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Bacteriophage effectiveness for biocontrol of foodborne pathogens evaluated via high-throughput settings

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TITLE:

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

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SUMMARY:

This protocol describes a robust method for using high-throughput settings to screen the antibacterial efficacy of bacteriophage cocktails.

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[1,2]. 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[3].

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[4,5]. 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[1]. 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[5] and changes in bacterial physiology due to exposure to environmental stressors[6].

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[7]. 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[8]. Furthermore, AKFV33, a T5-like phage, had greater efficacy than a cocktail of four phages in removing *E. coli* O157 from beef, despite the incubation temperatures applied[4]. Recently, it has been reported that the effectiveness of individual phages does not predict the efficacy of phage cocktails for control of O157[9], 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

1.1 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.

1.2 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.

1.2.1 Make 500 mL of Phosphate Buffered Saline (PBS; 4 g of NaCl, 0.1 g of KCl, 0.77 g of Na_2HPO_4, and 0.12 g of KH_2PO_4, 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.

1.3 Make 200 mL of 1 M $MgSO_4$ (49.294 g and 200 mL of ultrapure water) and sterilize via 0.22 µm polyethersulfone filter.

1.4 Make 500 mL of TSB with 10 mM $MgSO_4$ (mTSB; 15 g of TSB powder, 500 mL of ultrapure water, and 5 mL of 1 M $MgSO_4$) 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 $MgSO_4$; swirl gently to mix.

2. Preparation of bacterial culture

2.1 To prepare Bacterial Research Laboratory Culture (BRLC), remove glycerol stock vial(s) from the freezer inventory and transfer to the laboratory.

2.2 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.

2.3 Bag the prepared BRLC plates and store at 4 °C.

NOTE: General expiry period for BRLC: 14 days at 4 °C after generation.

2.4 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 log10 CFU/mL.

2.5 Prepare a serial dilution of the overnight cultures (the following morning), each *E. coli* O157 strain using mTSB containing 10 mM of $MgSO_4$ to achieve 4–5 log10 CFU/ml (or other desired inoculum level).

2.6 Mix an equal volume of an overnight culture of each strain to achieve 4–5 log10 CFU/mL in total (or as desired) to prepare the bacterial mixture.

2.7 Immediately place the diluted bacterial culture at 4 °C for pending use.

2.8 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

3.1 Propagate high-titer working stocks for each phage to be screened (≥10^8 PFU/mL) by following the standard methods[4].

NOTE: The general expiry period for the phage stocks is 3 months in a plastic bottle at 4 °C after generation.

3.2 To achieve the desired titer of, e.g., ~108 PFU/mL for lysis kinetics, dilute individual phage preparations with mTSB containing 10 mM of $MgSO_4$. 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

4.1 Prepare serial 10-fold dilutions of each phage 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**).

4.2 Place 180 µL of mTSB to wells from Columns 1 to 11 of the 96-well microplate.

4.3 Add 20 µL of diluted individual phages or phage cocktails (~10^8 PFU/mL) to Wells 1–8 of the top row of the microplate (Row A).

4.4 For the phage-free and Blank control, add 20 µL of mTSB to the top well of Columns 9, 10, and 11.

4.5 With a 12-channel pipette, dilute down the plate. Transfer 20 µL from row to row. Mix the well contents by gentle, repeated aspiration, ejection (at least five times), and changing tips between dilutions. Remove 20 µL from the last row (Row H).

NOTE: Using tips with filters is recommended to prevent cross-contamination.

4.6 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 µL of the diluted bacterial culture to each well. Change tips between each addition.

4.7 Cover and incubate the microplate at desired conditions (e.g., 37 °C for 10 h or 22 °C for 22 h).

4.8 At 2 h or other desired intervals, remove the microplates from the incubator.

5. Determination of optical density (OD_{600})

5.1 Examine for optical density at 600 nm (OD_{600nm}) using a Microplate Reader.

NOTE: It is advised to set up and save the assay protocol in the program before test plate preparation.

5.2 Turn on the microplate reader—open program.

5.3 Select the **Simple** mode in the **Startup Options** window and select **Create a New Protocol** in **Task Manager** (**Supplementary Figure 1a,b**).

5.4 From the **Select Plate Type** window, choose **96 Well Plate** from the drop-down list (**Supplementary Figure 2**).

5.5 Select **Absorbance** for detection method, the **Endpoint/Kinetic** option for read type, and **Monochromators** for optics type. Click on **OK** (**Supplementary Figure 3**).

5.6 For Read Step, enter **600 nm** for wavelengths and select **Normal** for read speed. Click on **OK** (**Supplementary Figure 4**).

5.7 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).

5.8 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**).

5.9 Click on the **Shake** checkbox in the **Procedure** window to set up the shake condition, if needed, for each kinetic read (**Supplementary Figure 6a**).

5.10 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**).

5.11 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.

5.12 After defining the settings for each well type, click on **Finish**.

5.13 Select a **Well ID** in the left-hand side interface, and then assign it to the matrix shown in the plate layout widow. Click on **OK** (**Supplementary Figure 8**).

5.14 Click on the **Read Plate** button and save the protocol as a .prt file and click on **Save** (**Supplementary Figure 9**).

5.15 Insert the plate and click on **OK**.

5.16 Once the experiment is completed, save the experiment as a .xpt file and click on **Save** (**Supplementary Figure 10**).

5.17 Export data to Excel by clicking on the **Yes** button in the message window box for further analysis (**Supplementary Figure 11**).

5.18 Click on the **Save Yes/No** selection and the **Don't Ask Again** checkbox to save preference.

6. Data analyses

6.1 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_{600} from each phage-treated and -free culture.

6.2 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 ($P < 0.05$).

6.3 Define superior phage efficacy based on OD_{600nm} value ≤ 0.01 corresponding to no detectable bacterial growth (limit of detection:300 CFU/mL).

6.3.1 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.

6.3.2 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)[9]. The percentage (%) of optical turbidity measurement ≤ 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_{600} 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_{600nm} 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**[9]. OD_{600} 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 ($OD_{600} \leq 0.01$), which warranted potential superior efficacy under experimental conditions (**Table 2** and **Table 3**).

FIGURE AND TABLE LEGENDS:

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.

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.

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[9] with permission).

Table 1: Odds ratios comparing the likelihood of superior phage efficacy against *E. coli* O157 at incubation temperatures, incubation times, MOIs, phages, and strain of *E. coli* O157 (adapted from Reference[9]).

Table 2: Overall phage efficacy against *E. coli* O157 at 37 °C (adapted from Reference[9]).

Table 3: Overall phage efficacy against *E. coli* O157 at 22 °C (adapted from Reference[9]).

Supplementary Figures 1–11: Snapshots of the microplate reader operation and procedures.

DISCUSSION:

This protocol described a robust approach for systemically evaluating phage efficacy against foodborne pathogens, including STEC[9] and *Salmonella*[10]. One critical step is when diluting overnight culture of bacteria, using pre-chilled medium and manipulating the dilution with an ice bucket are recommend 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^{2+} 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 µL, 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[11,12]. 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[13,14]. 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[13].

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 phage 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.

