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Precise Phage Mutagenesis with NgTET-Assisted CRISPR-Cas Systems

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Abstract

Bacteriophages, viruses that specifically target their bacterial hosts, hold significant potential for biotechnology and medicine, especially in combating multidrug-resistant infections. However, the molecular mechanisms underlying phage infection remain largely underexplored. Precise, site-specific mutagenesis of phages is a powerful tool to elucidate gene functions and phage-host interactions.

However, a major challenge in phage genome mutagenesis is the presence of phage DNA modifications that interfere with conventional genome editing tools like CRISPR-Cas.

While CRISPR-Cas systems have been used successfully for targeted mutagenesis in various organisms, their effectiveness in phage mutagenesis is often limited by DNA modifications such as cytosine glycosylation. To overcome this barrier, we developed an efficient method that temporarily reduces the abundance of phage DNA modifications, enabling efficient CRISPR-Cas targeting and precise mutation introduction into phage genomes. Specifically, we use the Ten Eleven Translocation (TET) methylcytosine dioxygenase from *Naegleria gruberi* (NgTET), which iteratively demodifies methylated and hydroxymethylated cytosines in DNA. By oxidizing hydroxymethylated cytosines within phage DNA, NgTET prevents subsequent cytosine modification like glycosylation and significantly enhances the efficiency of Cas-mediated DNA cleavage.

In conclusion, the scarless and precise genome-editing approach presented here enables the efficient introduction of point mutations while maintaining the native gene architecture in phage genomes. By preserving intact transcriptional and translational frameworks, this method minimizes unintended disruptions to complex regulatory networks. This is particularly important for investigating essential or multifunctional

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phage proteins. The ability to generate targeted genetic modifications without introducing extraneous sequences significantly expands the experimental toolkit for phage biology. This strategy not only facilitates detailed functional studies but also enhances the potential for rational engineering of phages for therapeutic and biotechnological applications.

Introduction

Bacteriophages are viruses that specifically target bacteria. They have attracted significant research interest due to their immense potential in biotechnology and medicine, particularly in the fight against multidrug-resistant bacteria[1,2,3]. Despite this, phage biology remains relatively understudied. A deeper understanding of their molecular mechanisms of hijacking their bacterial hosts is essential to fully harness their therapeutic and biotechnological potential[4]. To investigate the molecular mechanisms underlying the efficient phage infection and further to engineer the phages for specific applications, the ability to genetically modify phage genomes is essential. Yet it remains one of the most significant challenges in the field of phage biology[3,5,6].

Phage DNA is often modified by specific chemical alterations of the purine and pyrimidine bases, e.g., cytosine glycosylation and methylation. These modifications protect phage genetic material from recognition and degradation by host nucleases[6]. While these modifications are crucial for the phage fitness, they are a significant obstacle for phage genome engineering approaches that rely on DNA targeting systems[2,3,7].

Existing mutagenesis strategies, including restriction-modification systems and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated (Cas) protein-based technologies, face significant challenges when modifying phage DNA due to protective epigenetic modifications like cytosine glycosylation[8]. CRISPR-Cas-mediated genome editing, which is highly effective in bacterial and eukaryotic cells, relies on two key steps: the precise cleavage of DNA at targeted genomic loci by the Cas nuclease, followed by DNA repair through homologous recombination with donor DNA carrying the desired mutation[1]. However, in phages, DNA modifications often prevent Cas nucleases from binding or cleaving the genome efficiently. Additionally, the rapid and transient nature of phage replication can further limit the efficiency of homologous recombination. These barriers make it particularly difficult to apply DNA-targeting CRISPR-Cas technology for phage mutagenesis. Phage DNA modifications have been shown to impair phage DNA targeting with CRISPR-Cas both *in vitro* and *in vivo*[6,7].

To address the challenges posed by protective DNA modifications in phages, we introduce a genome mutagenesis strategy that harnesses the activity of the ten-eleven translocation (TET) methylcytosine dioxygenase, specifically NgTET. In eukaryotes, TET enzymes catalyze the stepwise oxidation of methylated cytosine through iterative processes: methylcytosine (5mdC) is first converted to hydroxymethylcytosine (5hmdC), then to formylcytosine (5fdC), and finally to carboxycytosine (5cadC) (**Figure 1**).

In our phage mutagenesis approach, NgTET is used to temporarily reduce the abundance of protective DNA

modifications, thereby enhancing the accessibility of phage DNA to genome editing tools such as Cas enzymes[9]. NgTET, was selected for modulating the bacteriophage genome due to previous reports of its successful expression in active soluble form in a heterologous bacterial host, particularly *E. coli*. This property is essential, as NgTET must remain active during phage infection[10].

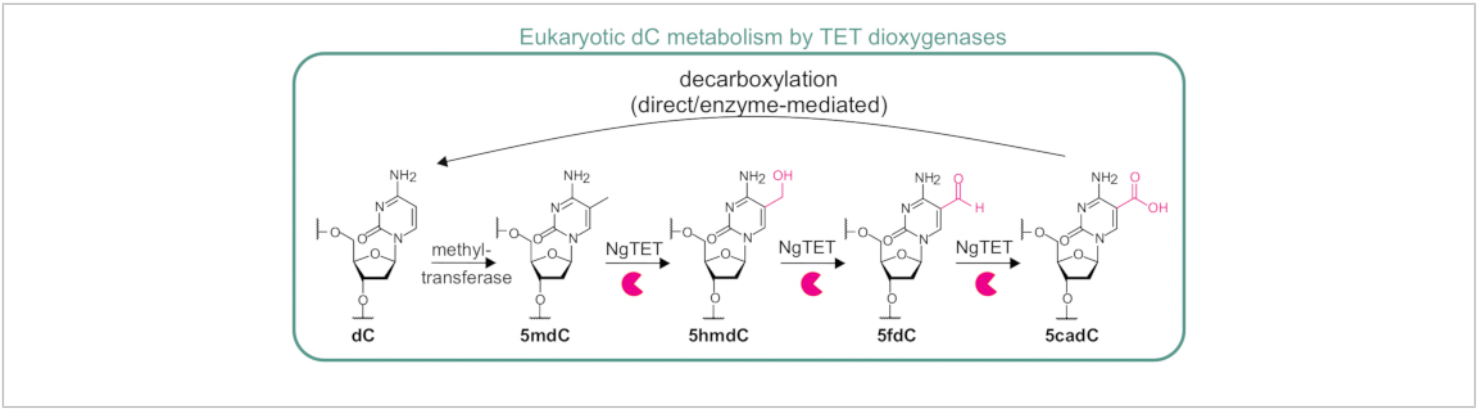


Figure 1: Stepwise Oxidation of 5-Methylcytosine by TET Dioxygenase[1]. The TET dioxygenase catalyzes the successive oxidation of 5-methyl-2´-deoxycytidine (5mdC) to 5hmdC, 5-formyl-2´-deoxycytidine (5fdC), and ultimately to 5-carboxyl-2´-deoxycytidine (5cadC). The final product, 5cadC, either spontaneously or enzymatically reverts to unmodified cytosine (dC), thereby contributing to dynamic epigenetic regulation. This multistep process is central to active DNA methylation in eukaryotes. Please click here to view a larger version of this figure.

Since methylcytosine and hydroxymethylcytosine are common precursors to phage DNA hypermodifications, including bulky protective structures like glycosylations, the oxidative activity of TET dioxygenase can be exploited to prevent the formation of these DNA hypermodifications[9]. In this TET-based genome mutagenesis approach, we heterologously express NgTET recombinantly in *E. coli* to oxidize hydroxymethylated cytosines in the phage genome upon infection, thereby preventing the formation of bulky glycosylation. By decreasing the abundance of these bulky DNA modifications, our approach can increase phage DNA accessibility to CRISPR-Cas nucleases. This enhanced accessibility facilitates efficient target recognition, precise DNA cleavage, and the introduction of scarless point mutations into the phage genome, distinguishing it from traditional phage mutagenesis methods that rely on gene deletions, reporter gene insertions, or the integration of artificial junctions for PCR-based mutant selection[1,11]. These conventional strategies have so far been the only available tools for targeted mutagenesis in phage genomes and therefore represent an important foundation for the method presented here. However, single phage proteins can possess multiple functions. Deletion of the entire gene encoding a protein of interest disrupts all associated functions. In contrast, the targeted introduction of point mutations permits the selective inactivation of specific functions, enabling detailed analysis of their roles. To date, the functions of many phage proteins remain largely uncharacterized; thus, we may overlook additional activities linked to a given gene. Therefore, a minimalistic approach that

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aims to remove only a single function while causing minimal changes to the overall protein is preferred[12,13]. The method described here enables precise genetic modifications without disrupting the overall genomic organization or function. Another major challenge of previous phage mutagenesis methods has been their low efficiency, which typically yielded mutation rates of around 0.1%[1,11]. With this technique, we present the first mutagenesis strategy capable of introducing single-codon changes, achieving a notable sevenfold increase in targeting efficiency. By combining it with an ONT-based high-throughput screening method, the detection of point mutations is simplified, eliminating the need to screen large phage populations to isolate one mutant. The TET-based mutagenesis strategy described here addresses a longstanding limitation in the field and enables more reliable genome editing[14].

