Chapter 25

Cell-Free Synthesis and Quantitation of Bacteriophages

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Abstract

Cell-free transcription-translation (TXTL) enables achieving an ever-growing number of applications, ranging from the rapid characterization of DNA parts to the production of biologics. As TXTL systems gain in versatility and efficacy, larger DNAs can be expressed in vitro extending the scope of cell-free biomanufacturing to new territories. The demonstration that complex entities such as infectious bacteriophages can be synthesized from their genomes in TXTL reactions opens new opportunities, especially for biomedical applications. Over the last century, phages have been instrumental in the discovery of many ground-breaking biotechnologies including CRISPR. The primary function of phages is to infect bacteria. In that capacity, phages are considered an alternative approach to tackling current societal problems such as the rise of antibiotic-resistant microbes. TXTL provides alternative means to produce phages and with several advantages over in vivo synthesis methods. In this chapter, we describe the basic procedures to purify phage genomes, cell-free synthesize phages, and quantitate them using an all-*E. coli* TXTL system.

Key words TXTL (cell-free transcription-translation), Bacteriophages, *E. coli*, Spotting assay, Kinetic infection assay

1 Introduction

Cell-free transcription-translation (TXTL) is becoming one of the most convenient and transformative technologies for many developing synthetic biology applications. As the scope of TXTL utilization is rapidly diversifying, the production of biologics and high-value chemicals remains TXTL's major strength [1–4]. Moreover, as cell-free gene expression systems are gaining in versatility and efficacy, expressing larger natural or synthetic DNAs in vitro promises to expand TXTL's capabilities in biomanufacturing to new areas of research. One promising area is the cell-free synthesis (CFS) of phages from their genomes and this is just starting to be exploited for synthetic biology applications [5–8]. Phages are a virtually limitless resource of bioactive materials that are exploited in biotechnology [9], nanotechnology [10], medicine [11],

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agriculture [12], and bioremediation [13]. Their host specificity has led to several phage therapies and commercial success in food and agriculture to control foodborne pathogens despite costly manufacturing. Phages are a promising solution to the threat of antibiotic-resistant microbes. CFS of phages has several major advantages compared to current industrial methods: (1) It avoids the use of dangerous pathogens, (2) it reduces the production cost of phages, and (3) it accelerates phage characterization and engineering. CFS of phages also offers an affordable and safe setting for student or biotechnician training, while providing a platform for hands-on practices in molecular biology, nextgeneration sequencing (NGS), and bioinformatics. This chapter provides a detailed description of basic procedures to extract and purify phage genomes, cell-free synthesize phages, and quantitate phage titers using an all-*E. coli* TXTL system. We use the *Escherichia coli*-specific phage T7 (40 kbp, 60 genes) as an example.

2 Materials

All the solutions should be prepared using dH_2O (ultrapure water, resistivity of 18 MΩ-cm at 25 °C, autoclaved when indicated) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Follow all waste disposal regulations when disposing of waste materials. Wear gloves, lab coat, and mask to prevent contamination of the solutions.

2.1 Phage Amplification

1. Phage lysate (from ATCC for phage T7).
2. Phage host strain. For T7: *E. coli* strain B.
3. Luria-Bertani (LB) broth liquid medium at room temperature (no antibiotic).
4. 1.5% LB agar plates.
5. 0.22 μm Cellulose-Acetate (CA) membrane filters and syringes.
6. Optional: SM buffer (5.8 g NaCl, 2.0 g $MgSO4{\cdot}7H_2O$, 50 mL 1 M Tris-HCl pH 7.4, in 1 L dH_2O. Autoclave and 0.02 μm filter-sterilize before use, and store at room temperature).
7. Optional: 100 kDa membrane Amicon Ultra-15 centrifugal filter units.

2.2 Phage DNA Extraction and Purification

1. Phage lysate (prepared in Subheading 3.1).
2. DNase I recombinant, RNAse-free.
3. RNase A 10 mg/mL.
4. 1 M $MgCl_2$ solution.
5. Proteinase K 20 mg/mL.

6. SDS, 20% wt/vol solution, RNase-free.
7. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (PCI) Saturated with 10 mM Tris-HCL, pH 8.0, 1 mM EDTA. Use PCI under a fume hood.
8. 500 mL Separatory funnel.
9. Chloroform.
10. 3 M sodium acetate solution, pH 5.2.
11. Ethanol absolute and 70% vol/vol ethanol.

2.3 Cell-Free Gene Expression

1. TXTL system: myTXTL Sigma 70 master mix kit.
2. Chi6 oligonucleotides.
3. Freshly extracted T7 genomic DNA (prepared in Subheading 3.2).
4. 1.5 mL tubes and a 30 °C static incubator.
5. Plasmid P70a-deGFP.

