FINAL SCRIPT: APPROVED FOR FILMING

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Title: Precise Phage Mutagenesis with NgTET-Assisted CRISPR-Cas Systems

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   Some filming locations are is separate rooms, but located on the same floor.

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length
Number of Steps: 21
Number of Shots: 43

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Katharina Höfer:** We investigate T4 phage infection of *E. coli* at the molecular level and translate these insights into applications for phage engineering and therapeutic strategies.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.1.1*

What are the most recent developments in your field of research?

1.2. **Katharina Höfer:** Recent advances include novel tools to edit and analyze phage genomes despite DNA modifications, and expanding insights into the highly dynamic host–phage interactions shaped by defense and counter-defense mechanisms.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What technologies are currently used to advance research in your field?

1.3. **Katharina Höfer:** Current research relies on CRISPR technologies, single-cell and high-throughput sequencing, cryo-EM, mass spectrometry, and advanced bioinformatics to dissect phage–host interactions and their underlying molecular mechanisms.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.4*

What are the current experimental challenges?

1.4. **Katharina Höfer:** Major challenges include phage DNA modifications hindering genome editing, difficulties in culturing diverse phages and hosts, and capturing dynamic defense–counterdefense interactions at single-cell resolution in real time.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.2*

What significant findings have you established in your field?

1.5. **Katharina Höfer:** We discovered NAD-capped RNAs and RNAylation during T4 phage infection and provided new insights into host–phage interactions, thereby expanding the toolkit for studying and engineering phages.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.6*

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions:

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Prof. Dr. Katharina Höfer, Group leader of the Max Planck Research Group "Bacterial Epitranscriptomics"(Max Planck Institute for terrestrial microbiology), Full Professor at the University of Marburg (Department of Pharmacy)**: (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Prof. Dr. Katharina Höfer, Group leader of the Max Planck Research Group "Bacterial Epitranscriptomics"(Max Planck Institute for terrestrial microbiology), Full Professor at the University of Marburg (Department of Pharmacy)** : (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

2. Sucrose Gradient Purification of Phage Particles for Downstream Analysis

Demonstrators: Katharina Höfer, Helene Keuthen

2.1. To begin, treat 500 microliters of phage suspension with 20 units of DNase I *(D-N-Ase-One)* **[1].** Add 2 microliters of RNase A/T1 *(R-N-Ase-A-T-One)* mix to the tube **[2]**. Incubate the treated mixture at 37 degrees Celsius for 30 minutes **[3]**.

2.1.1. WIDE: Talent adding DNase I into a microcentrifuge tube containing phage suspension.

2.1.2. Talent adding RNase A/T1 mix to the same tube.

2.1.3. Talent placing the tube in a pre-heated incubator set to 37 degrees Celsius.

2.2. Next, load 500 microliters of the treated phage solution on top of a prepared sucrose gradient **[1-TXT]**. Centrifuge the gradient at 70,000 *g* for 20 minutes at 4 degrees Celsius **[2]**.

2.2.1. Talent carefully pipetting the phage solution on top of the prepared gradient. **TXT: Gradient: 0 - 45 % sucrose in TM buffer**

2.2.2. Talent placing the gradient tube into the ultracentrifuge and initiating the run.

2.3. Light the centrifugation tube from the bottom to visualize the turbid phage band in the gradient **[1]**. Then, with a blunt cannula, carefully remove the phage-containing fraction into a new ultracentrifugation tube **[2]**.

2.3.1. Talent shining light at the base of the centrifugation tube to reveal the turbid band.

2.3.2. Talent extracting the turbid band using a blunt cannula. NOTE: 2.3.2 AND 2.4.1 were simultaneous actions, so combined

2.4. ~~Transfer the extracted phage fraction into a new ultracentrifugation tube **[1]**~~. Now add 30 milliliters of ice-cold TM buffer to the tube and centrifuge **[2-TXT].** Then resuspend the pellet in 500 microliters of TM buffer after discarding the supernatant **[3]**.