Overall, this method cannot only be applied to advance our understanding of bacteriophage infection mechanisms but also holds significant promise for synthetic biology. By enabling precise and efficient phage genome engineering, it paves the way for tailoring the phages to specific applications, thereby enhancing their potential for biotechnological and medical applications.

Protocol

A schematic demonstrating the generation of NgTET-treated T4 phage is shown in **Figure 2**.

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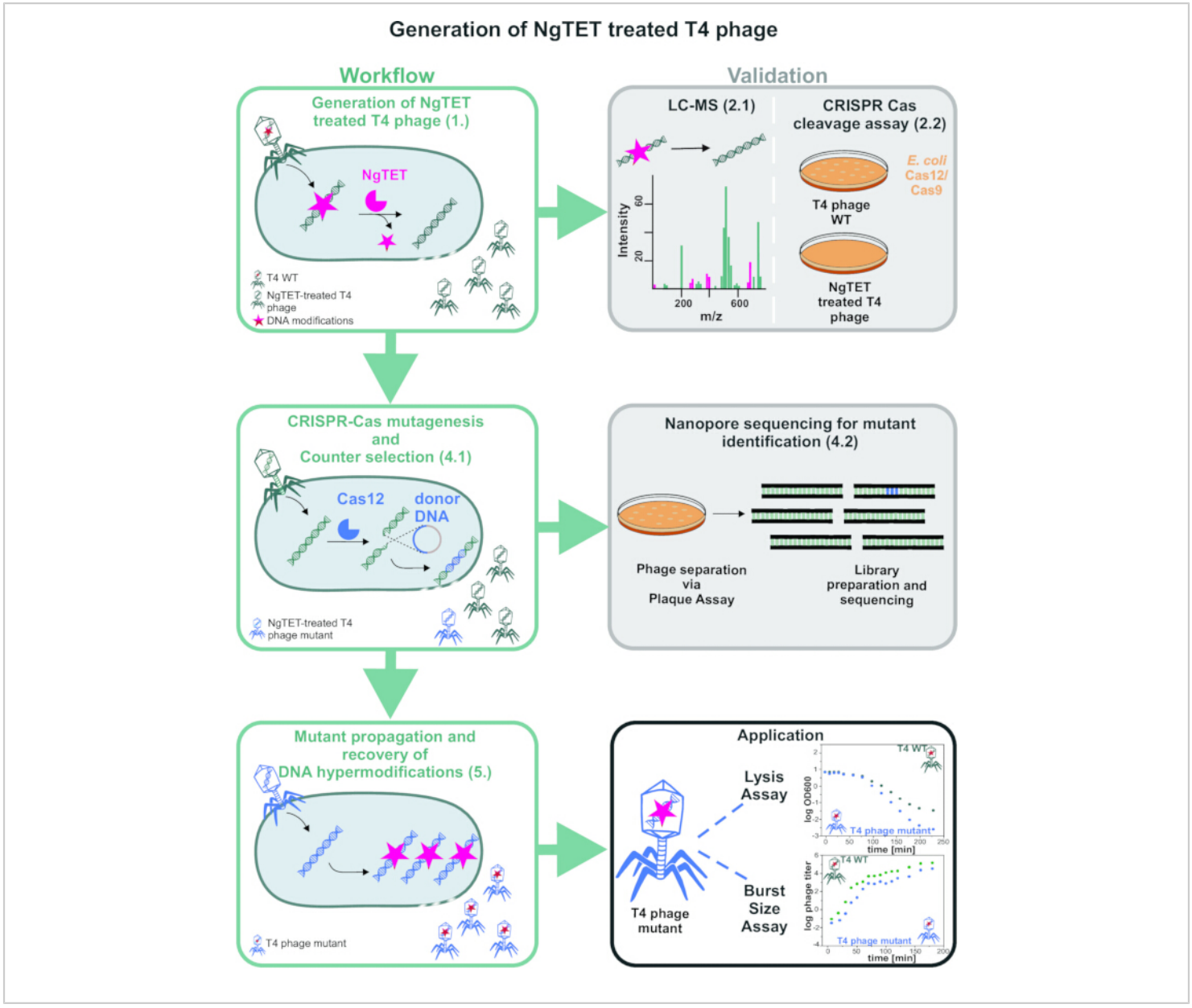


Figure 2: Schematic overview of the established workflow for T4 phage mutagenesis and mutant screening. The workflow includes the generation of the hypomodified T4 phage using NgTET, which can be validated either by LC-MS analysis or alternatively via CRISPR-Cas cleavage assay. Subsequently, the mutation of interest is introduced into the phage genome through CRISPR-Cas-mediated site-directed mutagenesis and identified by counterselection. The presence of the desired mutation can then be confirmed using Nanopore sequencing. Following validation, the mutant phage can be propagated to restore DNA hypermodifications. This enables the use of the engineered phage in downstream applications, including the analysis of infection phenotypes through lysis assays and burst size measurements. Each step in the figure is

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annotated with the corresponding protocol number in brackets to facilitate linking the protocol steps with the respective parts of the figure. Please click here to view a larger version of this figure.

1. Reducing phage DNA modifications using NgTET pre-treatment (~2 days)

NOTE: This step is critical for enabling efficient Cas nuclease targeting during mutagenesis by reducing the abundance of phage DNA modifications.

1. Inoculate the *E. coli* BL21 (DE3) strain transformed with pET28a_NgTET by transferring material from a glycerol stock into 10 mL of LB medium supplemented with 30 µg/mL kanamycin. Incubate overnight at 37 °C with shaking at 160 rpm.
2. The following day, use the overnight culture to inoculate a main culture to an initial OD_{600} of 0.1 in 50 mL of LB medium containing 30 µg/mL kanamycin. Induce the culture with 50 µM IPTG when an OD_{600} of 0.4 is reached.
3. Grow the culture at 37 °C with shaking at 160 rpm until it reaches an OD_{600} of 0.8.
4. Infect the culture with the phage of interest at a multiplicity of infection (MOI) of 0.1 and incubate for 4 h at room temperature with shaking at 120 rpm.
5. Harvest the cells by centrifugation at 4000 × *g* for 20 min at 4 °C.
6. Pass the supernatant through a 0.45 µm filter to obtain NgTET-treated phages with hypomodified DNA. Store the filtered phages at 4 °C for future use.
   NOTE: (PAUSE POINT) Filtered phages are stable for several months at 4 °C, stored in LB medium in a glass bottle.

2. Validation

NOTE: The aim of this step is to experimentally verify the reduced abundance of phage DNA modifications.

1. Treatment validation via LC-MS (~ 2 days)
   1. Phage purification via sucrose gradient
      1. To remove the residual nucleic acid from the lysate, treat 500 µL of the phage suspension (>10^{10} PFU/mL) with 20 U of DNase I and 2 µL of RNase A/T1 Mix (4 µg of RNase A, 10 U of RNase T1).
      2. Incubate the mixture at 37 °C for 30 min.
      3. Prepare a 0-45% sucrose gradient in TM buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5). Prepare the gradient using a gradient mixer or manually.
         NOTE: For manual preparation, start by pipetting 5 mL of TM buffer into a gradient tube. Then, using a blunt-end cannula, slowly pipette 5 mL of the 45% sucrose solution from the bottom of the tube, allowing the 0% solution to rise gradually.
      4. Load 500 µL of the treated phage solution on top of the gradient.
      5. Centrifuge at 70,000 × *g* for 20 min at 4 °C.
      6. Light the centrifugation tube from the bottom. This will allow for the visualisation of the phage, which appears as a turbid band in the gradient.

Remove the phage-containing fraction carefully using a blunt cannula.

7. Transfer the extracted phage fraction into a new ultracentrifugation tube.
8. Add 30 mL of ice-cold TM buffer and centrifuge at 100,000 × *g* for 1 h at 4 °C.
9. Discard the supernatant and resuspend the pellet in 500 µL of TM buffer.
10. Store the resuspended phages in a glass vial at 4 °C overnight.