2.4 Dilutions of Cell-Free Synthesized Phage Reactions

1. Cell-free expression reaction of phage T7 (prepared in Subheading 2.3).
2. LB liquid at room temperature (RT) (no antibiotic).
3. Thermomixer.
4. Filter tips.
5. Multichannel pipets.
6. 96-well plates.

2.5 Spotting Assay

1. Dilutions of cell-free synthesized phage reactions (prepared in Subheading 2.4).
2. Overnight *E. coli B* culture (5 mL LB broth, 37 °C, 250 rpm).
3. 1.5% wt/vol agar-LB plates solid at 37 °C (no antibiotic).
4. 1-well plates.
5. 0.7% wt/vol soft bacto-agar liquid at 50 °C (keep in water bath at 50 °C, no antibiotic).
6. Liquid LB broth at RT (no antibiotic).
7. Static incubator at 37 °C.
8. 14 mL round-bottom culture tubes.
9. Filter tips.
10. Multichannel pipets.
11. 96-well plates.

2.6 Kinetic Assay

1. Dilutions of cell-free synthetized phage reactions (prepared in Subheading 2.4).
2. Overnight *E. coli B* culture (5 mL LB broth, 37 °C, 250 rpm).
3. LB liquid at RT (no antibiotic).
4. Plate reader with absorbance measurement capabilities (e.g., Biotek H1m).
5. 96 bacterial culture well-plates (flat bottom transparent) with lids (e.g., Thermo Scientific™ Nunc MicroWell 96-Well Optical-Bottom Plates).

3 Methods

The whole cycle of procedures takes 3 days (Table 1). Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of a Phage Lysate

To get phage genomic DNA ready for TXTL reactions, one must first amplify the phage in cell cultures. The phage T7 is amplified in *E. coli* strain B liquid cultures using a commercial phage lysate (ATCC BAA-1025-B2).

Infectious phages can be also acquired from phage banks (*see* **Note 1**). The preparation of a phage lysate takes about 4 h provided simple steps at day-2 and day-1 are successful [14].

1. 2 days before Day 1: Plate the *E. coli* B strain on an LB-agar Petri dish to get colonies next day. Incubate the plate overnight at 37 °C.
2. The day before Day 1: From the agar plate, start an overnight culture of *E. coli* B strain (5 mL LB broth, 37 °C, 250 rpm), as late as possible.
3. On Day 1, start a culture of *E. coli* host B by adding 2 mL of an overnight *E. coli* B culture to 100 mL of LB broth and incubate at 37 °C (250 rpm).

Table 1
Agenda of the whole method presented in this chapter

	Procedures	Duration
Day 1	Preparation of phage lysate	~4 h
	Titration of phages in the lysate	~4 h
Day 2	Phage genome extraction and purification	~4–6 h
	Cell-free synthesis of phages	~3 h
Day 3	Dilutions of cell-free reactions	~1 h
	Phage kinetics assay	~4 h
	Phage spotting assay	~4 h

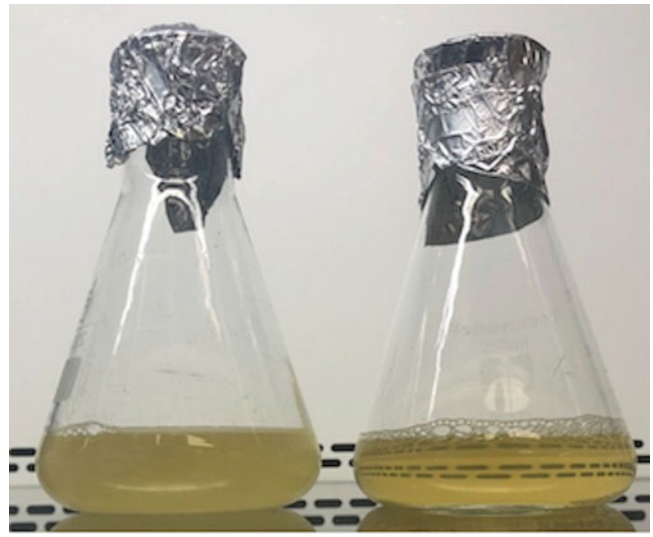
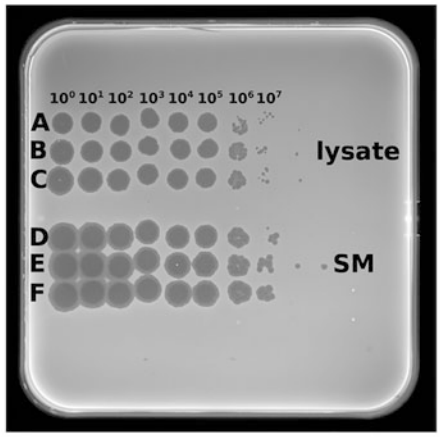