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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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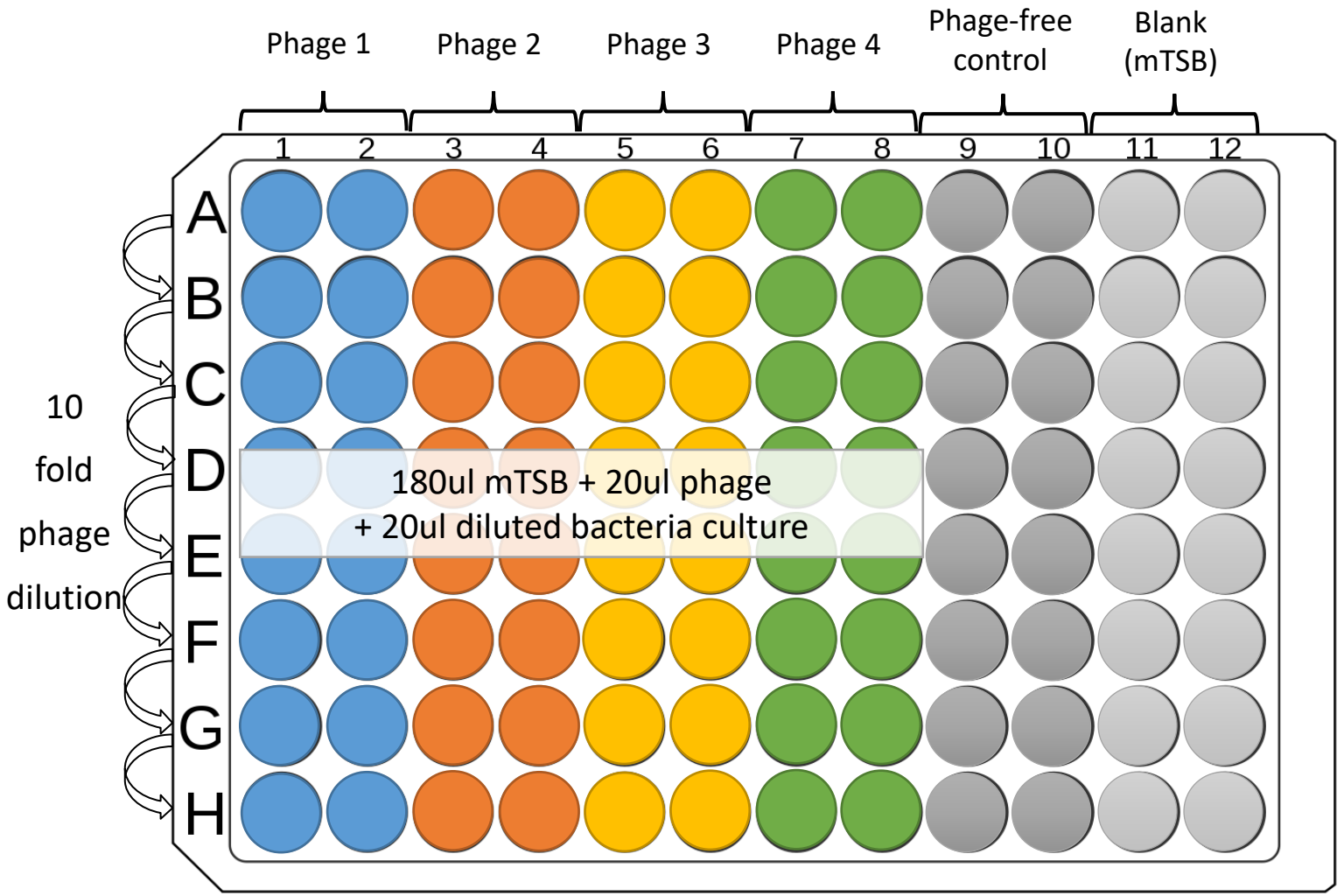

Figure 1. Suggested layout of 96-well microplate assay. Serial 10-fold dilutions of each phage are prepared in Columns 1-8 of sterile 96-well microplates. Four phages against 1 bacterial strain can be tested in duplicate, in adjacent columns, on each plate. The remaining columns are for controls.

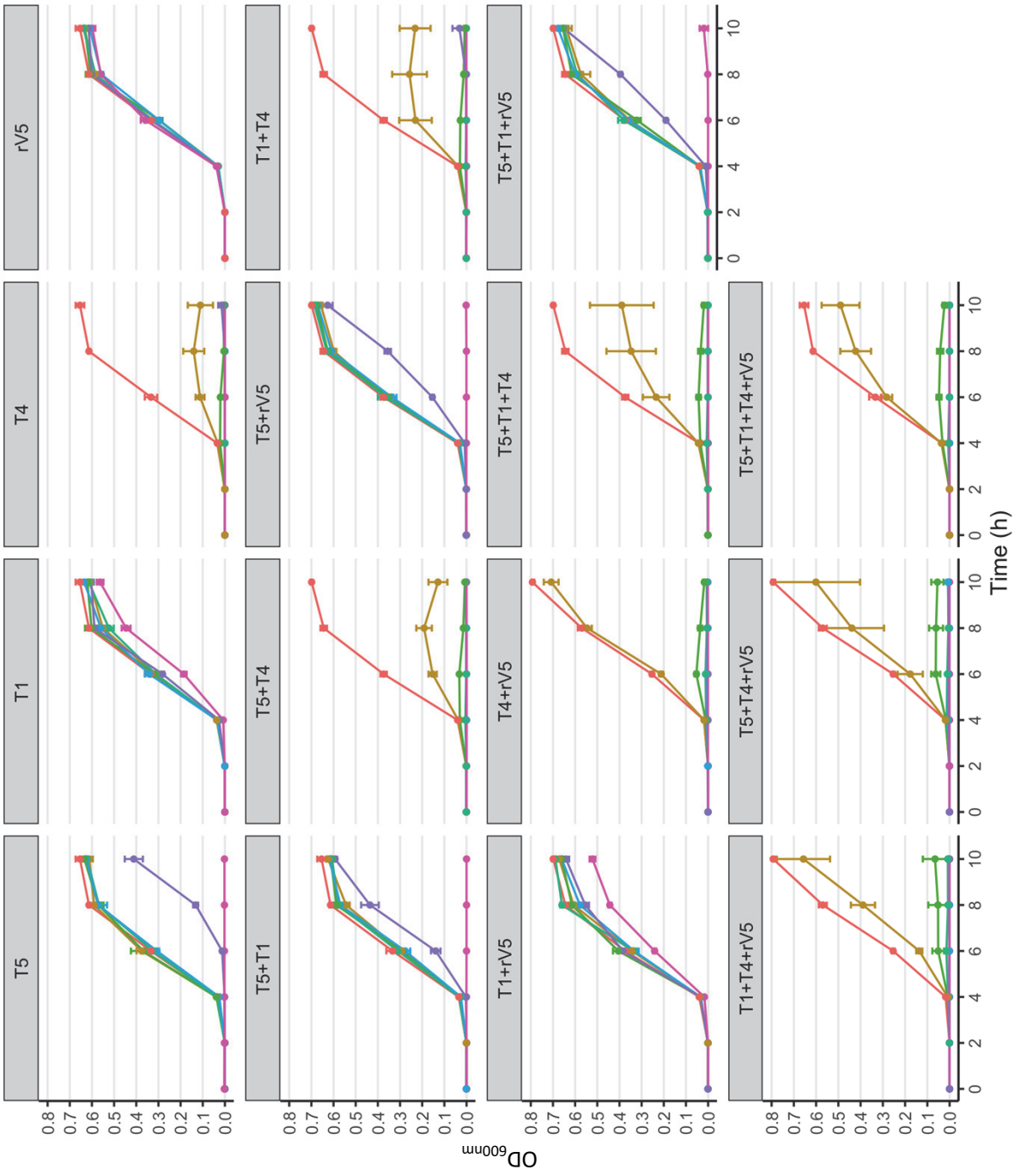

Figure 2. Growth curves of *E. coli* O157 strain 3081 at 37 °C treated and not treated with phages at each MOIs.

