condition. Based on this analysis, anti-O157 phage efficacy was maximized after 14, 16, or 18 h of incubation at 22 °C ( $P < 0.001$ ) and MOI = 1000 ( $P < 0.001$ ), with 75% and 89% of the growth of phage-treated culture being completely inhibited, respectively. In general, among 11 phage preparations, a cocktail of T1, T4, and rV5 was most effective ( $P < 0.05$ ) against O157. In addition, across incubation temperatures, times, MOIs, and phages tested, sensitivity to phages varied, with O157

strains tested with strain CO281-31N being most sensitive ( $P < 0.001$ ) and 3081 ( $P < 0.001$ ) being least sensitive to phages.

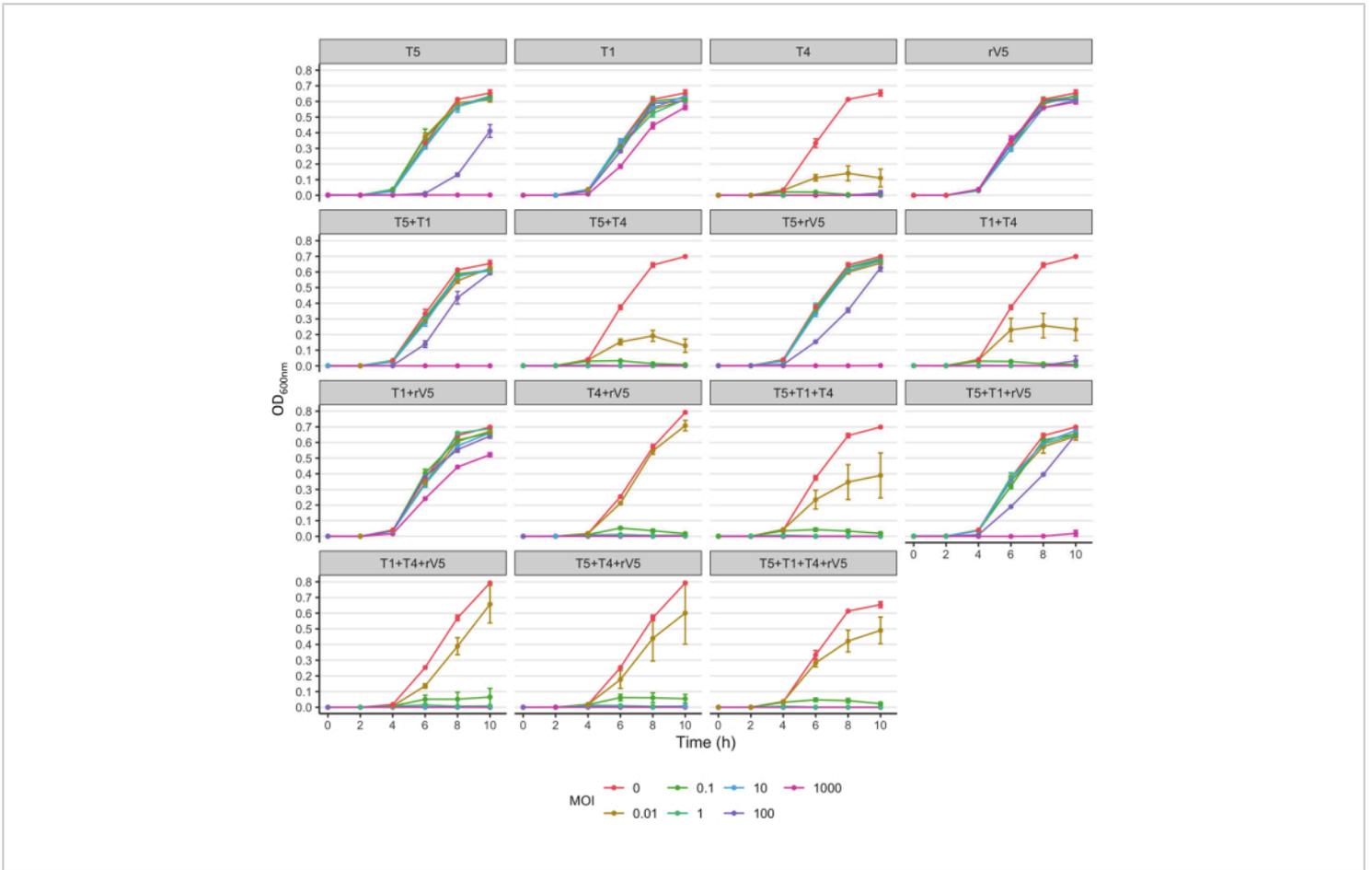
For understanding the phage-killing kinetics of each bacterial strain tested, OD<sub>600</sub> value was plotted against each sampling time, MOIs, and phage treatment at each incubation temperature. Representative results from the efficacy of phages against *E. coli* O157 3081 at 37 °C are shown in **Figure 2**. It is ensured that the growth curve of phage-free control culture is normal before assessing inhibition of growth curve of phage-treated culture. According to **Figure 2**, the inclusion of T4 completely inhibited bacterial growth at 37 °C, at each sampling time at an MOI as low as 1. To overview how phage inhibited bacterial growth over time at various temperatures, irrespective of MOIs, the mean OD<sub>600nm</sub> value averaged from all the MOIs (MOI > 0) was plotted against each sampling time and phage treatment (**Figure 3**). Compared to 37 °C, particular phage killing efficacy, e.g., phages T4 and T1 + T4, was enhanced over 22 °C. Another approach to summarize and compare overall anti-