Fig. 1 Picture of *E. coli* strain B cultures after 2 h of incubation at 37 °C. On the left, 100 mL of an *E. coli* strain B culture not infected with phage T7, and on the right, 100 mL of an *E. coli* strain B culture cleared after infection by the phage T7 (phage lysate)

4. Monitor the culture optical density at 600 nm (OD). When the OD reaches 0.3, add 1 mL of phage lysate at 10^9 PFU/mL to start the culture with an initial multiplicity of infection (MOI) between 0.01 and 0.1. This will roughly allow 2–3 bacterial cycles before complete lysis and increase the titer of the lysate. The complete lysis is obtained when there is as many bacteria in the culture as phages.
5. Monitor the lysis by OD absorbance. For the phage T7, after approximately 2 h of incubation, complete lysis of the bacterial culture is obtained (phage lysate). The bacterial culture is completely cleared (Fig. 1), and the OD is <0.01 (phages eliminated almost all the bacteria).
6. Filter-sterilize the phage lysate with a low binding 0.22 μm filter membrane (e.g., cellulose acetate membranes) to remove the remaining bacterial cells.
7. Aliquots and store the lysate at 4 °C.
8. Optional: Change the buffer of the lysate by passing 20 mL phage lysate into an Amicon ultra-15 centrifugal filter. Alternate the concentrations and dilutions of the phage lysate in SM buffer by 3–4 rounds of 5-min centrifugations at 2000× *g* (*see* **Note 2**).
9. Titer the phage lysate obtained by the spotting assay (Fig. 2), described in Subheading 3.5.



Phage stock	# plaques	PFU/mL	Mean PFU/mL
A: lysate	$7\ 10^7$	$2.33\ 10^{10}$	$2.00\ 10^{10} \pm 3\ 10^9$
B: lysate	$5\ 10^7$	$1.67\ 10^{10}$	
C: lysate	$6\ 10^7$	$2.00\ 10^{10}$	
D: SM buffer	$6\ 10^7$	$2.00\ 10^{10}$	$3.00\ 10^{10} \pm 5\ 10^9$
E: SM buffer	$9\ 10^7$	$3.00\ 10^{10}$	
F: SM buffer	$8\ 10^7$	$2.67\ 10^{10}$	

Fig. 2 Results of the spotting assay for phage T7 lysate dilutions. Lines A, B, and C are three parallel ten-fold serial dilutions of lysate and lines D, E, and F for phages in SM buffer. The dilution level is indicated as powers of 10 (10^0–10^7). 3 μL of each dilution was spotted on a soft agar bacterial layer and incubated at 37 °C overnight. The average PFU/mL and standard deviations are calculated based on the single plaque counts of the last dilution with visible plaques (number of plaques >1)

3.2 Phage DNA Extraction

From 10 mL of phage lysate at 10^9–10^{11} PFU/mL (*see* Subheading 3.1), one can extract 100 μL of T7 genomic DNA at a concentration of about 10 nM in water. The extraction and purification of a phage genome takes about 4–6 h [15]. Alternatively, various phage genome extraction kits based on silica spin column DNA extraction can be used (*see* **Note 3**).

1. Add 10 mL phage lysate to a 50 mL tube.
2. Add 100 μL of 1 M MgCl2 (10 mM MgCl2).
3. Add 400 U of DNase I and 750 μg RNase A (*see* **Note 4**). Mix by gently vortexing the tube.
4. Incubate 30 min at 37 °C.
5. Incubate 15 min at 75 °C to inactivate the DNase/RNase reactions.
6. Add 100 μg/mL of proteinase K and 0.5% wt/vol SDS to lyse the phage capsids and release the genomes.
7. Mix the solution by inverting the tube gently several times (do not vortex). Incubate at 55 °C for 30 min. Mix the lysate by inverting the tube 2–3 times during incubation (do not vortex).
8. Add 10 mL of the pre-treated lysate to a separatory funnel (Fig. 3).
9. Perform **steps 9–15** in a fume hood. Add 10 mL of Phenol: Chloroform:isoamyl alcohol (PCI).
10. Close the stopper and invert slowly. Hold the separatory funnel tightly at the stopper and the stopcock. Release the pressure by