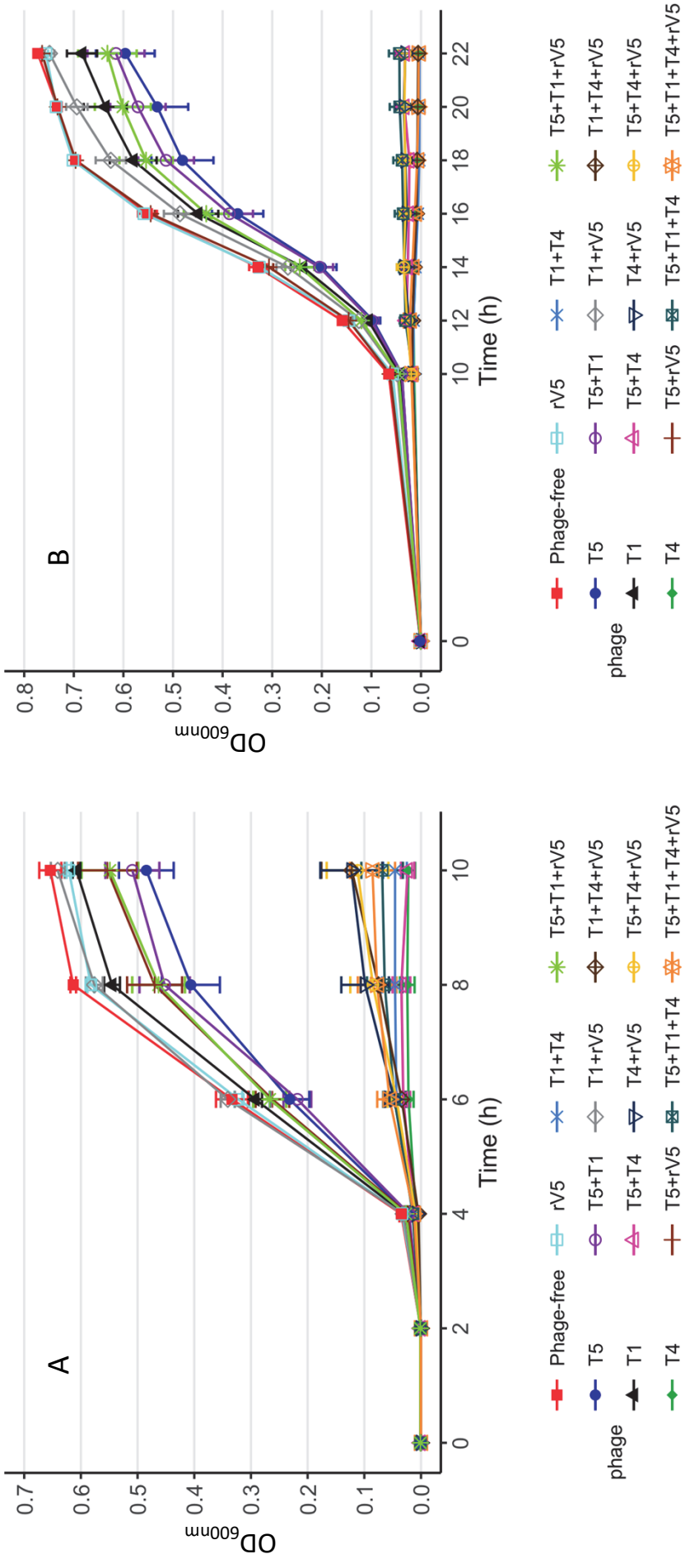

Figure 3. Growth curves of *E. coli* O157 strain 3081 at 37 °C (A) and 22 °C (B) in individual and mixed cultures treated and not treated with phages across MOIs.

Table 1: Odds ratios comparing the likelihood of superior phage efficacy against *E. coli* O157 at different incubation temperatures, incubation times, MOIs, phages and strain of *E. coli* O157 (adapted from Ref. 10).

Factors[§]	Percentage (%) of optical turbidity measurement < 0.01	Odds ratio	95% CI[#]	*p* -value[*]
Temperature, time				
22°C, 16 h	75[a]	2.96	2.58−3.40	<0.001
22°C, 18 h	75[a]	2.88	2.51−3.31	<0.001
22°C, 14 h	75[a]	2.8	2.44−3.21	<0.001
37°C, 8 h	72[b]	2.4	2.10−2.76	<0.001
22°C, 12 h	72[bc]	2.32	2.02−2.66	<0.001
22°C, 10 h	71[bcd]	2.13	1.86−2.43	<0.001
22°C, 20 h	71[cd]	2.04	1.79−2.34	<0.001
37°C, 6 h	70[d]	1.87	1.63−2.14	<0.001
37°C, 10 h	67[e]	1.55	1.36−1.77	<0.001
22°C, 22 h	61[h]	Referent		
MOIs				
1000	88[a]	23.39	20.62−26.52	<0.001
100	81[b]	11.57	10.35−12.94	<0.001
10	79[c]	9.2	8.26−10.25	<0.001
1	73[d]	5.97	5.39−6.64	<0.001
0.1	61[e]	2.65	2.41−2.92	<0.001
0.01	44[f]	Referent		
Phages				
T1+T4+rV5	89[a]	109.12	88.88−133.98	<0.001
T5+T1+T4+rV5	87[ab]	90.57	74.21−110.52	<0.001
T5+T1+T4	86[b]	83.03	68.21−101.07	<0.001
T5+T1	86[b]	81.64	67.10−99.32	<0.001
T5+T1+rV5	83[c]	60.3	49.95−72.78	<0.001
T1+T4	82[c]	56.37	46.78−67.94	<0.001
T5	81[c]	51.61	42.91−62.07	<0.001
T5+T4	79[d]	41.66	34.78−49.89	<0.001
T5+T4+rV5	67[e]	17.74	14.99−20.99	<0.001
T4+rV5	65[ef]	15.95	13.49−18.85	<0.001
T5+rV5	64[ef]	15.45	13.08−18.25	<0.001

T4	63[f]	14.41	12.21−17.02	<0.001
T1+rV5	59[g]	11.34	9.62−13.37	<0.001
T1	52[h]	7.44	6.32−8.74	<0.001
rV5	19[i]	Referent		
Bacteria				
E. coli O157:H7 CO281-31N (H, PT8)	88[a]	27.95	24.15−32.34	<0.001
E. coli O157:H7 H4420N (B, PT87)	80[b]	11.42	10.06−12.96	<0.001
E. coli O157:H7 EDL933(H, PT21)	78[c]	9.88	8.73−11.19	<0.001
E. coli O157:H7 E318N (H, PT4)	74[d]	7.06	6.26−7.96	<0.001
E. coli O157:H7 R508N (B, PT14)	73[d]	6.45	5.72−7.26	<0.001
E. coli O157:NM E32511 (H, PT31)	71[e]	5.6	4.98−6.30	<0.001
All 7 mixture	61[f]	2.97	2.66−3.32	<0.001
E. coli O157:H7 3081 (B, PT43)	43[g]	Referent		

[§] H: Human; B: Bovine; PT: phage type;

[#] CI, confidence interval for the odds ratio.