O157 effectiveness among phages is shown in **Table 2** and **Table 3**<sup>9</sup>. OD<sub>600</sub> value was averaged from each sampling time and MOI and ranked phage efficacy from highest (A) to lowest (F: 37 °C and D: 22 °C). This table facilitates the identification of the best phage treatment. For example, for strain 3081, phages T4 and T5 + T4 (Panel A) were the most effective treatment at 37 °C, whereas in addition to phage T4, phages T1 + T4, T1 + T4 + rV5, and 4 phage cocktails (Panel A) were the most effective treatment at 22 °C.

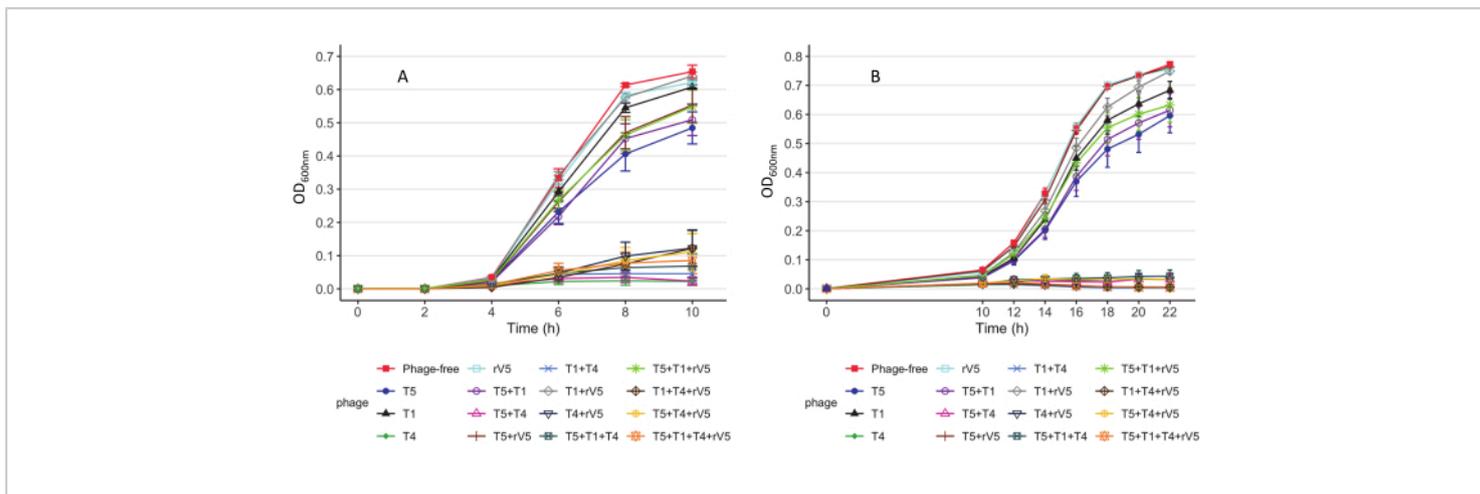
This protocol also enabled us to compare phage efficacy against various bacterial strains targeted and customize phage preparation. For example, when selecting phages, at 37 °C, excluding strain 3081, phage T1 + T4 + rV5 had the most significant effectiveness against all other strains, regardless of their phage types. At 22 °C, the 4-phage cocktail was the most effective against all strains tested. The best MOIs resulted in complete lysis (OD<sub>600</sub> ≤ 0.01), which warranted potential superior efficacy under experimental conditions (**Table 2** and **Table 3**).