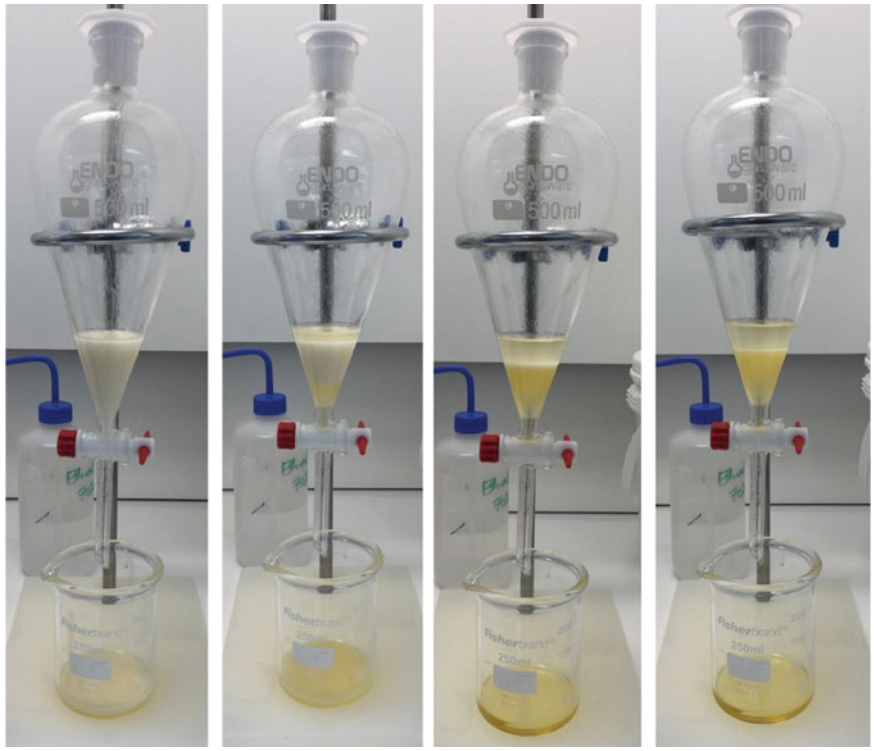


Fig. 3 Time series of a mixture of PCI and phage lysate in a separatory funnel. Right after mixing, an off-white emulsion is obtained. Upon decanting, the three phases slowly separate until the interphase becomes clearly separated from the upper phase, and the upper phase becomes transparent

opening the stopcock towards the back of the hood. Close the stopcock and shake the funnel gently. Vent it again. Repeat this step until no more gas escapes (*see* **Note 5**).

11. Allow the layers to separate for about 20–30 min until 3 phases are clearly separated (*see* **Note 6**).
12. Remove the stopper and drain the bottom layer and the interphase into a waste container.
13. Add 10 mL of PCI and repeat the procedure (**steps 9–12**).
14. Add 10 mL of PCI and repeat the procedure again (**steps 9–12**). At this step the interphase should be very thin and the aqueous phase very clear.
15. Add 10 mL of pure chloroform to remove phenol traces. Shake, vent, decant, and drain the bottom layer (chloroform).
16. Recover the aqueous phase (~10 mL) containing trace chloroform and DNA in a 50 mL glass tube.
17. To the aqueous phase, add 0.3 M of sodium acetate and twice the volume of pre-chilled (4 °C) 100% ethanol.
18. Gently mix by inverting, then precipitate at −80 °C for 1 h (*see* **Note 7**).

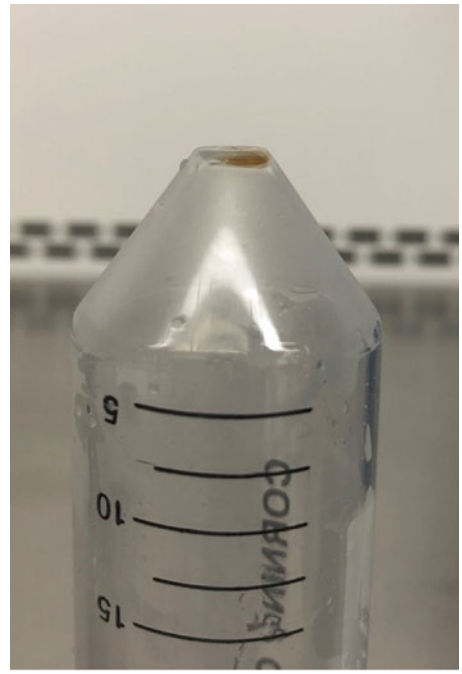


Fig. 4 T7 genome dried pellet after precipitation and wash. The pellet can be white to brown

19. Recover the tube from −80 °C and centrifuge the precipitated DNA for 20 min at 4000× *g* at 4 °C (*see* **Note 8**).
20. Discard the supernatant by inverting the tube and wash the DNA pellet with 1 mL 70% ethanol four times without disturbing the pellet.
21. Carefully pipet all remaining ethanol droplets around the DNA pellet and let the pellet dry 15 min at room temperature under a fume hood (Fig. 4). Do not let the pellet dry for more than 30 min as it may be difficult to resuspend it in water.
22. Resuspend the pellet in 100 μL of deionized water.
23. Transfer the resuspended DNA to a 1.5 mL centrifuge tube and measure the DNA concentration using a Nanodrop. Test the phage DNA to ensure the absence of phages by the spotting and kinetic assays (Subheadings 3.5 and 3.6) (*see* **Note 9**).