2. Phage DNA isolation

1. Add 1 µg of Proteinase K to the resuspended phages.
2. Incubate for 30 min at 37 °C.
3. Add 1 volume of phenol/chloroform/isoamyl alcohol (P/C/I) mixture. Mix by inversion. Separate the phases by centrifugation at 15,000 x *g* for 1 min at 4 °C. Transfer the aqueous phase into a new reaction tube. Repeat the extraction three times.
   CAUTION: Phenol/chloroform/isoamyl alcohol (P/C/I) is highly toxic, corrosive, and volatile. Always handle it inside a fume hood while wearing appropriate protective equipment, including a lab coat, safety goggles, and double nitrile gloves. Avoid skin and eye contact, and store in a tightly sealed container in a designated chemical cabinet. Dispose of waste through approved hazardous waste channels. In case of contact, rinse immediately with water and seek medical attention.
4. To remove residual phenol, perform three times chloroform back-extractions with 1 volume of chloroform.
5. Precipitate DNA overnight with 0.1 volume of 3 M NaOAc (pH 5.5) and 2.5 volume of ethanol at -20 °C.
6. Next day, pellet the DNA by centrifugation at 15,000 × *g* for 1 h at 4 °C.
7. Wash the DNA pellet twice with 200 µL of 70% ethanol, gently shaking, centrifuging at 15,000 × *g* for 15 min at 4 °C, and carefully removing the supernatant.
8. Resuspend the purified DNA in ultrapure water.
9. Store the DNA at -20 °C until further use.
   NOTE: (PAUSE POINT) Precipitated DNA can be stored at -20 °C for several months.

3. Preparation of phage DNA for LC-MS analysis

1. Digest the purified DNA into single nucleosides using a Nucleoside Digestion Mix.
2. Analysis of DNA composition via LC-MS.
   NOTE: This step allows for validation of the effectiveness of the NgTET treatment. The protocol is adapted to the specific equipment and column available in the lab, but can be adjusted based on available resources and conditions.
3. Use digested DNA isolated from purified phage (step 2.1.10).
4. Include the following commercially available standards for the measurements: dA, dT, dG, dC, 5hmdC, 5fdC, 5cadC. If available, include

the standard of the cytosine hypermodification present on the phage DNA.

5. Use a HPLC system equipped with a C18 column (150 × 2.1 mm, 100 Å, 3 µm) and a 20 × 2.1 mm guard column.

6. Set the column temperature to 40 °C and the eluent flow rate to 0.2 mL/min.

7. Prepare eluents as follows:
   Eluent A: 10 mM Ammonium Acetate in water (pH 4.5).
   Eluent B: 0.1% formic acid in methanol.
   CAUTION: 0.1% formic acid in methanol is flammable and can irritate skin, eyes, and the respiratory tract. Always use in a well-ventilated area or fume hood. Wear appropriate protective equipment, including a lab coat, safety goggles, and nitrile gloves. Keep away from heat, sparks, and open flames. Store in a flammable storage cabinet. Dispose of via approved hazardous waste protocols.

8. Apply the following mobile phase profile: 0-1 min: Constant at 5% B; 1-5 min: Gradient from 5% to 90% B; 5-7 min: Constant at 90% B; 7-7.1 min: Gradient from 90% to 5% B; 7.1-12 min: Constant at 5% B.

9. Use a mass spectrometer in negative and positive ionization modes (separate injections) with a high-temperature electrospray ionization (H-ESI) source under the following conditions: H-ESI spray voltage: 3400 V (+), 2400 V (-), Sheath gas: 35 arbitrary units, Auxiliary gas: 7 arbitrary units, Sweep gas: 0 arbitrary units, Ion transfer tube temperature: 300 °C, Vaporizer temperature: 275 °C, Detection mode: Full scan using the Orbitrap mass analyzer, Mass resolution: 120,000, Mass range: 200-450 (m/z).

10. Extract ion chromatograms of the $[M-H]^-$ (dA, dT, dC, 5hmdC, 5fdC, 5cadC) and $[M+H]^+$ (5ghmdC) forms using Tracefinder software.

11. Calculate the relative abundance of each modification by normalizing the peak area of each signal to the dG peak area within the sample, using dG as a sample-specific internal standard.

2. Validation with CRISPR-Cas cleavage assay (~2.5 days)
   NOTE: This step enables the phenotypic assessment of the success of the NgTET treatment and its impact on CRISPR-Cas cleavage. If NgTET is active, no plaques should form from the treated phage DNA, or the phage titer should be reduced, as the removal of protective DNA modifications renders the genome susceptible to CRISPR-Cas12 or 9 cleavage. In contrast, if the phage DNA is untreated, the number of the formed plaques will correspond to the titer of the phages, indicating that the hypermodified T4 phage genome is resistant to CRISPR-Cas12 or 9 cleavage.

   1. Inoculate *E. coli* BL21 (DE3) from an overnight culture, transformed with both the pET28a_NgTET plasmid and a Cas12 or Cas9 expression plasmid (e.g., DS-spCas or pCpf1 without spacer). All plasmids with additional information can be found in **Table 1**.

      1. Grow the cells in LB medium supplemented with the appropriate antibiotics at 3 °C with shaking, starting from an OD_{600} of 0.1. Once the culture

reaches an OD_{600} of approximately 0.4, induce NgTET expression by adding 0.05 mM IPTG and incubate for an additional 2 h at 3 °C.

2. As a control, compare *E. coli* BL21 (DE3) strains with and without NgTET overexpression. In the absence of NgTET activity, a higher number of plaque-forming units is expected, indicating reduced CRISPR-Cas efficiency due to the presence of glycosylated 5ghmdC in the phage DNA.

2. Transfer 300 µL of the *E. coli* cultures into a sterile tube.

3. Infect the culture with T4 WT or NgTET-treated phage with MOI 0.01. For this experiment, apply a low MOI to ensure that bacteria are infected by only one phage at a time. This maximizes the efficiency of the CRISPR-Cas cleavage screening and simplifies interpretation of the results. Mix gently to ensure even distribution of the phages.

4. Incubate the bacteria-phage suspension at 37 °C for 7 min.

5. Add the bacteria-phage mixture to 4 mL of LB soft agar (0.75%) supplemented with antibiotics. Mix thoroughly but gently to avoid introducing bubbles.

6. Pour the soft agar mixture onto a pre-warmed LB agar plate.

7. Allow the plates to solidify briefly at room temperature. Incubate the plates at 37 °C overnight.

8. The next day, count the resulting plaques to determine plaque-forming units (PFU).

3. CRISPR-Cas mutagenesis (~ 1 week)

NOTE: CRISPR-Cas mutagenesis is based on the principle that a specific site within the phage genome is targeted by a Cas nuclease during phage infection, resulting in a double-strand break. This break is repaired through homologous recombination using donor DNA, which is provided on a second plasmid present in the infected host strain during mutagenesis. The plasmid that contains the donor DNA also encodes NgTET, which is essential to maintain accessibility of the phage DNA for the Cas nuclease by reducing the abundance of modified cytosines, as described previously. In this step, target mutations are introduced into the genome of NgTET-treated T4 phages upon infection of *E. coli*[15]. To ensure consistently low levels of DNA modifications in the T4 genome, NgTET dioxygenase is expressed by the addition of 50 µM IPTG (analogously to step 1.2) throughout the mutagenesis process.

1. Generation of pET28a_NgTET_donor DNA plasmids via golden gate cloning[15]

    1. For the generation of the donor DNA, order the desired sequence as an entire gene or amplify from a genome, for example, from the phage genome, and introduce the desired mutation.

    NOTE: For donor DNA design, include homologous regions flanking the target site (≈200 bp on each side for point mutations; longer regions for larger inserts). The linear donor fragment should carry BsaI recognition sites that generate compatible overhangs with the plasmid upon cleavage. *In silico* cloning is recommended to verify the construct design. In the representative results, all critical

components for the donor DNA are illustrated in an example.