* P – value for the likelihood of superior phage efficacy as affected by different incubation temperatures, incubation times, MOIs, phages and strains of *E. coli* O157.

[a-i] within same factors, means different letters differs ($P < 0.05$)

Table 2: Overall phage efficacy against *E. coli* O157 at 37°C (adapted from Ref. 10)

3081				CO281-31N				E318N
Panel[§]	**Phage treatment**	**Mean OD**	**Best MOIs**[*]	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**	**Panel**
A	T4	0.018	>0.01	A	T1+T4+rV5	0	Any	A
	T5+T4	0.025	>0.01		T5+T4	0	Any	
B	T1+T4	0.041	>0.01		T5+T1+T4	0	Any	
	T5+T1+T4	0.055	>0.1		T5+T1+rV5	0	Any	B
	T1+T4+rV5	0.069	>0.1		T1+T4	0	Any	
	T5+T1+T4+rV5	0.07	>0.1		T5+rV5	0	Any	
	T5+T4+rV5	0.075	>0.1		T5+T4+rV5	0	Any	
C	T4+rV5	0.085	>0.1		T4+rV5	0	Any	
D	T5	0.371	1000	B	T5	0.004	Any	C
	T5+T1	0.39	1000		T5+T1	0.005	Any	
E	T5+T1+rV5	0.421	1000		T4	0.005	Any	
	T5+rV5	0.424	1000		rV5	0.005	Any	
F	T1	0.479	NA		T5+T1+T4+rV5	0.006	Any	D
	rV5	0.506	NA	C	T1+rV5	0.01	>0.01	
	T1+rV5	0.515	NA	D	T1	0.336	NA	
Average Mean OD		0.236		**Average Mean OD**		0.025		**Average Me**
Significance between panels				**Significance between panels**				**Significance**
A vs B $P < 0.01$				A vs B $P < 0.001$				A vs B $P <$ (
B vs C $P < 0.01$				B vs C $P < 0.05$				B vs C $P <$ (
C vs D $P < 0.001$				C vs D $P < 0.001$				C vs D $P <$ (
D vs E $P < 0.001$								
E vs F $P < 0.001$ (F: did not work as compared to control ($P > 0.1$))								

EDL933				H4420N				R508N
Panel	**Phage treatment**	**Mean OD**	**Best MOIs**	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**	**Panel**
A	T1+T4+rV5	0	Any	A	T1+T4+rV5	0	Any	A
	T5+T1	0.004	Any		T5	0	Any	
	T4	0.004	Any		T5+T1	0	Any	
	T5+rV5	0.004	Any	B	T5+rV5	0	>0.01	
	T5+T1+rV5	0.005	Any		T5+T1+T4+rV5	0	>0.01	
	T1+T4	0.005	Any		T5+T1+T4	0	>0.01	B

	T5	0.006	Any		T5+T1+rV5	0.001	>0.01	
	T5+T4	0.006	Any	C	T5+T4	0.013	>0.1	
	T5+T1+T4+rV5	0.006	Any		T5+T4+rV5	0.021	>0.1	
B	T5+T1+T4	0.014	≠10		T4	0.027	NA	
C	T5+T4+rV5	0.053	>0.01		T1+T4	0.027	>0.01	
	T4+rV5	0.058	>0.01		T4+rV5	0.035	>0.1	
D	T1	0.232	NA[y]	D	T1+rV5	0.052	NA	C
	T1+rV5	0.24	NA	E	T1	0.063	1000	D
E	rV5	0.417	NA		rV5	0.075	NA	
Average Mean OD		0.07		**Average Mean OD**		0.021		**Average Me**
Significance between Panels				**Significance between Panels**				**Significance**
A vs B $P < 0.05$				A vs B $P < 0.05$				A vs B $P <$ (
B vs C $P < 0.001$				B vs C $P < 0.05$				B vs C $P <$ (
C vs D $P < 0.001$				C vs D $P < 0.001$				C vs D $P <$ (
D vs E $P < 0.001$				D vs E $P < 0.01$				

[§] Panels A−G were assigned to each phage treatment of which overall anti-O157 efficacy across time and MOIs statistically diff

[*] Best MOIs represented complete inhibition of bacterial growth (OD600 $\leq$ 0.01) at each sampling time; NA: Not applicable – Symbol > indicates all concentrations greater than that listed, symbol ≠ indicates all other MOIs were superior to that listed.

E32511						
Phage treatment	**Mean OD**	**Best MOIs**	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**
T1+T4+rV5	0	Any	A	T1+T4+rV5	0	Any
T5	0.005	Any		T5+T4+rV5	0.002	Any
T5+T1	0.006	Any		T4+rV5	0.002	Any
T5+rV5	0.008	≠100		T5	0.003	Any
T5+T4	0.009	>0.1		T5+rV5	0.004	Any
T5+T1+T4+rV5	0.015	>1	B	T5+T1+rV5	0.005	>0.01
T4	0.015	>10		T5+T1	0.006	>0.01
T5+T1+rV5	0.021	0.1, 1		T4	0.007	>0.01
T1+T4	0.023	>10		T5+T4	0.008	>0.1
T5+T4+rV5	0.025	>0.1		T5+T1+T4+rV5	0.011	>0.1
T4+rV5	0.026	>0.1		T1+T4	0.012	>0.1
T1+rV5	0.033	1000		T5+T1+T4	0.012	0.1,1,10
T5+T1+T4	0.076	>1	C	rV5	0.098	100
T1	0.08	NA	D	T1+rV5	0.106	>1
rV5	0.086	NA	E	T1	0.375	NA
ean OD	0.029		**Average Mean OD**		0.043	
: between panels			**Significance between panels**			
0.01			A vs B $P < 0.01$			
0.01			B vs C $P < 0.001$			
0.01			C vs D $P < 0.01$			
			D vs E $P < 0.001$			

			All 7 mixture			
Phage treatment	**Mean OD**	**Best MOIs**	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**
T1+T4+rV5	0	Any	A	T1+T4+rV5	0	Any
T4+rV5	0	Any	B	T5+T1	0.007	> 0.01
T5+T4+rV5	0	Any		T5	0.007	> 0.01
rV5	0.002	Any		T5+rV5	0.008	> 0.01
T5	0.002	Any		T5+T1+rV5	0.008	> 0.01
T4	0.005	>0.01		T1+T4	0.014	> 0.1

T5+T1+rV5	0.005	>0.01		T4+rV5	0.016	> 0.1
T5+T1	0.005	>0.01		T5+T1+T4	0.02	> 0.1
T1+rV5	0.005	>0.01		T5+T4+rV5	0.021	> 0.01
T5+rV5	0.006	≠1	C	T4	0.025	1000
T1+T4	0.006	>0.1		T5+T4	0.026	10, 1000
T5+T1+T4	0.008	>0.1		T5+T1+T4+rV5	0.038	> 0.01
T5+T4	0.026	1000	D	rV5	0.143	NA
T1	0.042	NA		T1	0.149	NA
T5+T1+T4+rV5	0.077	≠1 and 0.01		T1+rV5	0.194	NA
ean OD	0.013		**Average Mean OD**		0.045	

between Panels	**Significance between Panels**
0.05	A vs B $P < 0.001$
0.001	B vs C $P < 0.001$
0.001	C vs D $P < 0.001$

ered ($P < 0.05$) within each strain.

bacterial growth was not completely inhibited at any MOI.