3.3 TXTL of Phages

The goal is to synthesize the phage T7 from its purified genome (Subheadings 3.1 and 3.2) in a TXTL reaction. This has already been demonstrated with an all-*E. coli* TXTL system [5, 7, 16], now sold by Arbor Biosciences under the name myTXTL. The transcription and translation are performed by endogenous molecular components provided by an *E. coli* cytoplasmic extract. A typical TXTL reaction is composed of 33% (v/v) of *E. coli* extract. The other 66% of the reaction volume is composed of an energy mixture, amino

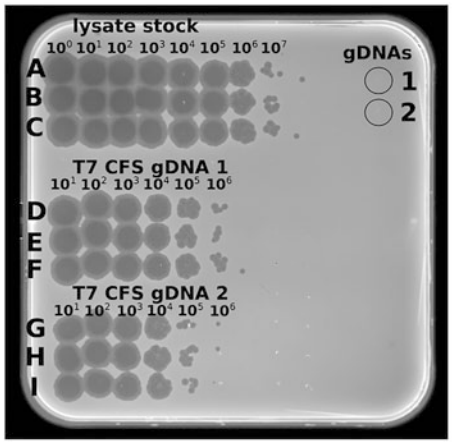
acids, ions (magnesium and potassium), a molecular crowder (PEG8000) and the DNAs to be expressed. A complete description of this system has been published [16, 17]. For cell-free expressing T7 phage, 3 μM of chi6 DNA are added to the mix to inhibit degradation of the linear dsDNA genome by the RecBCD complex [18]. The myTXTL Sigma 70 master mix kit provides 75 μL aliquots at 75% of the final volume (e.g., 9 μL of the mix for a final reaction volume of 12 μL). The myTXTL Sigma 70 master mix kit contains all the necessary components except chi6 and the DNAs to be expressed. The cell-free synthesis of phages takes about 3 h.

1. Thaw a 75 μL myTXTL Sigma 70 master mix kit aliquot on ice. Vortex the mix gently.
2. Split the mix into 9 μL aliquots in sterile 1.5 mL tubes.
3. Add 1 μL Chi6 DNA at 36 μM (3 μM final, *see* **Note 10**) to each of the 9 μL reactions.
4. Add 1.2 μL T7 genome at 10 nM to one tube (1 nM final concentration), bring to 12 μL by adding 0.8 μL water.
5. To a second tube, add 1.2 μL plasmid P70a-deGFP at 50 nM (5 nM final concentration), bring to 12 μL by adding 0.8 μL water. This reaction is a control (synthesis of deGFP).
6. To a third tube, add 2 μL water (negative control).
7. Vortex each reaction gently.
8. Incubate the reactions overnight at 29 °C.
9. To perform the spotting (Subheading 3.5) and the kinetic assay (Subheading 3.6) the next day, start an overnight culture of *E. coli* host B (5 mL LB broth, 37 °C, 250 rpm), as late as possible.

3.4 Serial Dilutions of Cell-Free Synthesized Phages

Ten-fold serial dilutions of the reactions are prepared in LB to determine (by the spotting assay and/or the kinetic assay) the concentration of infectious T7 phages synthesized overnight in TXTL. The serial dilution takes about 1 h using multichannel pipets.

1. Recover the TXTL reaction tubes from the incubator. Add 108 μL of LB to each of the 12-μL reactions (1st ten-fold dilution).
2. Place the tube on a thermomixer, mix for 20 min at 1000 rpm and 37 °C (*see* **Note 11**).
3. Serially dilute the reactions ten-fold by adding 10–90 μL of LB (*see* **Notes 12** and **13**).
4. Store the cell-free phage dilutions at 4 °C for spotting and optical density kinetic assays.