~~2.4.1. Talent transferring the extracted phage solution to a clean ultracentrifuge tube.~~ NOTE: 2.4.1 is same AS 2.3.2, so VO merged

2.4.2. Talent pouring ice-cold TM buffer into the tube containing phage extract. **TXT:**

Centrifugation: 100,000 x *g*, 1 h, 4 °C

2.4.3. Talent resuspending the pellet in TM buffer by gentle pipetting.

2.5. Store the resuspended phages in a glass vial **[1]** at 4 degrees Celsius overnight **[2]**. **NOTE**: VO added for the extra shot, 2.5.1 and 2.5.2 were shot together one time and also separately

2.5.1. Talent transferring the resuspended phage solution to a labeled glass container.

Added shot: Talent Placing container in a refrigerator.

3. Purification of Phage DNA via Organic Extraction and Ethanol Precipitation

3.1. Add 1 microgram of Proteinase K to the resuspended phages **[1]**. Incubate the mixture at 37 degrees Celsius for 30 minutes **[2]**.

3.1.1. Talent pipetting Proteinase K into the tube containing resuspended phages.

3.1.2. Talent placing the tube in the incubator set at 37 degrees Celsius.

3.2. Then add an equal volume of phenol/chloroform/isoamyl alcohol *(Phenol-chloroform-iso-amyl-alcohol)* mixture to the sample **[1]**. Invert to mix well and centrifuge **[2-TXT]**. Transfer the aqueous phase to a new reaction tube and repeat the extraction three times **[3]**.

3.2.1. Talent adding phenol/chloroform/isoamyl alcohol mixture to the sample tube.

3.2.2. Talent gently inverting the tube to mix. **TXT: Centrifugation: 15,000 x *g*, 1 min, 4 °C** **NOTE**: 3.2.1 and 3.2.2 are combined

3.2.3. Talent transferring the upper aqueous layer to a new tube. **Videographer's NOTE:** 3.2.3 is uploaded in two versions

3.3. Perform three chloroform back-extractions **[1]** using an equal volume of chloroform to remove residual phenol **[2]**. **NOTE**: VO adjusted

3.3.1. Talent adding chloroform to the sample.

Added shot: Transferring the aqueous phase to a clean tube after each extraction.

3.4. Then precipitate the DNA by adding 0.1 volume of 3 molar sodium acetate at pH 5.5 and 2.5 volumes of ethanol **[1]**. Incubate the mixture overnight at minus 20 degrees Celsius **[2]**. The next day, centrifuge the sample at 15,000 *g* for 1 hour at 4 degrees Celsius to pellet the DNA **[3]**.

3.4.1. Talent adding sodium acetate and ethanol to the tube.

3.4.2. Talent placing the tube in a freezer set to minus 20 degrees Celsius.

3.4.3. Talent placing the tube into the centrifuge and initiating the spin.

3.5. Wash the DNA pellet twice with 200 microliters of 70 percent ethanol **[1]**. Gently shake the tube and centrifuge again **[2-TXT]**.

3.5.1. Talent adding 200 µL ethanol to the DNA pellet.

3.5.2. Talent shaking the tube and placing it in a centrifuge. **TXT: Centrifugation: 15,000 x *g*, 15 min, 4 °C**

3.6. Carefully remove the supernatant **[1]**, then resuspend the purified DNA in ultrapure water **[2]**. Store the DNA at minus 20 degrees Celsius until further use **[3]**.

3.6.1. Talent removing the supernatant without disturbing the pellet.

3.6.2. Talent pipetting ultrapure water into the tube and mixing gently.

3.6.3. Talent placing the DNA sample into a labeled tube and storing it in a minus 20 degrees Celsius freezer.

4. Validation of Phage Editing via CRISPR-Cas Assays and Cas13a-Based Counterselection

4.1. Inoculate *Escherichia coli* BL21 (DE3) *(B-L-Twenty-One-D-E-Three)* cells that are transformed with both the pET28a_NgTET *(p-E-T-Twenty-Eight-A-N-G-T-E-T)* plasmid and a Cas12 *(Kas-Twelve)* or Cas9 *(Kas-Nine)* expression plasmid **[1]**.

4.1.1. Talent inoculating *E. coli* from an overnight culture into fresh LB medium containing selective antibiotics. **TXT: Initial OD_{600}: 0.1**

~~4.1.2. The main culture was inoculated to an initial OD_{600} of 0.1. (added shot)~~ **NOTE**: Cannot use this extra shot as no proper narration is given, added on-screen text

4.2. Grow the culture in LB medium with supplemented with appropriate antibiotics at 37 degrees Celsius with shaking **[1-TXT]**. Induce NgTET expression by adding 0.05 millimolar IPTG *(I-P-T-G)* **[2]** and incubate for an additional 2 hours at 37 degrees Celsius **[3]**. **NOTE**: VO adjusted

4.2.1. Talent placing the flask on a shaking incubator~~.~~ **TXT: Start culture from OD_{600} : 0.1; Continue until culture reaches 0.4** **NOTE:** They separated 4.2.1 into two steps, but just show what is described in the shot

4.2.2. Talent adding IPTG to the flask.

Added shot: Talent returning flask to the incubator.