Table 3: Overall phage efficacy against *E. coli* O157 at 22°C (adapted from Ref.10)

3081				CO281-31N			
Panel	**Phage treatment**	**Mean OD**	**Best MOIs[z]**	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**
A	T4	0.009	>0.1	A	T5+T1+T4	0.001	Any
	T1+T4	0.009	>0.1		T1+T4+rV5	0.001	Any
	T1+T4+rV5	0.012	>0.1		T1	0.001	Any
	T5+T1+T4+rV5	0.013	>0.1		T1+T4	0.001	Any
B	T5+T4	0.027	>0.1		T5+T1+T4+rV5	0.001	Any
	T4+rV5	0.03	>0.1		T5+T1	0.001	Any
	T5+T4+rV5	0.031	>0.1		T5	0.001	Any
	T5+T1+T4	0.036	>0.1		T1+rV5	0.001	Any
C	T5	0.33	1000		T5+T1+rV5	0.001	Any
	T5+T1	0.347	1000		T5+T4	0.002	Any
	T5+T1+rV5	0.377	1000	B	T4	0.008	>0.01
D	T1	0.391	NA[y]	C	T5+T4+rV5	0.016	>0.1
	T1+rV5	0.429	NA	D	T4+rV5	0.042	>0.01
	T5+rV5	0.464	NA		T5+rV5	0.046	>0.1
	rV5	0.468	NA	E	rV5	0.253	NA
Average Mean OD		0.198		**Average Mean OD**		0.025	
Significance between Panels				**Significance between Panels**			
A vs B P <0.001				A vs B P <0.001			
B vs C P < 0.001				B vs C P < 0.001			
C vs D P < 0.001				C vs D P < 0.001			
T5+rV5 and rV5 did not work as compared to the control (P > 0.1)				D vs E P < 0.001			

EDL933				H4420N			
Panel	**Phage treatment**	**Mean OD**	**Best MOIs**	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**
A	T5+T1+rV5	0.002	Any	A	T1	0.001	Any
	T5+T1+T4	0.003	Any		T5+T1+T4	0.001	Any
	T5+T1	0.003	> 0.01		T1+T4+rV5	0.001	Any

	T5+T1+T4+rV5	0.003	>0.01		T5+T1+T4+rV5	0.001	Any
	T1+rV5	0.004	>0.01		T1+T4	0.001	Any
B	T1	0.005	>1		T5+T1+rV5	0.001	Any
	T1+T4	0.005	>0.1		T5+T1	0.001	Any
	T5	0.005	>1		T1+rV5	0.001	Any
	T5+T4	0.005	≠1 and 10	B	T5	0.004	>0.01
C	T1+T4+rV5	0.007	>0.1	C	T5+T4	0.008	>0.01
	T4	0.008	>0.01	D	T5+T4+rV5	0.018	>0.1
	T4+rV5	0.008	>0.01	E	T5+rV5	0.046	>0.1
	T5+T4+rV5	0.011	>0.1		T4	0.05	>0.1
	T5+rV5	0.012	0.1	F	T4+rV5	0.061	>0.1
D	rV5	0.353	NA	G	rV5	0.293	NA
Average Mean OD		0.029		**Average Mean OD**		0.033	
Significance between Panels				**Significance between Panels**			
A vs B < 0.01				A vs B < 0.05			
B vs C < 0.05				B vs C < 0.001			
C vs D < 0.001				C vs D < 0.001			
				D vs E < 0.001			
				E vs F < 0.001			
				F vs G < 0.001			

[§] Panels A−G were assigned to each phage treatment of which overall anti-O157 efficacy across time and MOIs statisti

[*] Best MOIs represent complete inhibition of bacterial growth (OD600 ≤ 0.01) at each sampling time; NA: Not applica

Symbol > indicates all concentrations greater than that listed, symbol ≠ indicates all other MOIs were superior to that l

E318N				E32511			
Panel	**Phage treatment**	**Mean OD**	**Best MOIs**	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**
A	T5+T1	0.002	Any	A	T5	0.003	>0.01
	T5+T1+T4 +rV5	0.003	Any		T5+T1	0.004	>0.01
	T5+T1+T4	0.004	Any		T5+T4	0.005	>0.01
	T5+T4	0.004	> 0.01		T5+T1+T4 +rV5	0.005	>0.01
	T5	0.004	≠1		T5+T1+T4	0.006	>0.01
	T5+T1+rV 5	0.005	Any		T5+T1+rV 5	0.007	>0.01
	T1+T4+rV 5	0.007	>0.01	B	T4	0.013	>0.1
	T4	0.007	>0.01		T5+rV5	0.017	>0.1
B	T5+T4+rV 5	0.011	>0.1		T5+T4+rV 5	0.018	>0.1
	T1	0.011	>10	C	T1+T4+rV 5	0.026	>0.1
	T1+rV5	0.011	NA		T4+rV5	0.03	>0.1
	T1+T4	0.012	1000	D	T1+T4	0.05	>0.1
C	T5+rV5	0.021	>0.1	E	rV5	0.17	>1
D	T4+rV5	0.048	>0.01	F	T1+rV5	0.2	>10
E	rV5	0.426	NA	G	T1	0.245	NA
Average Mean OD		0.038		**Average Mean OD**		0.053	
Significance between Panels				**Significance between Panels**			
A vs B P <0.001				A vs B P <0.05			
B vs C P < 0.001				B vs C P < 0.01			
C vs D P < 0.001				C vs D P < 0.001			
D vs E < 0.001				D vs E P < 0.001			
				E vs F P < 0.001			
				F vs G P < 0.001			

R508N				All 7 mixture			
Panel	**Phage treatment**	**Mean OD**	**Best MOIs**	**Panel**	**Phage treatment**	**Mean OD**	**Best MOIs**
A	T5+T1+T4	0.001	Any	A	T5+T1+rV 5	0.003	Any
	T1	0.002	Any		T5+T1	0.003	Any
	T5+T1	0.002	Any		T5+T1+T4 +rV5	0.004	Any

	T5+T1+T4+rV5	0.002	Any		T5+T1+T4	0.004	Any
	T5+T1+rV5	0.002	Any		T5	0.004	Any
	T1+rV5	0.002	Any		T1+T4	0.004	>0.01
	T1+T4+rV5	0.003	Any		T1+T4+rV5	0.006	Any
	T1+T4	0.003	Any		T5+T4	0.008	>0.01
B	T5	0.067	>0.01	B	T4	0.029	1, 10
C	T5+T4	0.072	>0.1		T1	0.043	0.01, 0.1
D	T5+rV5	0.136	>1		T4+rV5	0.045	10
E	T5+T4+rV5	0.172	>10		T5+T4+rV5	0.054	1, 100, 1000
F	rV5	0.267	NA		T5+rV5	0.056	> 0.1
G	T4+rV5	0.285	NA		T1+rV5	0.064	0.01
H	T4	0.336	NA	C	rV5	0.324	NA
Average Mean OD		0.09		**Average Mean OD**		0.043	
Significance between Panels				**Significance between Panels**			
A vs B < 0.001				A vs B < 0.001			
B vs C < 0.001				B vs C < 0.001			
C vs D < 0.001							
D vs E < 0.001							
E vs F < 0.001							
F vs G < 0.001							
G vs H < 0.001							

cally differed ($P < 0.05$) within each strain.