2. Introduce the donor DNA fragment downstream of the NgTET coding sequence and terminator in the pET28a_NgTET backbone using Golden Gate assembly.
    1. Prepare the assembly reactions by combining 70 fmol of pET28a_NgTET plasmid, 140 fmol of donor DNA insert, 1 µL of BsaI (20 U/µL), 5 U of T4 DNA ligase, and 2 µL of 10× ligase buffer, with nuclease-free water added to a final volume of 20 µL.
    2. Run reactions in 25 cycles in a PCR-cycler with the following settings: 1) Restriction, 37 °C for 1.5 min, 2) Ligation, 16 °C for 3 min, 3) Final restriction, 37 °C for 5 min, and 4) Denaturation, 60 °C for 10 min.
3. Add 6 µL of the reaction mixture (from step 3.1.2) to 100 µL of chemically competent *E. coli* DH5α, incubate on ice for 10 min, and heat shock at 42 °C for 45 s. Immediately place the cells on ice for 1 min, then add 750 µL LB medium. Recover at 37 °C with shaking (500 rpm) for 1 h. Plate on LB agar containing kanamycin (30 µg/mL) and incubate overnight at 37 °C.
4. Pick a single colony and inoculate 20 mL of LB medium supplemented with kanamycin (30 µg/mL). Incubate overnight at 37 °C with shaking at 160 rpm.
5. From 5 mL of the overnight culture, pellet the cells and isolate plasmid DNA using a standard plasmid extraction method. Elute the plasmid in 40 µL of nuclease-free water.
6. Confirm correct cloning by sequencing the insertion site of the plasmid using Sanger sequencing.

2. Construction of spacer-containing CRISPR-Cas plasmids (pCpf1-sp as a Model)[15]

NOTE: The plasmid described in this chapter enables the first step of mutagenesis by introducing a site-specific double-strand break in the phage genome. While the procedure is demonstrated here with the CRISPR-Cas12 system (pCpfl-sp plasmid), alternative nucleases such as CRISPR-Cas9 (DS-spCas plasmid) can also be employed[15] .

1. Design a 20-nt spacer targeting the mutagenesis site (PAM: TTTV). Identify and design suitable protospacers and corresponding spacers using bioinformatic tools.
2. Synthesize two 5′-phosphorylated oligonucleotides. Ensure each carries 10 nt of the desired spacer sequence (together forming the full 20 nt spacer) and an additional 10 nt region complementary to the insertion site within the pCpf1-sp plasmid.
3. Insert the designed CRISPR-Cas spacer into the pCpf1-sp plasmid by PCR amplification using the primers from step 2. Set up the reaction as follows (final volume 50 µL): 5 µL 10× GC buffer, 1 µL 10 mM dNTPs, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 2.5 µL DMSO, plasmid template corresponding to 2 fmol, 1 µL Phusion DNA polymerase (2 U/µL), and nuclease-free water to 50 µL.
4. Place the reaction tubes in a thermocycler and run the following program: initial denaturation at 98 °C for 30 s; 25 cycles of 98 °C for 10 s, annealing at the appropriate temperature (X °C) for 30 s, and

extension at 72 °C for 6 min; followed by a final extension at 72 °C for 10 min. Hold the samples at 4 °C until further processing.

5. Mix 5 µL of the PCR reaction with DNA loading buffer (10×) and load onto a 1% agarose gel. Run the gel at 130 V for 20 min and visualize the DNA bands using UV trans illumination. If amplification is successful, purify the PCR product using a suitable cleanup kit and elute in 20 µL of nuclease-free water.

6. Circularize the plasmid by ligation using 100 ng of the amplified product mixed with 1× T4 DNA ligase buffer and 60 U T4 DNA ligase in a total reaction volume of 15 µL. Incubate for 1 h at room temperature. Transform 100 µL of chemically competent *E. coli* with the ligation product as described in step 3.1.3. Plate the cells on LB agar containing 50 µg/mL streptomycin and incubate overnight at 37 °C.

7. Pick a single colony from the plate and inoculate it into 20 mL of LB medium supplemented with 50 µg/mL streptomycin. Incubate overnight at 37 °C with shaking at 160 rpm. From 5 mL of the overnight culture, harvest the cells by centrifugation and isolate plasmid DNA using a plasmid extraction method of choice. Elute the plasmid in 40 µL of nuclease-free water.

8. Confirm successful cloning by sequencing the plasmid at the insertion site using Sanger sequencing.

3. Phage mutagenesis

1. Transform *E. coli* BL21 (DE3) cells with two plasmids: pET28a_NgTET_donor-DNA and pCpf1-sp (Cas12)/DS-SPcas (Cas9) spacer (generated in steps 3.1 and 3.2). Mix 1 µL of each plasmid with 100 µL of chemically competent *E. coli* BL21 (DE3). Follow the protocol described in step 3.1.3. Plate the transformed cells on agar containing 50 µg/mL streptomycin and 30 µg/mL kanamycin, and incubate overnight at 37 °C.

2. Inoculate the transformed *E. coli* strain (*E. coli* BL21 (DE3) pET28a_NgTET_donor-DNA and pCpf1-sp(Cas12)/DS-SPcas(Cas9)_spacer) into 10 mL of LB medium supplemented with 30 µg/mL kanamycin and 50 µg/mL streptomycin. Incubate overnight at 37 °C with shaking at 160 rpm.

3. The next day, dilute the overnight culture to an initial OD_{600} of 0.1 in 50 mL of LB medium containing 30 µg/mL kanamycin and 50 µg/mL streptomycin.

4. Grow the culture at 37 °C with shaking at 160 rpm until an OD_{600} of 0.8 is reached. Lower the temperature to room temperature (RT) and reduce the agitation speed to 130 rpm.

    1. To enhance phage adsorption and DNA injection, add $MgCl_2$ and $CaCl_2$ to final concentrations of 1 mM each, as these divalent cations facilitate efficient phage-host interactions.

    2. Infect the culture with NgTET-pretreated T4 phages, and as a negative control, with NgTET-untreated T4 phages at an MOI of 0.1. Continue incubation at RT for 4 h.

5. After incubation, transfer the cultures to a 50 mL conical tube and centrifuge at 4000 × *g* at 4 °C for 20 min to pellet the cells. Filter the supernatants through a 0.45 µm filter to remove any residual bacterial cells. Use the filtered supernatants for

plaque assays (described in step 2.2) to determine phage concentration or perform counterselection.

4. Mutant identification

NOTE: The purpose of counterselection is to decrease the proportion of wild-type phages remaining in the population after mutagenesis. For this, phages obtained from the mutagenesis step (step 3.3) are used to infect *E. coli* carrying the same CRISPR-Cas13 system, which is encoded by another plasmid pBA560 (additional information in **Table 1**) and the same spacer sequence applied during mutagenesis. In this setup, Cas nucleases selectively recognize and cleave wild-type phage DNA, while phages carrying the desired mutations remain unaffected. This selective pressure suppresses wild-type replication and enriches the population for mutant phages[14].

1. Counterselection with Cas13
    1. Infect *E. coli* Cas13a_spacer (plasmid pBA560) with the phages for counter-selection. This step facilitates counterselection by degrading phage RNA that has not undergone the intended mutation.
    2. Perform the counter-selection under the same conditions used during mutagenesis (see Steps 3.3.1-3.3.5). Use *E. coli* expressing a non-targeting Cas13a spacer as a negative control to confirm that plaque reduction is spacer-specific.
    3. After incubation, filter the supernatant containing counter-selected phages through a 0.45 µm filter to remove bacterial debris.

        NOTE: (PAUSE POINT) Filtered phages are stable for several months at 4 °C, stored in LB medium in a glass bottle.
    4. Use the counterselected and filtered phages for a plaque assay on an *E. coli* B strain to isolate individual plaques for downstream validation.
2. Next generation sequencing (~ 1 week)

    NOTE: The method for the highly multiplexed sequencing approach involves a two-step PCR process to generate multiplexed amplicon DNA suitable for long-read next-generation sequencing[16].
    1. Separate the phages from the mutagenesis by plating them out as described in step 2.2. Include proper controls by using phages obtained from mutagenesis performed both in the presence and absence of NgTET expression. As a control, CRISPR-Cas9 and Cas12 targeting without NgTET should not result in the introduction of point mutations. In contrast, co-expression of NgTET is expected to enable successful editing, leading to higher mutagenesis efficiencies.
    2. To isolate the single phages, pick individual plaques from the plate and transfer them into a tube containing 100 µL of Pi-Mg buffer (26 mM Na_2PHO_4, 68 mM NaCl, 22 mM KH_2PO_4, 1 mM $MgSO_4$, pH 7.5) and 1 µL of $CHCl_3$.

        CAUTION: Chloroform is toxic, volatile, and a suspected carcinogen, even in small volumes. Handle only in a fume hood and wear full protective equipment, including a lab coat, safety goggles, and nitrile gloves. Avoid inhalation and skin contact. Store in a tightly sealed container in a ventilated, flammable chemical cabinet. Dispose of chloroform waste via hazardous waste collection.
    3. Use 1 µL of isolated phage T4 in Pi-Mg buffer as the template for the screening.