4.3. As a control, compare *E. coli* BL21 (DE3) strains with and without NgTET overexpression **[1-TXT]**. Transfer 300 microliters of the *E. coli* cultures into a sterile tube **[2]**.

4.3.1. Talent labeling two sets of cultures for comparison and preparing for downstream plaque analysis. **TXT: In the absence of NgTET, a higher number of plaque-forming units are expected** **NOTE**: 4.3.1 and 4.3.2 were combined

4.3.2. Talent pipetting the bacterial culture into a sterile microcentrifuge tube.

4.4. Infect the culture with either T4 wild-type or NgTET-treated phage at a multiplicity of

infection of 0.01 **[1]**. Mix gently to ensure even phage distribution then incubate again **[2-TXT]**.

4.4.1. Talent adding phage solution into the tube with *E. coli*.

4.4.2. Talent gently flicking or pipetting the tube to mix the contents. **TXT: 37 °C, 7 min**

4.5. Add the bacteria-phage mixture to 4 milliliters of LB soft agar containing 0.75 percent agar and antibiotics **[1]**. Mix thoroughly but gently to avoid bubble formation **[2]**.

4.5.1. Talent pipetting the mixture into molten LB soft agar tube. **NOTE**: 4.5.1 and 4.5.2 are in one clip as well.

4.5.2. Talent gently inverting or swirling the tube to mix.

4.6. Pour the soft agar mixture onto a pre-warmed LB agar plate **[1]**. Let the plate solidify briefly at room temperature **[2]** and incubate overnight at 37 degrees Celsius **[3]**.

4.6.1. Talent pouring the soft agar evenly over the LB agar plate.

4.6.2. Plate resting on the bench while the agar solidifies.

4.6.3. Talent placing the plate in a 37 degrees Celsius incubator.

4.7. The next day, count the plaques to determine the number of plaque-forming units **[1]**.

4.7.1. Shot of agar plate showing distinct plaques.

4.8. For counter-selection, infect *E.coli* Cas13a spacer using the phages to be counter-selected **[1-TXT]**.

4.8.1. Talent pipetting phage solution into *E. coli* Cas13a_spacer culture. **TXT: This facilitates RNA degradation in phages without desired mutation**

4.9. Perform the counter-selection under the same conditions used for mutagenesis **[1-TXT]**. ~~Include *E. coli* with a non-targeting Cas13a spacer as a negative control to confirm spacer-specific plaque reduction **[2]**~~.

4.9.1. Talent replicating infection and incubation conditions used in prior mutagenesis steps. **TXT: Negative control: *E. coli* with a non-targeting Cas13a spacer**

4.9.2. ~~Talent labeling and preparing control cultures with non-targeting spacers~~.

4.10. After incubation, filter the supernatant through a 0.45-micrometer filter to remove bacterial debris **[1]**. Use the counter-selected and filtered phages for a plaque assay on an *E. coli* B strain **[2]** to isolate individual plaques for downstream validation **[3]**. **NOTE**: VO adjusted

4.10.1. Talent using a vacuum setup to filter the culture supernatant.

4.10.2. Talent pipetting the filtered phage preparation onto *E. coli* for initiating infection for plaque assay.

Added shot: E. coli/ phage mix is pipetted onto soft agar overlay for plaque assay.