ıble – bacterial growth was not completely inhibited at any MOI.

isted.

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June 18, 2020
Dr. Vidhya Iyer
JoVE

Re: 62812

Dear Dr. Vidhya Iyer

Thank you for your May 31 email regarding our manuscript entitled “Bacteriophage effectiveness for biocontrol of foodborne pathogens evaluated via high-throughput settings”. ‘Clean’ versions of our revised manuscript including main body and Tables of Materials are uploaded with this letter.

Reviewer’s comments are copied below, with the authors’ responses inserted between.

The authors thank the reviewers for their constructive comments, and look forward to publishing future work in the JoVE Journal.

Thank you very much!

With kind regards,

Sincerely,

Dongyan Niu, PhD

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
   Thank you. We have asked Dr. John Kastelic, a professional Editor, to proofread the manuscript.
2. Please provide an email address for each author.
   Thank you. Added.

3. Please increase the word count of your abstract to be 150-300 words.

Thank you. Added new content in the abstract, meet the word requirement now.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Thank you. Corrected as suggested.

5. Please revise the following lines to avoid overlap with previously published work: 25-27, 32-33, 61-65, 113-118, 119-126, 134-139, 155-160, 198-203, 205-211, 219-225, 227-228, 230-232.

Thank you. Reworded.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: BioTek, Winooski, VT, USA; GLIMMIX; SAS (Version 9.4, 1999);

Removed BioTek, Winooski, VT, USA ; But we kept GLIMMIX; MIXED and SAS, as they are standard name of program model and seems no alternative name can be replaced.

7. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Thank you. Revised.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Thank you. Revised as suggested.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add

references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Thank you. More steps and details added.

10. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Thank you. highlighted in yellow.

11. In the text and figures, ensure that you have a space between the numbers and units (except for %). Write µL not uL.

Thank you. corrected as suggested.

12. Ensure that you cite figures and tables in order in the text—Table 1 is cited after Table 2.

Thank you. The tables should be in the right order now

13. Please include a table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Thank you. Added material table.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The present study evaluated a proposed method for the development of phage cocktails, considering factors such as the phage genera and various combinations, multiplicity of infection, temperature, and time.

Major Concerns:

While the approach and documentation of a systematic protocol is of importance, and the authors were throughout with the analysis and results, the use of overnight culture is particularly problematic (lines 194-196), as usually bacterial cultures at log phase are used in order to maximize the number of bacteria that are viable for phage infection. How would results change if log phase cultures were used instead.

Thank you. We explained why using overnight culture instead of log culture in line 299. We enumerated diluted overnight culture each time when doing the assay. So the bacterial number for phage infection

are consistent (~10^4-10^5 CFU/mL) across different trials. We are not sure how results would change if using log culture, but can test it in the future.

In addition, the protocol itself is not completely novel. The title does not reflect what was done in the sense of the bacterial strains tested. Since only E. coli O157 was used, the title should reflect this, as well as the abstract. More info of E. coli O157 as a foodborne pathogen may be needed to benefit readers.

The protocol was also successfully executed in phage-killing kinetics against Salmonella in our published work. We added the discussion in line 287.

Minor Concerns:

Line 56: The authors may have forgotten to add the date in the Bourdin reference.

Thank you. The reference was added now.

Line 73: Is incubation with shaking needed at this stage?

No shaking is required. We added “statically”.

Line 76: Explain the importance of adding MgSO4

Thank you. We explained use of MgSO4in the discussion, Line 295-296.

Line 85: Why were phage stocks diluted in mTSB and not a buffer (e.g., PBS)?

Thank you. We need to make E. coli grow as well.

Lines 134 and as needed throughout the text: Add the word "phage" before describing the designations T4, T5, T1, etc. so it reads "phage T5" instead of just "T5"

Thank you. Added as suggested.

Line 149: Strain 3081?

Yes, we added strain.

Reviewer #2:

Manuscript Summary:

The manuscript is pretty straight forward and describes a strategy for optimal use of phage cocktails for biocontrol. Such descriptive protocols are useful for not only in this setting but also in other scenarios such as clinical applications in human phage therapy. The authors describe a systematic approach of using phage and bacterial dilutions and mixes for assessing the efficacy of the individual and cocktails. Some additional questions that could be addressed are: do suboptimal phage concentrations (low MOIs) induce resistance?

Yes, it may be, but the current protocol was not able to monitor the anti-phage resistance. We discussed the limitation in lines 306.

What is the ideal number of phages in a cocktail to obtain maximum benefit of biocontrol?

It depends the bacteria target and how much to kill and environmental condition. Usually MOI (phage to bacteria ratio) > 10 are required.

These experiments are done buffer system. What happens with these cocktails in actual biocontrol settings? These questions are not to be addresses here for additional experiments. But perhaps, discussed for the benefit of the readers.

We discussed in the lines 307-309.

Major Concerns:

None

Minor Concerns:

None

Reviewer #3:

Manuscript Summary:

In the manuscript "Bacteriophage effectiveness for biocontrol of foodborne pathogens evaluated via high-throughput settings", the authors provided a method for screening high-efficiency phages. The procedure was well described and easy to follow.

Minor Concerns:

1. Please check the references carefully and format them according to the JoVE.
   Thank you, References corrected.
2. Lines 187-188, please make sure whether the abbreviation month "mo" is acceptable by JoVE.
   We changed to months

Lines 199, "37C" should be "37°C". Please go through the manuscript for such minor errors.

Thank you. Corrected.

3. I am confused about the tables. "Panels A–G were assigned to each phage treatment", why some panels have more than one treatment? Some panels have more treatments than others? What kind of statistical analysis was used? The first row was not assigned properly. The E. coli strains also should be mentioned in the legends.

Number of panels were originated from statistical analysis of each phage treated bacteria culture.

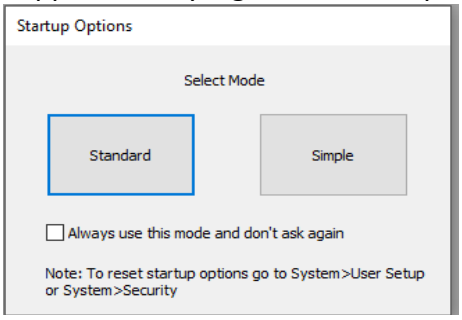
As sensitivity of strain to phage was not uniform, the different assignment of panels reflects this variance. The statical analysis was described as the following

5. Data analyses

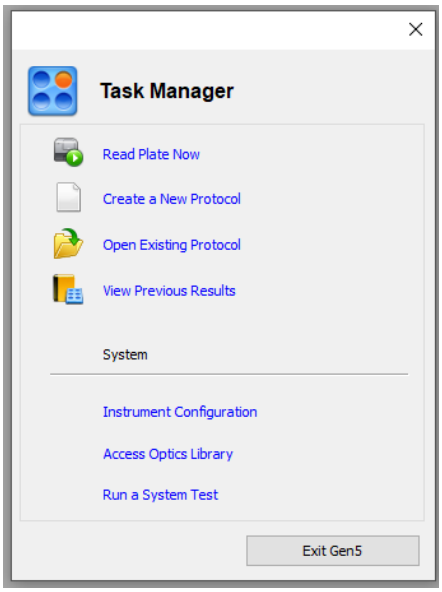
1. *Repeat at least 2 independent experiments as described above. Compile results from all the independent trials. Calculate the average and standard deviation of the OD_{600} from each phage-treated and -free culture.*
2. *For each bacterial strain and temperature, square-root transform the OD values at 600 nm and analyze using an appropriate statistical model. For SAS software, select the MIXED model and least-squares to differentiate means ($P < 0.05$). 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_{600} value ≤ 0.01 that is corresponding to no detectable bacterial growth (limit of detection:300 CFU/mL). Analyze effects of incubation temperature, time, MOIs, E. coli O157 strains and phage types on phage efficacy using an appropriate statistical model. For SAS software, use GLIMMIX with random measures. Calculate Odds Ratios to compare superior efficacy for different environmental and biological factors of interests.*

Corrected others.