Phage stock	# plaques	PFU/mL	Mean PFU/mL
A: lysate	$5\ 10^7$	$1.67\ 10^{10}$	$2.00\ 10^{10} \pm 3\ 10^9$
B: lysate	$7\ 10^7$	$2.33\ 10^{10}$	
C: lysate	$6\ 10^7$	$2.00\ 10^{10}$	
D: gDNA 1	$4\ 10^5$	$1.33\ 10^8$	$2.00\ 10^8 \pm 9\ 10^7$
E: gDNA 1	$5\ 10^5$	$1.67\ 10^8$	
F: gDNA 1	$9\ 10^5$	$3.00\ 10^8$	
G: gDNA 2	$1\ 10^5$	$3.33\ 10^7$	$3.00\ 10^7 \pm 0$
H: gDNA 2	$1\ 10^5$	$3.33\ 10^7$	
I: gDNA 2	$1\ 10^5$	$3.33\ 10^7$	

Fig. 5 Results of the spotting assay for the cell-free synthesis of T7 phages. Lines A, B, and C are 3 parallel ten-fold serial dilutions (prepared in Subheading 3.4) of phage lysate (prepared in Subheading 3.1). gDNA 1 and 2 are the spotting of 5 μL of T7 genomic DNA stocks from 2 different DNA extractions prepared on two different days. No plaques are detected. Lines D, E, and F are the spotting of cell-free phage dilutions of 3 independent TXTL reactions (*see* Subheading 3.4) prepared from gDNA 1 and lines G, H, and I from gDNA 2. The dilution factors are indicated as powers of 10. 3 μL of each dilution were spotted on a soft agar bacterial layer and incubated at 37 °C overnight. The average PFU/mL and standard deviations are calculated based on the plaque counts of the last dilution with visible plaques (1–20 plaques)

3.5 Phage-Host Infection Spotting Assay

The spotting assay consists of adding a small volume of each of the dilutions of a phage solution to a lawn of the host strain. After several hours, the lawn of bacteria is lysed wherever phages are present, forming translucent circles into the lawn. At low phage concentrations, single plaques are observed that enable titration of phages (Figs. 2 and 5). In the case of T7, the spotting assay takes about 1 h to set up and 3–4 h to incubate.

1. Warm up 1.5% agar-LB plates at 37 °C for at least 1 h.
2. Make 100 mL of 0.7% soft agar solution. Add 2.5 g LB and 0.7 g Bacto-agar to a 100 mL bottle. Fill to 100 mL with de-ionized water. Autoclave at 121 °C for 15 min. Set water bath to 50 °C.
3. Once autoclave is finished, incubate the soft agar solution at 50 °C in the water bath. Wait at least 15 min so that the temperature equilibrates.
4. Plate the soft-agar layer as follows: to a 14 mL round-bottom culture tube, add 5 mL liquid soft agar (from the 50 °C water bath) and 100 μL of the overnight culture of *E. coli* host B (5 mL LB broth, 37 °C, 250 rpm). Vortex gently. Using a 5 mL pipette, slowly dispense 2.5 mL of solution onto each plate (avoid bubbles). Slowly let the solution out of the pipette and onto the center of the petri dish. Gently tilt the dish such that the solution coats the entire top surface of the agar plate. Let

the soft-agar plates solidify for 15 min at room temperature on a flat surface. The dryness of the agar is very important; if too moist, the drops will run and coalesce, if too dry, the bacteria will grow poorly.

5. Spotting: for each phage dilution, add 3 μL on the soft agar. Upon phage spotting, touch the surface of the soft agar with the tip to mark the position of the center of the droplet. Space the spots evenly (it is helpful to use a 96-well plate lid with condensation rings as a guide under the soft-agar plate).
6. Let the plate dry 15 additional minutes on the bench to make sure all the droplets are absorbed by the soft agar. Label your plate and mark the positions of the spotting underneath the plate.
7. Place the plates at 37 °C, facing down, for 4 h (you should start seeing lysis after 2 h of incubation for T7 phage).
8. The first dilutions (typically 10^1–10^5 dilutions) typically yield clear and uniform area slightly bigger than the dispensed droplet for T7 spots on *E. coli* host. Plaques become progressively countable within the spotted droplet area with higher dilutions.
9. Count the plaques at the dilution where each spot has approximately 1–20 plaques.
10. PFU/mL: example for 12 plaques at 10^7 dilution. There are 12 PFU in 3 μL so 4×10^3 PFU/mL at a 10^7 dilution. This means 4×10^{10} PFU/mL initially. A more accurate phage titer is obtained by increasing the number of spots and calculating mean and standard deviation (*see* **Note 14**).