4. Prepare the reaction mix with 0.125 µM of each primer (around the insertion position) and 1x high-accuracy polymerase master mix, adjusting the total volume of the amplification reaction to 10 µL. Run a PCR reaction with 30 amplification cycles.
5. Perform a second PCR to attach the barcodes. Therefore, use 1 µL of a 1:10 dilution of the product from the first PCR as a template.
6. Prepare the reaction mix with 0.3 µM barcoding primers and a high-fidelity hot-start polymerase mix in a total volume of 7 µL. Perform PCR with 20 amplification cycles.
   NOTE: (PAUSE POINT) PCR-Products can be stored at -20 °C for several weeks.
7. Pool all barcoded PCR products and purify them using magnetic bead-based cleanup according to standard protocols for next-generation sequencing library preparation.
8. Capture DNA using magnetic beads, wash twice with 80% ethanol, and elute in 100 µL of elution buffer (5 mM Tris-HCl, pH 8.5).
9. Measure the DNA concentration using spectrophotometric and fluorometric methods, applying either broad-range or high-sensitivity assay as appropriate.
   NOTE: (PAUSE POINT) DNA can be stored at -20 °C for several weeks.
10. Generate sequencing libraries using a ligation-based protocol according to the manufacturer's instructions, starting with 1 µg of input DNA.

5. Mutant propagation recovery of DNA modifications and proof

NOTE: Once the desired phage mutant is identified, it is propagated in wild-type *E. coli* to restore DNA modification levels similar to those of the WT phage. This ensures that the mutant phage can be utilized in subsequent biological experiments without any compromise, apart from the introduced mutation.

1. Prepare a 10 mL culture of *E. coli* B strain in LB medium and incubate overnight at 37 °C with shaking at 160 rpm.
2. The following day, inoculate a fresh 50 mL LB medium with the overnight culture to achieve an initial OD_{600} of 0.1.
3. Allow the culture to grow at 37 °C with shaking at 160 rpm until it reaches an OD_{600} of 0.8.
4. Add 50 µL of the mutant phage stock (4.2.2) to the culture. Remove the phase carefully to avoid transferring $CHCl_3$.
5. Incubate the infected culture overnight at room temperature with gentle shaking at 120 rpm.
6. After incubation, centrifuge the culture at 4000 × *g* to remove bacterial cells. Filter the supernatant through a 0.45 µm filter to collect the phage.
7. Transfer the filtered mutant phage to a glass container and store at 4 °C until further use.
   NOTE: (PAUSE POINT) Filtered phages are stable for several months at 4 °C, stored in LB medium in a glass bottle.
8. As an optional validation of the regeneration of DNA modifications, specifically glycosylated cytosines, perform an LC-MS analysis as described in step 2.1.

1. For this purpose, purify the recovered phage DNA from the phage progeny as outlined in step 2.1.1, isolate as in step 2.1.2, and subsequently prepare for LC-MS analysis as described in step 2.1.3.
2. Analyze ion chromatograms and calculate the relative abundance of each modification by normalizing the peak area of each signal to the dG peak area within the sample, using dG as a sample-specific internal standard as described in steps 2.1.3.

   NOTE: Including T4 wild-type phage, T4 phage treated with NgTET, and T4 phage treated with the inactive NgTET D234A variant as controls is recommended, in order to directly validate and compare the abundance of 5ghmdC[10].

Representative Results

Identification of the phage band in the gradient.

The visibility of the phage band in the gradient depends on the phage concentration in the initial sample loaded on the gradient. When the phage is sufficiently concentrated (~>1 × 10^{11} PFU/mL), the band can be clearly seen by illuminating the gradient from the bottom with a light source (see **Figure 3**).

However, at low phage concentrations, the band may not be easily visible. In this case, we recommend repeating the visualization attempt by illuminating the tube from below in the dark, which can enable detection of the phages.

If the phage band remains still not visible, carefully remove gradient fractions from the top using a blunt cannula and collect 1.5 mL per fraction. These fractions can then be analyzed via plaque assay to determine phage concentration. The two fractions with the highest phage concentration should be combined for subsequent DNA extraction.

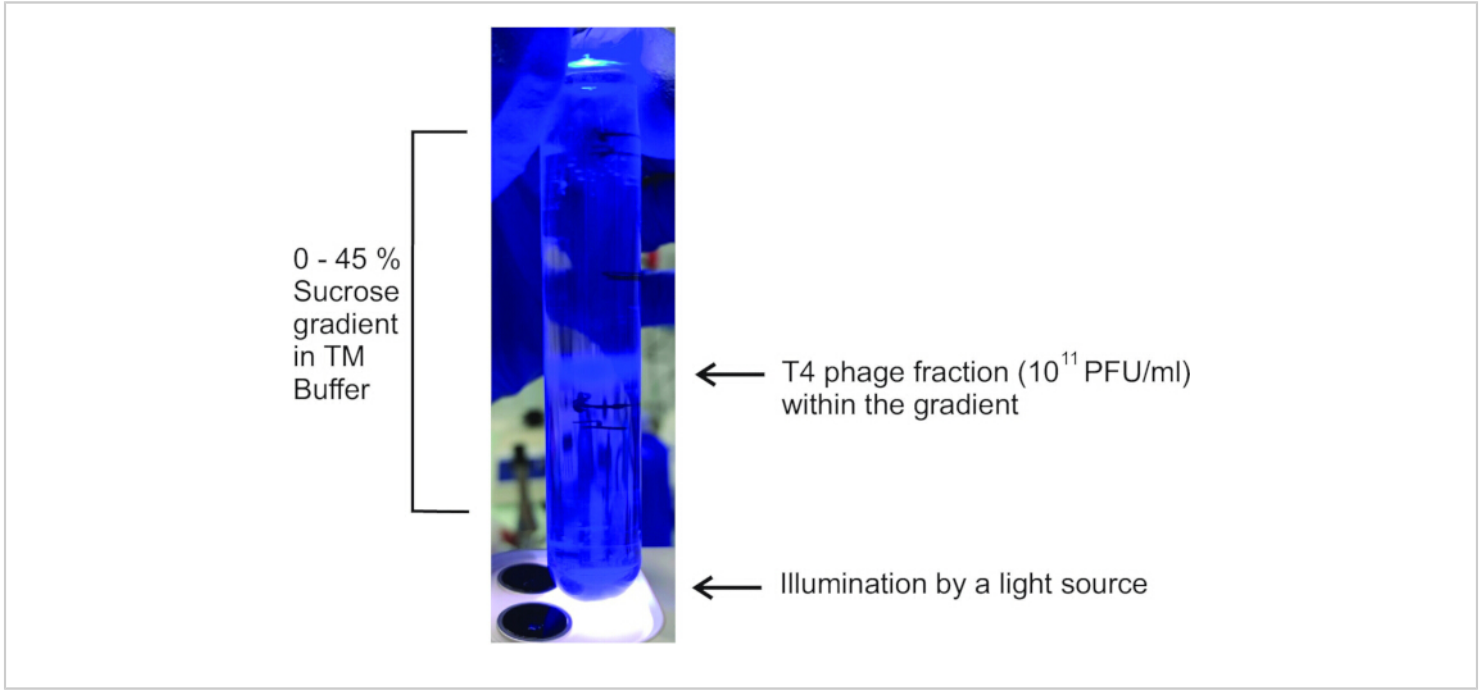


Figure 3: Visualization of the T4 phage band in a CsCl gradient. The visibility of the band depends on the initial phage concentration. At high concentration (> 10^{11} PFU/mL), the band is readily visible when the gradient is illuminated from below. Please click here to view a larger version of this figure.

Verification of NgTET expression

In this study, NgTET was heterologously expressed in *E. coli*. However, when expressing NgTET in different bacterial species or different *E. coli* strains, we recommend verifying protein expression using SDS-PAGE before performing the reduction of phage DNA modifications. Moreover, to assess whether the protein is expressed in soluble form, we suggest analyzing both soluble and insoluble fractions of the cells after cell lysis and fractionation via centrifugation (see **Figure 4** for an example of expression verification in *E. coli*).