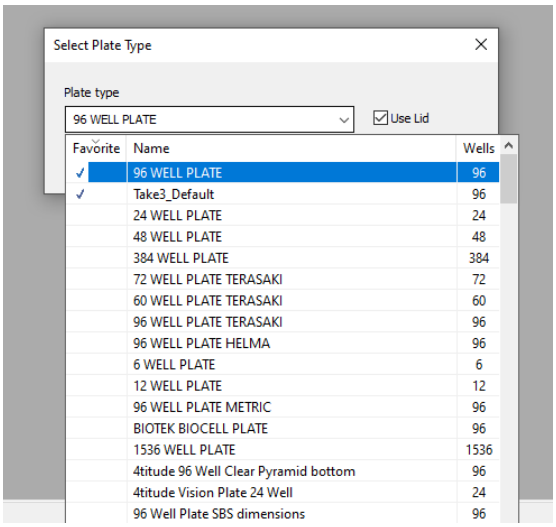
Supplementary Figure 1a. Startup Options window.



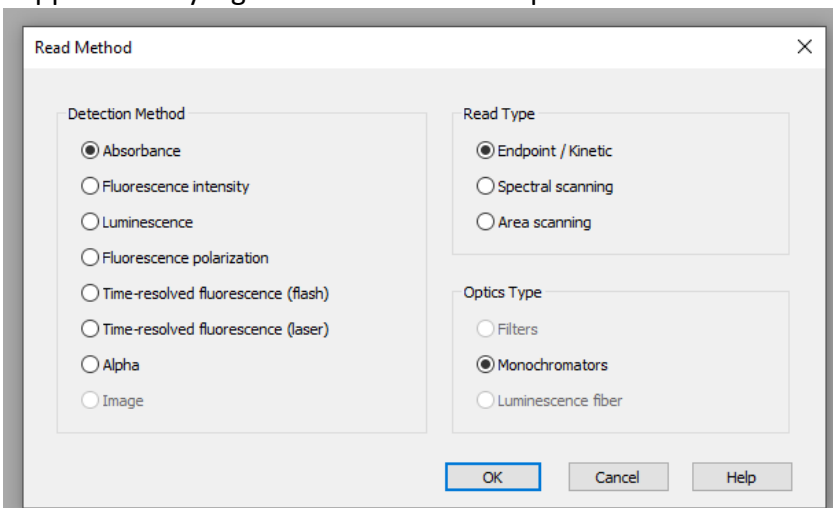
Supplementary Figure 1b. Task Manager window for creating a protocol.



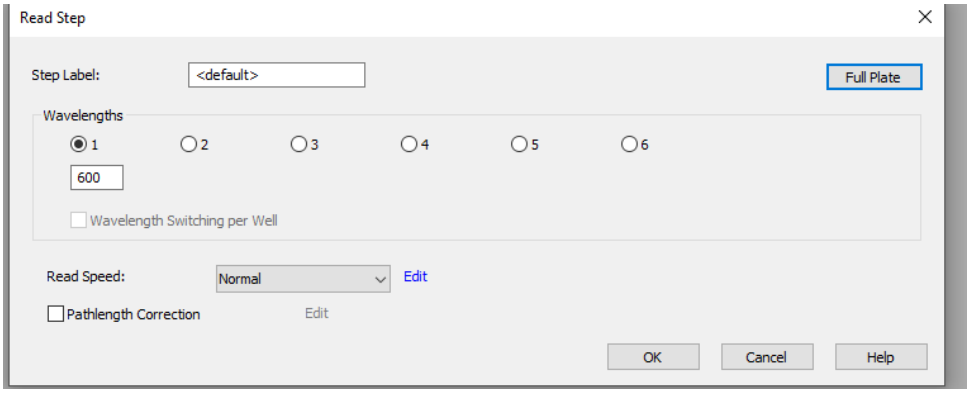
Supplementary Figure 2. Select plate type using a drop-down list.



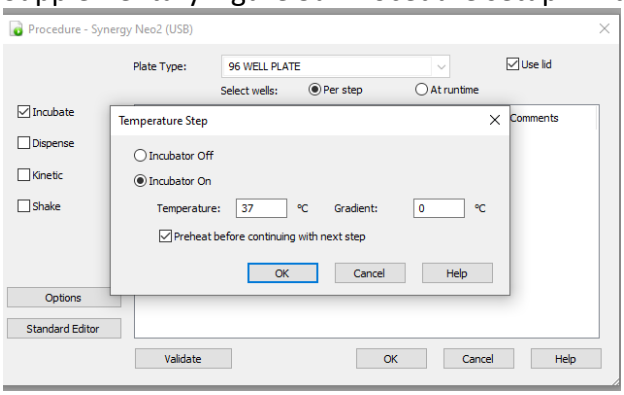
Supplementary Figure 3. Read Method options window.



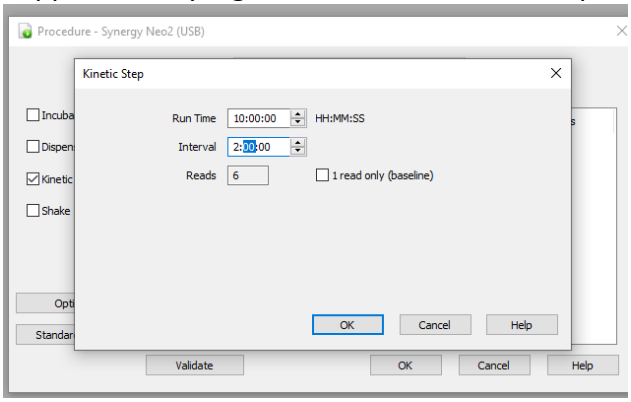
Supplementary Figure 4. Read Step selection window.



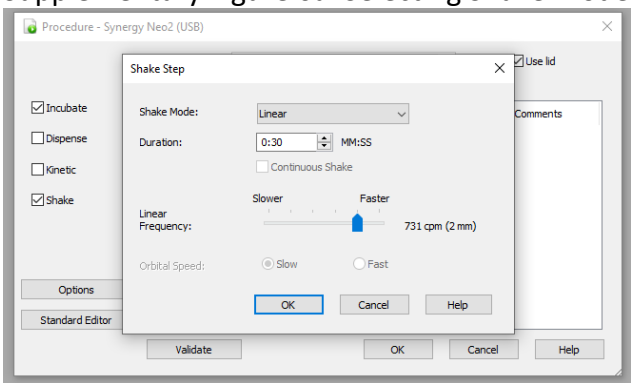
Supplementary Figure 5a. Procedure setup window.