**Figure 1: Suggested layout of 96-well microplate assay.** Serial 10-fold dilutions of each phage are prepared in Columns 1-8 of sterile 96-well microplates. Four phages against one bacterial strain can be tested in duplicate, in adjacent columns, on each plate. The remaining columns are for controls. [Please click here to view a larger version of this figure.](#)



**Figure 2: Growth curves of *E. coli* O157 strain 3081 at 37 °C treated and not treated with phages at each MOIs. The data were compiled from two independent trials. Bars present standard deviation. [Please click here to view a larger version of this figure.](#)**



**Figure 3: Growth curves of *E. coli* O157 strain 3081 strains. (A) Growth curves at 37 °C. (B) Growth curves at 22 °C.** Each curve represents individual and mixed cultures, treated and not treated with phages across MOIs. The data were compiled from two independent trials. Bars present the standard error of the mean (adapted from Reference<sup>9</sup> with permission). [Please click here to view a larger version of this figure.](#)

**Table 1: Odds ratios comparing the likelihood of superior phage efficacy against *E. coli* O157 at incubation temperatures, incubation times, MOIs, phages, and strains of *E. coli* O157 (adapted from Reference<sup>9</sup> with permission).** [Please click here to download this Table.](#)

**Table 2: Overall phage efficacy against *E. coli* O157 at 37 °C (adapted from Reference<sup>9</sup> with permission).** [Please click here to download this Table.](#)

**Table 3: Overall phage efficacy against *E. coli* O157 at 22 °C (adapted from Reference<sup>9</sup> with permission).** [Please click here to download this Table.](#)

**Supplementary Figures 1-11: Snapshots of the microplate reader operation and procedures.** [Please click here to download this File.](#)

## Discussion

This protocol described a robust approach for systemically evaluating phage efficacy against foodborne pathogens, including STEC<sup>9</sup> and *Salmonella*<sup>10</sup>. One critical step is when diluting overnight culture of bacteria, using pre-chilled medium and manipulating the dilution with an ice bucket are recommended to minimize potential bacterial growth. In addition, phage dilution was prepared before diluting bacterial culture. The enumeration step 2.8 provided actual numbers of bacterial inoculum for calculation of the final MOI applied. For phage preparation, crude phage lysates prepared by filter phage infected in 4-6 h of bacterial culture is generally used. The critical step associated with phage infectivity is always to use phage work stocks prepared within 3 months. Extremely accurate pipetting (particularly when using a multichannel pipette) and uniformity of approach are also essential to obtain comparable and interpretable results. Modified TSB supplemented with 10 mM of Mg<sup>2+</sup> was used to dilute phages,

bacterial culture, and base medium to optimize adsorption and infection of phages.

As bacteria proliferate during the log phase, even below incubator temperature, it is recommended to use diluted overnight culture instead of log-phase culture, to minimize potential bacterial growth.

The proposed protocol has limitations. First, because microplate can only hold 200  $\mu$ L, prolonged incubation may cause substantial evaporation and is not recommended. In this case, the assay may not be suitable for slow-growing bacteria. Second, the proposed protocol was not able to monitor the amplification of phages. Third, this protocol could not monitor the development of phage resistance over time, a critical factor that determines the outcome of phage treatment<sup>11,12</sup>. Follow-up experiments are required to assess the further performance of the most influential cocktail in the screening in preventing the emergence of anti-phage mutants in an extensive broth culture system and other biological matrices.

In contrast to conventional antimicrobials, the biological nature of phages affects the complexity of biocontrol and therapeutic use in practical settings. Conventionally, rational selection of phage cocktails is primarily based on lytic activity and the host range of phages. Phage candidates with the strongest lytic activity and broadest host range are often recommended<sup>13,14</sup>. However, based on the current study, phages such as rV5 and T1, although alone not as virulent as T4 and T5, greatly facilitated overall biocontrol outcome when combined with T4 and/or T5. Consequently, to achieve superior efficacy of phage cocktails, systemic screening of antibacterial activity of potential phage combinations against targeted host strains under desired environmental conditions is recommended. In addition, the determination of receptors

for phage candidates and the inclusion of phages with various receptors may prevent competition for host attachment, thwart rapid development of anti-phage mutants, and improve biocontrol outcomes<sup>13</sup>.

This method enabled accurate quantification of phage lysis kinetics in a high-throughput format. Furthermore, it allowed systematic evaluation of various biological and environmental factors on the antibacterial efficacy of an assortment of phages, thereby facilitating the formulation of phage cocktails with optimized outcomes. The method's future applications and development are assumed to involve *in situ* monitoring the efficacy of each phages within phage cocktails by fluorescence labeling of phages. In addition to the proposed protocol, understanding genetic determinants that promote synergistic and facilitated effects between phages when co-infecting one host would facilitate the formulation of appropriate phage cocktails with superior efficacy.

## Disclosures

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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