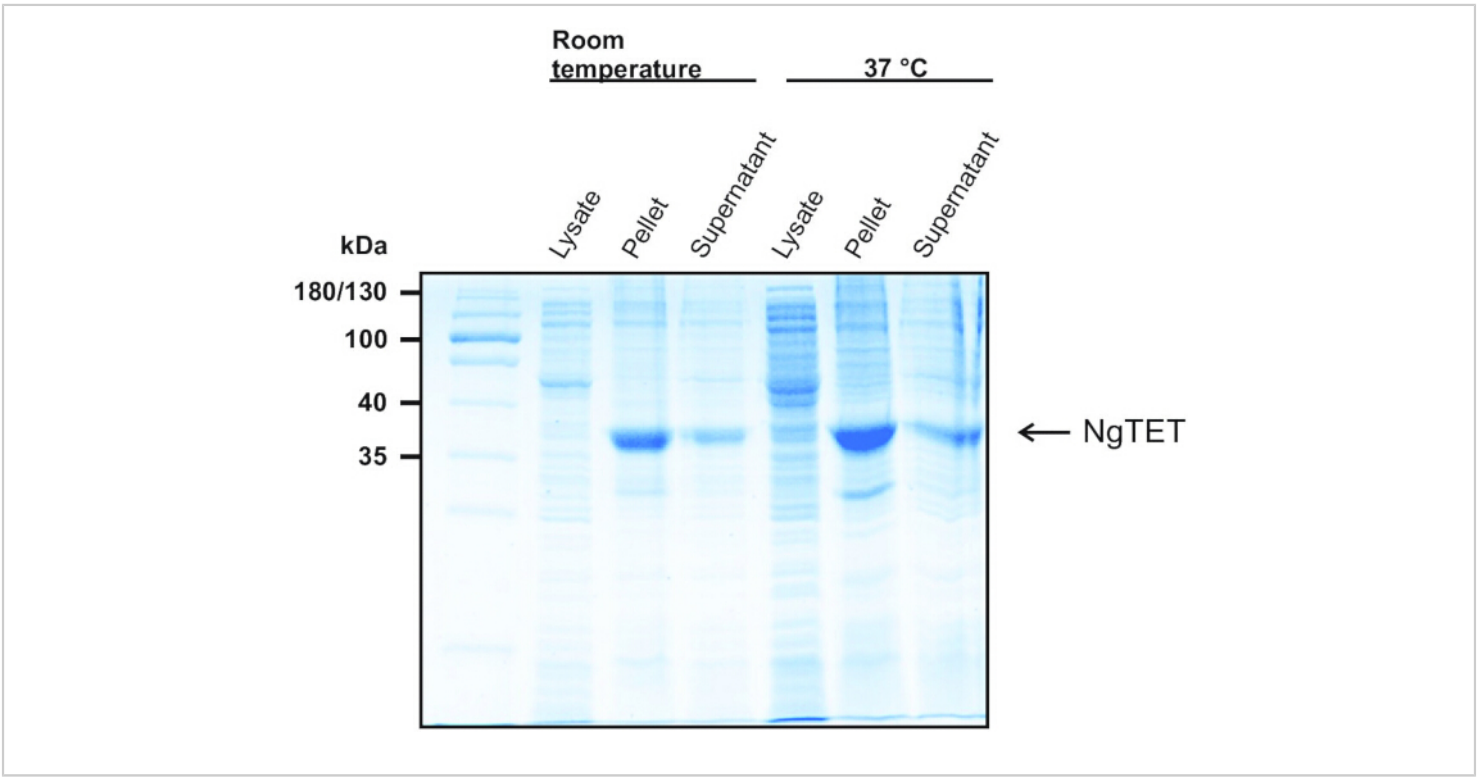


Figure 4: Expression of soluble and functionally active NgTET in *E. coli*. NgTET was expressed at 3 °C and room temperature to evaluate the impact of expression conditions on solubility and yield. For each condition, lysate, pellet, and supernatant fractions were collected and analyzed by SDS-PAGE. Samples were separated by electrophoresis and visualized using Coomassie staining. The expected molecular weight of NgTET is 38.7 kDa. This analysis allowed assessment of total expression levels and solubility of the recombinant protein under different temperature conditions. Please click here to view a larger version of this figure.

Proving the activity of NgTET on phage DNA via LC-MS Analysis[1]

The generation of hypomodified T4 phages through NgTET treatment was validated by LC-MS analysis, aimed at

verifying the reduced abundance of DNA modifications. To this end, purified genomic DNA from T4 phages treated or untreated with NgTET was analyzed[1].

A crucial control is the inclusion of untreated T4 DNA to allow direct comparison of nucleotide abundances. As shown in **Figure 5**, the extracted ion chromatograms reveal that the relative abundance of unmodified nucleotides such as dA, dG, and dT remains consistent between the NgTET-treated and untreated T4 phage DNA samples, indicating that NgTET treatment does not affect the overall DNA composition[1].

In contrast, the abundance of modified cytosine derivatives, including 5hmdC, 5fdC, and 5cadC, is significantly reduced in the NgTET-treated phage DNA compared to the untreated sample. This confirms that NgTET specifically demodifies 5hmdC without altering other canonical nucleotides, thereby validating the efficiency and specificity of the NgTET-based treatment for generating hypomodified T4 phage genomes[1].

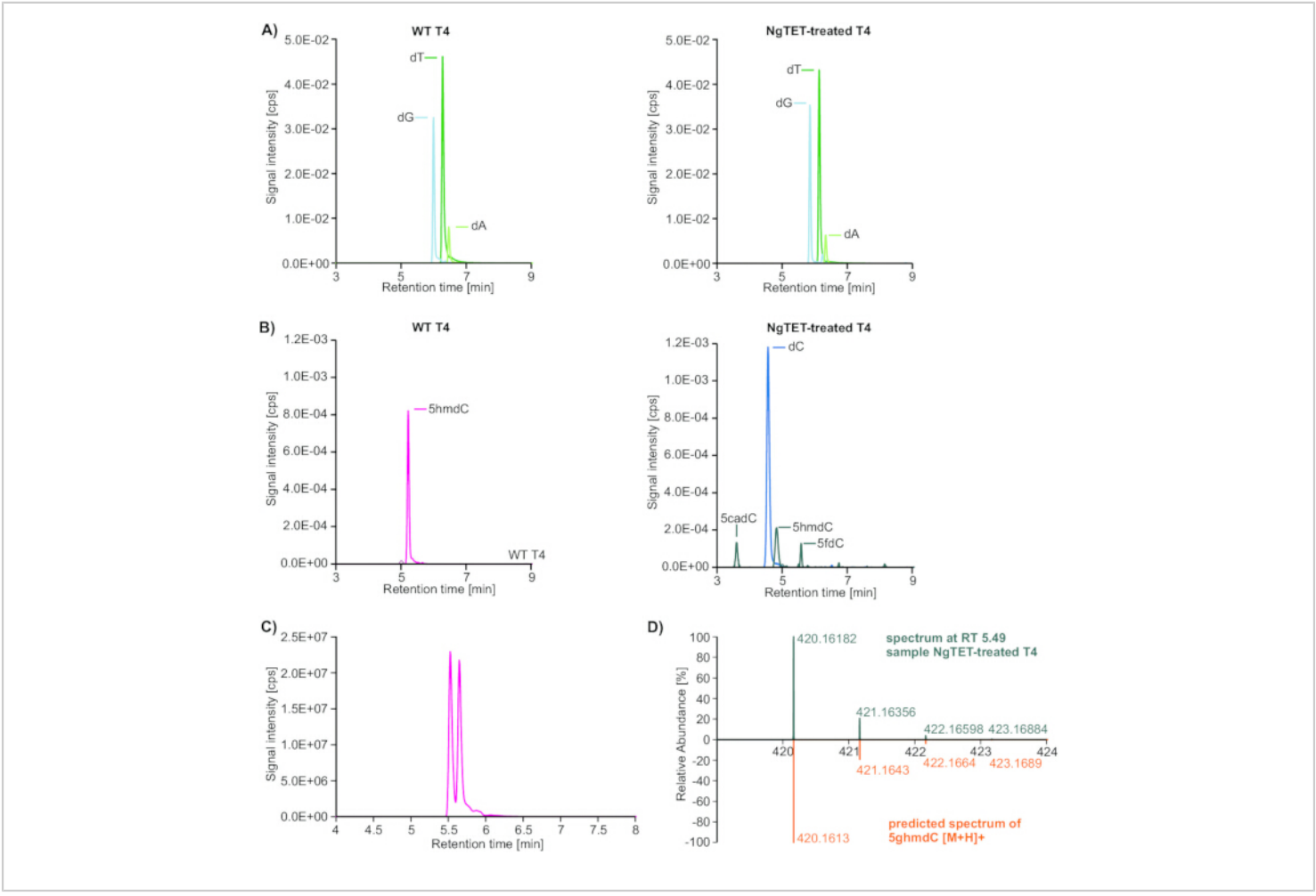


Figure 5: Validation of hypomodified T4 phage DNA via LC-MS analysis[1]. (**A**,**B**) Extracted ion chromatograms showing normalized mass traces of nucleotides from untreated T4 phage DNA (front) and NgTET-treated T4 DNA (back). Unmodified nucleotides dA, dG, and dT (**A**) display similar relative abundance profiles across both samples, confirming that NgTET

treatment does not affect the overall nucleotide composition. In contrast, the modified cytosine derivatives dC, 5hmdC, 5fdC, and 5cadC (**B**) show a marked decrease in the NgTET-treated sample, demonstrating specific demodification of 5hmdC-containing cytosines[1]. Signal intensities were normalized to total nucleotide content to account for variations in sample input and injection volume. (**C**) Extracted ion chromatogram of presumed 5ghmdC, observed in untreated T4 DNA. (**D**) The MS/MS fragmentation pattern of the presumed 5ghmdC corresponds well with the in silico predicted spectrum, supporting its structural assignment[1]. Please click here to view a larger version of this figure.