3.6 Phage-Host Infection Kinetics Assay

The spotting assay enables a direct count of the plaques, hence a precise phage titer in PFU/mL for either phage in lysate, phage in SM buffer or cell-free synthesized phages. The kinetic assay, based on optical density readings over time in a welled-plate, also enables quantifying the concentration of phages in a solution, such as a TXTL reaction (Figs. 6 and 7). The kinetic assay has the advantage of providing the time course of infection of each phage dilution [19]. The more concentrated the phages, the quicker and less variable the lysis. Upon dilutions, less and less phages are initially present in each well, increasing the variability between the wells. Despite this, comparing the titers from the spotting assay with the liquid infection assay for T7 phage in *E. coli* B, we can show that the liquid assay sensitivity (the sensitivity is observed for the last dilution at which the three wells lyse) is around 10 PFU/mL. In the case of T7, the kinetics assay takes about 2–4 h. The duration of the kinetics assay must be tested for other phages.

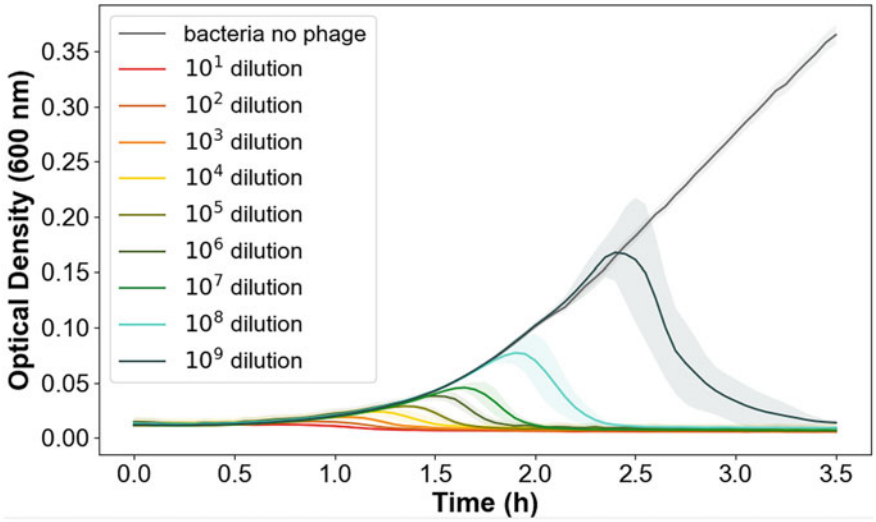


Fig. 6 T7 stock lysate kinetic assay. Serial dilution of the stock lysate (amplified in Subheading 3.1). Each curve is the result of three independent serial dilutions of the stock (prepared in Subheading 3.4). Only the dilutions where the three wells lysed are represented. The stock concentration was titered at 2×10^{10} PFU/mL in Subheading 3.5. This indicates that the kinetic assay is sensitive until ~20 PFU/mL

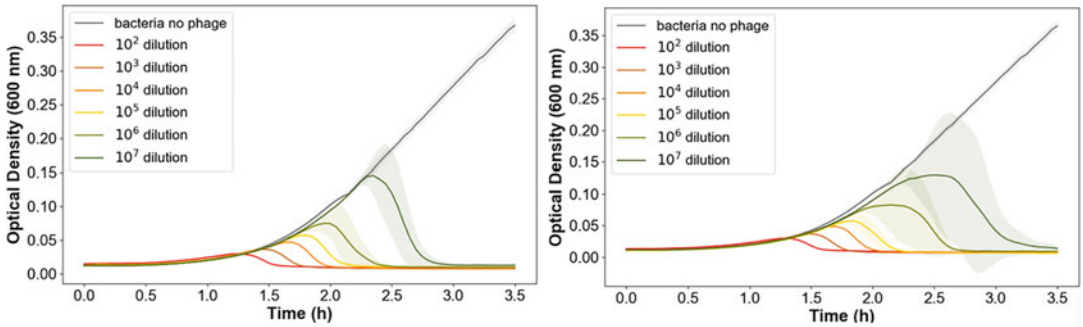


Fig. 7 Cell-free T7 phage expression from purified T7 genomes (first preparation) kinetic assay. Serial dilution of the cell-free expressed T7 phages (prepared in Subheading 3.4). Each curve is the result of three parallel independent cell-free expression reactions. Only the dilutions where the three wells lysed at the same dilution are represented here. Left: the stock concentration was titered at 2 10^8 PFU/mL in Subheading 3.5. This indicates that the kinetic assay is sensitive until ~20 PFU/mL. Right: the stock concentration was titered at 3×10^8 PFU/mL in Subheading 3.5. This indicates that the kinetic assay is sensitive until ~3 PFU/mL

1. Program the plate reader:
   (a) Set the temperature to 37 °C.
   (b) Continuous double orbital shaking at 90–180 rpm.
   (c) Set kinetic read for 5 h (T7 phage and *E. coli* B) with absorbance at 600 nm reads every 2 min.
   (d) Plot the mean and standard deviation of each condition. Subtract all the conditions by the negative control to get the real OD value.