Results

5. Results

5.1. The phage band was clearly visible when the T4 phage sample was sufficiently concentrated **[1].** Expression of NgTET protein in *E. coli* was confirmed by SDS-PAGE, showing its presence in both soluble and insoluble fractions following cell lysis and centrifugation **[2].**

5.1.1. LAB MEDIA: Figure 3. *Video editor: Highlight the phage band at the label "T4 phage fraction (10^{11} PFU/ml)"*

5.1.2. LAB MEDIA: Figure 4. *Video editor: Highlight the row of bands labeled "NgTET"*

5.2. Ion chromatograms showed that the relative abundances of deoxyadenosine, deoxyguanosine, and thymidine were unchanged between wild-type and NgTET-treated T4 phage DNA **[1].** The abundance of modified cytosine derivatives was significantly reduced in the NgTET-treated phage DNA compared to the untreated sample **[2].**

5.2.1. LAB MEDIA: Figure 5A. Video editor: Highlight the peaks labeled dA, dG, and dT in both chromatograms

5.2.2. LAB MEDIA: Figure 5B . *Video editor: Highlight the peaks labeled 5hmdC, 5fdC, and 5cadC in the NgTET-treated chromatogram*

5.3. A plasmid containing the NgTET gene and homology regions for targeted mutagenesis was constructed using Golden Gate cloning **[1].**

5.3.1. LAB MEDIA: Figure 6A.

5.4. PCR screening confirmed successful amplification of the target gene modA in lanes 2 through 9 of the agarose gel, indicating positive hits among the picked plaques **[1].**

5.4.1. LAB MEDIA: Figure 7. *Video editor: Highlight the lanes labeled Plaque 2 to Plasque 9 showing visible bands at the expected size for modA*

5.5. In wild-type T4 phage DNA, the relative abundance of 5-glycosylhydroxymethyl-2'-deoxycytidine was high **[1].** In NgTET-treated T4 phage DNA, levels of 5-glycosylhydroxymethyl-2'-deoxycytidine were strongly reduced compared to wild-type **[2].**

5.5.1. LAB MEDIA: Figure 8A. *Video editor: Highlight the pink bar for 5ghmdC*

5.5.2. LAB MEDIA: Figure 8B. *Video editor: Highlight the pink bar for 5ghmdC*

5.6. T4 phage DNA treated with the catalytically inactive mutant NgTET D234A retained high levels of 5-glycosylhydroxymethyl-2'-deoxycytidine **[1].** Recovered T4 phage

progeny showed nearly restored levels of 5-glycosylhydroxymethyl-2'-deoxycytidine compared to wild-type **[2].**

5.6.1. LAB MEDIA: Figure 8C. *Video editor: Highlight the pink bar for 5ghmdC*

5.6.2. LAB MEDIA: Figure 8D. *Video editor: Highlight the pink bar for 5ghmdC*

1. DNase

Pronunciation link: https://www.merriam-webster.com/dictionary/dnase
IPA (American): /ˈdiːˌneɪs/
Phonetic spelling: dee-nays

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2. RNase

Pronunciation link: https://www.merriam-webster.com/dictionary/rnase
IPA (American): /ˈɑːrˌneɪs/
Phonetic spelling: ar-nays

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3. Ultracentrifuge

Pronunciation link: https://www.merriam-webster.com/dictionary/ultracentrifuge
IPA (American): /ˌʌltrəˈsɛntrəˌfjuːdʒ/
Phonetic spelling: uhl-truh-sen-truh-fyoohj

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4. Phenol / Chloroform / Isoamyl

- **Phenol**
  Pronunciation link: https://www.merriam-webster.com/dictionary/phenol
  IPA: /ˈfiːnɔl/
  Phonetic: fee-nol
- **Chloroform**
  Pronunciation link: https://www.merriam-webster.com/dictionary/chloroform

IPA: /ˈklɔːrəˌfɔrm/
Phonetic: klor-uh-form

- **Isoamyl**
  I did *not* find a confirmed entry at Merriam-Webster or Oxford specifically for "isoamyl," but "iso-" + "amyl" would be pronounced:
  IPA (approx): /ˌaɪsoʊˈæmɪl/
  Phonetic: eye-soh-am-il

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5. Centrifuge

Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːdʒ/
Phonetic: sen-truh-fyoohj

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6. Glycosylhydroxymethyl (as part of "5-glycosylhydroxymethyl-2′-deoxycytidine")

This is a long, compound technical chemical name. I could not find a single entry, but you can break it down:

- glycosyl → /ˈglaɪkəzɪl/ → gly-kuh-zil
- hydroxy → /ˌhaɪˈdrɒksi/ → hydroksee
- methyl → /ˈmɛθəl/ → meth-uhl

So "glycosylhydroxymethyl" in IPA might approximate:
IPA (approx): /ˈglaɪkəzɪlˌhaɪˈdrɒkˌsiˈmɛθəl/
Phonetic: gly-kuh-zil hy-drok-see meth-uhl