Proving the activity of NgTET on phage DNA via phenotype analysis

The most reliable and quantitative method to prove NgTET activity on phage DNA is DNA composition analysis via LC-MS, which allows for the determination of relative changes in single deoxynucleoside abundance in phage DNA (**Figure 5**).

However, if LC-MS is not accessible, NgTET activity can be assessed indirectly using a CRISPR-Cas12/9-based targeting efficiency assay. This method involves comparing the efficiency of CRISPR-Cas12/9 cleavage of phage DNA before and after NgTET treatment, as described in step 2.2. Following CRISPR-Cas12/9 treatment, a plaque assay is performed. If NgTET is active, no plaques should be formed from the treated phage DNA, or the phage titer should appear reduced, as the removal of protective modifications renders the genome susceptible to CRISPR-Cas12/9 cleavage. In contrast, if the phage DNA is untreated or NgTET is catalytically inactive, the number of formed plaques will correspond to the titer of the phages, indicating that the hypermodified T4 phage genome is resistant to CRISPR-Cas12/9 cleavage.

CRISPR Cas mutagenesis of T4 phage ModA[1,15]

For CRISPR-Cas mutagenesis, the target gene *modA* was selected to introduce a point mutation resulting in the amino acid substitution ModA E165A. To achieve this, the mutation ModA E165A was introduced by amplifying the gene from the T4 phage genome using primer pairs carrying the desired mutation (**Table 1**). The primers were additionally designed with BsaI recognition sites in opposite orientation, and five extra bases were included to generate sticky ends after BsaI digestion, enabling seamless plasmid assembly. **Figure 6** illustrates the essential components required within the donor DNA. Following successful cloning, the donor plasmid (pET28a_NgTET_donorDNA) was validated by Sanger sequencing[15].

Alongside this donor plasmid, a pCpf1-sp plasmid (for the CRISPR-Cas12 system) was constructed to introduce a site-specific double-strand break in the phage genome. For this purpose, a 20 nt spacer sequence targeting the mutagenesis site (PAM: TTTV) was designed. Two 5′-phosphorylated DNA oligos (sequences can be found in **Table 1**, exemplary for ModA E165) were synthesized, each encoding 10 nt of the spacer sequence (together forming the 20 nt spacer) as well as 10 nt complementary to the insertion site of the pCpf1-sp plasmid [15]. The spacer was integrated into the plasmid by PCR amplification using the designed primers. After PCR amplification and plasmid circularization, the construct was again validated by Sanger sequencing. With this strategy, both plasmids required for phage mutagenesis were successfully generated (**Table 1**).

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Figure 6: CRISPR-Cas Mutagenesis[15]. (**A**) Visualization of the key components required for generating the pET28a_NgTET_donor-DNA plasmid, highlighting the structure and essential elements of the donor DNA design.(**B**)

Schematic representation of plasmid construction using Golden Gate cloning[15]. Please click here to view a larger version of this figure.

Mutant identification via next generation sequencing

The highly multiplexed sequencing approach is based on a two-step PCR process to generate amplicon libraries suitable for long-read next-generation sequencing. For this purpose, individual plaques obtained after phage mutagenesis were picked and subjected to a PCR reaction as described in step 4.2.4, using the corresponding forward and reverse primers for the target gene *modA*. Following the first PCR, the amplicons were analyzed on a 1% agarose gel and evaluated based on their expected fragment size. For the target gene *modA*, the expected product was detected in lanes 2-9 (**Figure 7**), confirming the presence of positive hits within the picked plaques. These identified samples were subsequently qualified for the second PCR step, during which barcode primers were added to the PCR products to enable multiplexing in next-generation sequencing and identify the desired mutation of the gene.

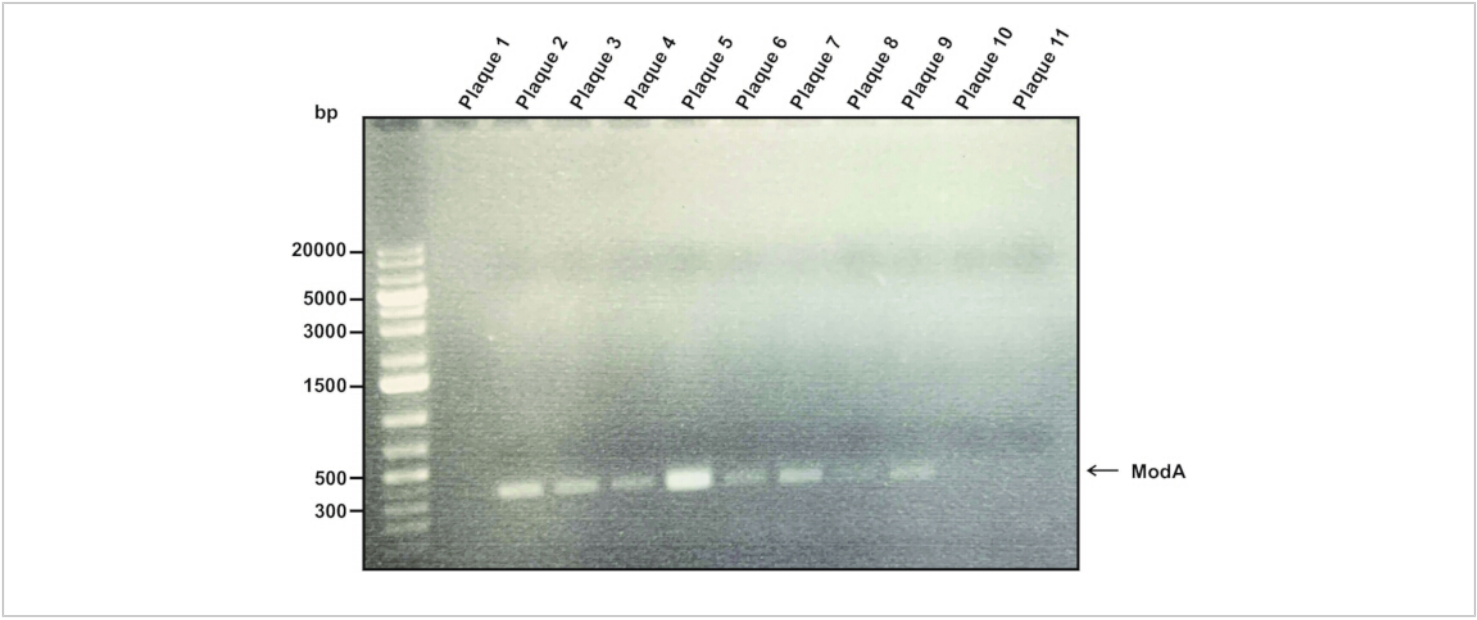


Figure 7: Mutant identification via next generation sequencing. Visualization of a 1% agarose gel stained with nucleic acid dye, showing amplification of the *modA* gene from plaques obtained after phage mutagenesis. Lanes 2-8 display bands corresponding to the expected size of *modA*, thereby identifying candidates for subsequent next-generation sequencing. Please click here to view a larger version of this figure.