2. Dilute the overnight *E. coli* B culture to an OD of 0.033 and determine the total volume of culture needed. For a whole 96-well plate $V_{host\_0.033} = 96 \times 200$ µL ~20 mL. From a starting culture at OD = 1, put 660 µL host in 19,340 µL LB to obtain 20 mL at OD 0.033.
3. Dispense 180 µL of the host culture to the wells of a 96 flat-bottom well plate.
4. Add 20 µL of different dilutions of the overnight TXTL reactions to each well containing the host culture for a final volume of 200 µL per well. For each condition, do four replicates. The controls are:
   - Positive control = 180 µL host + 20 µL LB (>4 replicates).
   - Negative control = 200 µL LB (>4 replicates).
5. Close the 96-well plate with a lid to reduce evaporation.
6. Insert the source plate into the preset plate reader.
7. Lysis shows up as an abrupt drop in the growth curves. Lysis occurs quickly for the first dilutions (usually 10^1, 10^2, 10^3, and 10^4 within the two first hours) and is progressively delayed for the higher dilutions. The first dilution at which the growth is not always inhibited (typically 10^{10}) indicates that initially less than one phage per well was present, providing a first indicator of the initial phage titer ~10^{10}–10^{11} PFU/mL (less than 1 phage at 10^{10} is >1 phage at a 10^9 dilution; 1 phage in 20 µL is ~50 PFU/mL diluted 10^9 times gives ~5×10^{10} PFU/mL) if the OD drops at 10^9 and not at 10^{10}. More accurate titer estimations can be done by running a calibration experiment from a known stock concentration of phages (*see* **Notes 15** and **16**).

4 Notes

1. The Felix d'Hérelle Reference Center for Bacterial Viruses, Leibniz Institute—DSMZ (German Collection of Microorganisms and Cell Cultures), Bacteriophage Bank of Korea, American Type Culture Collection (ATCC) Bacteriophage Collection, and National Collection of Type Cultures (NCTC) Bacteriophage Collection.
2. Buffer exchange slightly increases DNA purity. It is also relevant for other experiments requiring a pure phages solution free of small bacterial effectors.
3. Kits as an alternative to phenol/chloroform such as Norgen Biotek (46800), bioWORLD (10760112) are available commercially. However, genome DNA might be fragmented by the silica columns and hence reduce cell-free phage expression.

4. $MgCl_2$ is a DNase/RNase cofactor.
5. Significant pressure might build up in the separatory funnel during the first extraction.
6. The bottom layer is the phenol phase containing lipids and cellular debris, the interphase is an emulsion containing aggregated proteins, and the upper layer is the aqueous phase containing the genomic DNA.
7. The mixture should not freeze at −80 °C.
8. A free-floating DNA precipitate should be visible when recovering the tube from the −80 °C freezer.
9. TXTL systems are sensitive to the presence of solvents. Notably ethanol and phenol strongly inhibit cell-free gene expression. It is critical to obtain a genome as pure as possible.
10. Chi6_sense: TCACTTCACTGCTGGTGGC CACTGCTGGTGGCCACTGCTGGTGGC CACTGCTGGTGGCCACTGCTGGTGGC CACTGCTGGTGGCCA
    Chi6_antisense: TGGCCACCAGCAGTGGCCACCAG CAGTGGCCACCAGCAGTGGCCACCAGCAGTGGCCAC CAGCAGTGGCCACCAGCAGTGAAGTGA.
11. Do not try to pipet the reaction as it is typically very viscous. There is no need to use the thermomixer after the first dilution.
12. Use filter pipet tips to pipet up and down a dozen of times to ensure proper mixing at each step of the serial dilution.
13. A convenient way to do the additional dilutions is to use a 96 well-plate and multichannel pipets.
14. The T7 genomes prepared in Subheading 3.2 are also spotted to control for the presence of residual phages in the DNA.
15. The percentage of inhibition (PI) can be calculated from the OD curves as follow:

$$\mathrm{PI} = \frac{(A\mathrm{control} - A\mathrm{blank}) - (A\mathrm{phage} - A\mathrm{blank})}{(A\mathrm{control} - A\mathrm{blank})} * 100$$

where Acontrol is the area under the curve of PI, Ablank is the area under the curve of negative control, and Aphage is the area under the curve of a given phage dilution. These areas under the curve are calculated between two time points arbitrarily defined to start before the first lysis (SPD) and after the last one (EPD).

16. The typical titers obtained with T7 are 10^9–10^{12}. Titers for other phages have been measured [5, 16]. For instance, MS2 and phix174 (both *E. coli*) have titers of about 10^{12}.

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