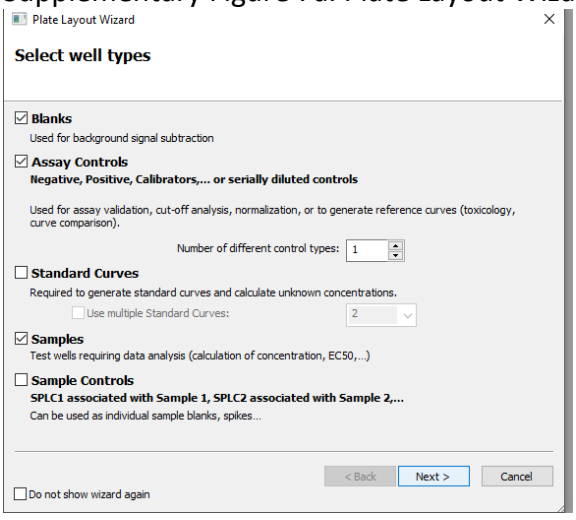
Supplementary Figure 5b. Kinetic mode setup window.



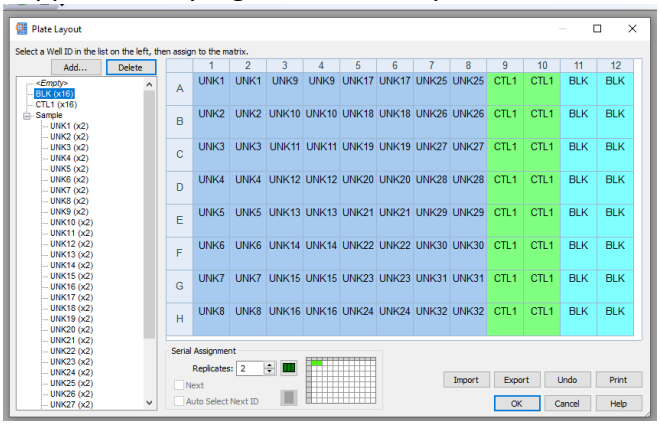
Supplementary Figure 6a. Selecting Shake Mode in the Shake Step window.



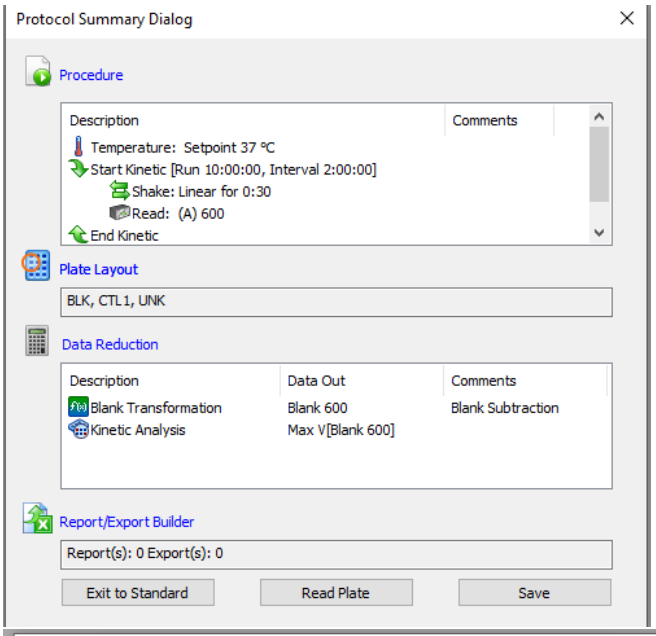
Supplementary Figure 7a. Plate Layout Wizard window.

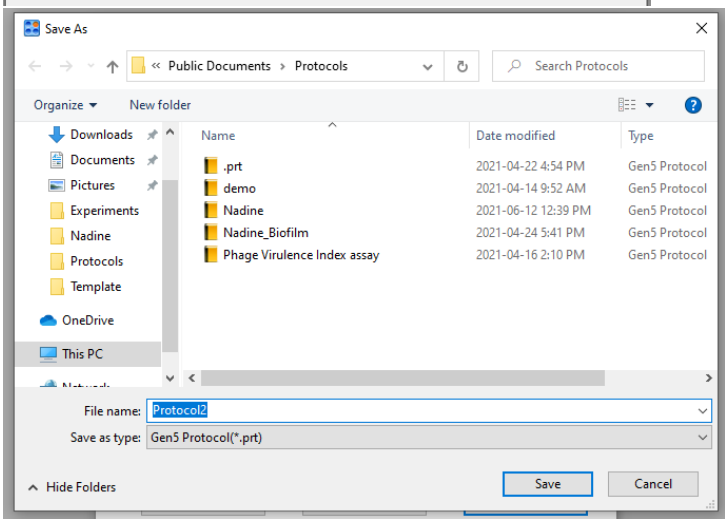


Supplementary Figure 8. Plate Layout window.

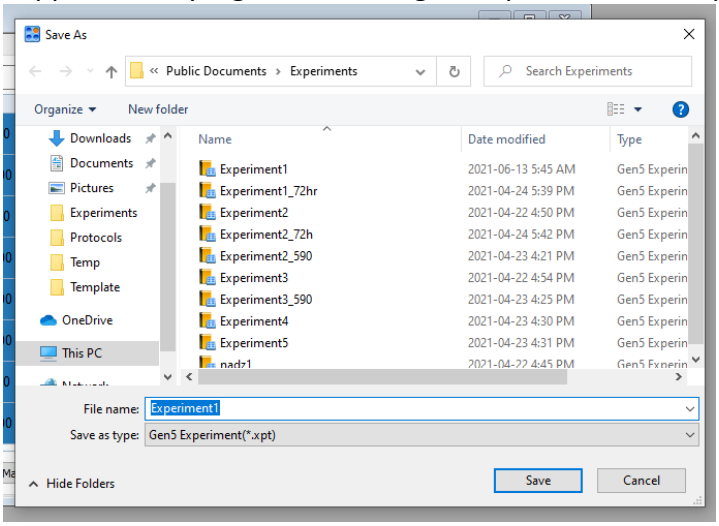


Supplementary Figure 9. Protocol Summary Dialog box.

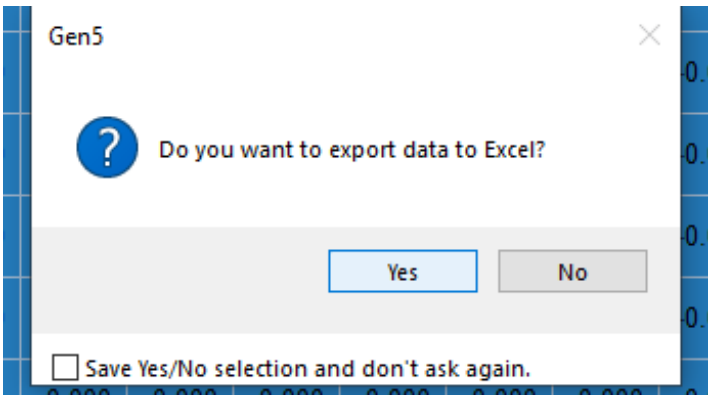




Supplementary Figure 10. Saving an experiment as a .xpt file.



Supplementary Figure 11. Exporting experimental results to Excel.



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