Recovery of DNA modifications via LC-MS[1]

As an optional validation of the regeneration of DNA modifications, specifically 5ghmdC, LC-MS analysis can be performed as described in step 5.8. For this purpose, recovered phage DNA from progeny phages is purified as outlined in step 2.1.1, isolated as described in step 2.1.2, and subsequently prepared for LC-MS analysis following step 2.1.3. Using this workflow, the relative abundances (%) of 2′-deoxycytidine metabolites were determined for T4 wild-type phage (**Figure 8A**), NgTET-treated T4 phage (**Figure**

8B), NgTET D234A-treated T4 phage (**Figure 8C**), and recovered T4 phage progeny (**Figure 8D**)[1]. The results clearly show that recovered T4 phages restore the abundance of 5ghmdC, which is strongly reduced in NgTET-treated phages. Furthermore, when compared with the negative control (NgTET D234A treatment) and the T4 wild-type reference, the levels of 5ghmdC in recovered phages are nearly fully restored[1]. This demonstrates that T4 phage DNA is reversibly demodified and that following NgTET treatment and CRISPR-Cas mutagenesis, its initial DNA modification state can be re-established.

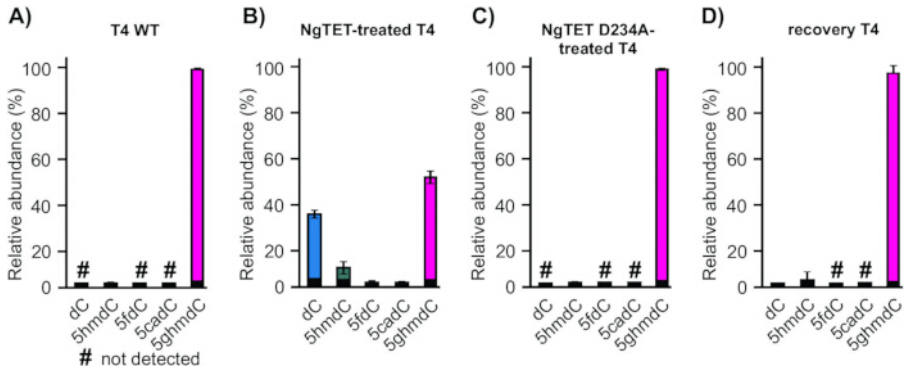


Figure 8: Recovery of DNA modifications via LC-MS[1]. Relative abundance (%) of 2´-deoxycytidine metabolites determined via LC-MS analysis in different T4 phage strain: Phage DNA samples include T4 WT (**A**), NgTET-treated T4 (**B**), T4 treated with the inactive mutant NgTET D234A (**C**), and recovered T4 progeny (**D**). The small dC signals observed in the recovered T4 DNA (D) likely originate from residual NgTET-treated input phages that failed to infect *E. coli* and thus were not propagated. The hashtag indicates nucleosides that were not detected in a given sample. Data represent three independent biological replicates[1]. Please click here to view a larger version of this figure.

Table 1: Table of plasmids and primers. Please click here to download this Table.

Discussion

This study presents a robust and adaptable strategy for introducing precise point mutations into bacteriophage genomes, including single-nucleotide changes. A central element of the workflow is the integration of stringent validation steps at each stage, which is essential for ensuring both the accuracy and efficiency of genome editing. This is particularly critical given the multistep nature of the workflow and the dynamic interactions between phages and their bacterial hosts, which introduce numerous variables that can directly affect the success of phage mutagenesis. These factors include, among others: I) the accessibility of the target DNA regions, II) the activity of CRISPR-associated nucleases, III) the stability of the donor DNA during CRISPR-Cas mediated mutagenesis in the *E. coli* host strain, IV) the loss of the desired mutation due to natural selection or evolution, and V) the temporal constraint imposed by the short phage infection cycle. These parameters may ultimately compromise the overall efficiency of the described genome editing approach.

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In the following, we discuss these potential influencing factors, highlight critical steps within the workflow, and propose possible solutions to address these challenges.

The first essential validation step is the immediate verification of DNA demodification following NgTET treatment, which can be achieved using either liquid chromatography-mass spectrometry (LC-MS) or CRISPR-Cas cleavage assays. This step is critical for confirming enzymatic activity and for facilitating subsequent genome editing, as the removal of DNA modifications is a prerequisite for efficient targeting by CRISPR nucleases. Our results demonstrate that transient removal of DNA modifications by NgTET substantially increases the efficiency of CRISPR-Cas9 and Cas12 systems, enabling successful mutagenesis through single amino acid substitutions at previously inaccessible loci with efficiencies of up to 6%[1].

Despite these advances, additional factors influence the overall efficiency of phage genome editing. For instance, beyond the inherent difficulties associated with targeting modified phage DNA, degradation of the donor DNA can further limit genome editing efficiency. This donor DNA, typically provided as a plasmid during CRISPR-Cas-mediated mutagenesis in the *E. coli* host strain, is susceptible to degradation, thereby limiting mutagenesis success[1,17,18]. To address the generally low editing efficiencies, enrichment strategies for phage populations carrying the desired mutation are highly useful. One such approach involves counterselection using RNA-targeting Cas13 nucleases. However, this method is more effective for mutations larger than three nucleotides[19,20,21]. Due to Cas13's tolerance for mismatches, additional downstream screening is often necessary to differentiate true mutants from wild-type phages[19,20,21]. As a potential solution, engineered Cas13 variants with increased sensitivity to single-nucleotide mismatches, or DNA-targeting nucleases such as Cas12, may enhance the specificity and stringency of counterselection[19].

In addition to molecular-level factors related to phage and host DNA, general biological constraints such as natural selection also play a critical role, particularly in cases where introduced mutations reduce phage fitness. Reversion to the wild-type sequence can occur rapidly through competitive exclusion, especially in the case of non-synonymous mutations[22]. Therefore, we strongly recommend Sanger sequencing phage mutants after each propagation cycle to verify the presence of the intended mutation and to detect possible reversion to the wild-type phage sequence[1].

Phage propagation poses further challenges not only in terms of evolutionary selection pressure but also due to the inherently short duration of the infection cycle. For example, in phage T4, the entire cycle from DNA injection to progeny release occurs within approximately 20 min[23]. Thus, all editing steps, including DNA demodification via NgTET, CRISPR-Cas-mediated cleavage, and homologous recombination, must be completed within this limited timeframe[1]. This highly condensed and tightly regulated process emphasizes the importance of optimizing each step, particularly the recombination step, to ensure successful genome editing.

Following the discussion of key steps, associated challenges, and potential solutions, it is important to emphasize that this approach was developed using *E. coli* and phage T4. Consequently, its broader applicability remains to be evaluated. Expression and activity of NgTET in alternative bacterial hosts will require further investigation, including codon optimization and verification of enzymatic function.

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We hypothesize that this strategy can be extended to other phages with cytosine modifications, such as T2- and T6 phages, such as methylation or hydroxymethylation, which are known substrates of TET dioxygenases[9,24]. Moreover, the field of phage epigenetics remains largely underexplored, and it is likely that many phages harbor DNA modifications that have yet to be identified. Prior to initiating genome editing, bioinformatic analyses can be employed to predict the presence of DNA-modifying enzymes. This can include identifying homologs to known cytosine-methylating or hydroxymethylating enzymes through sequence or structural comparisons. These cytosine-modifying enzymes are widely distributed among diverse phages, including those infecting clinically and biotechnologically relevant bacterial genera such as *Klebsiella*, *Salmonella*, and *Serratia*[1]. For instance, in phage T4, key proteins involved in cytosine hypermodification include deoxycytidylate 5-hydroxymethyltransferase (UniProt ID: P08773), alpha-glucosyltransferase (UniProt ID: P04519), and beta-glucosyltransferase (UniProt ID: P04547)[1]. These proteins can serve as references for homology-based searches in other phage genomes. Nonetheless, experimental validation beyond the *E. coli*-T4 model system remains essential. Given the widespread occurrence of cytosine-modifying enzymes among diverse bacteriophages, this approach holds substantial potential for both basic research and applied biotechnology. Validating this method in additional phage systems with cytosine-modified genomes will be crucial for assessing its generalizability.

In summary, the application of TET dioxygenases to enhance CRISPR-Cas-mediated genome editing in phages represents a major advancement in phage engineering. By overcoming the limitations imposed by phage DNA modifications, this approach provides a precise and efficient platform for the genetic manipulation of bacteriophage genomes[1]. As interest in phage-based therapeutics and industrial applications continues to grow, this strategy offers a valuable foundation for the development of tailored phages in both clinical and biotechnological contexts.

Disclosures

KH and NP filed a European Patent Application for "Engineering of Phages", European Patent Application No. 23 175 257.7. The other authors declare no competing interests.

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