

11-16-2021

## Expression and purification of phage T7 ejection proteins for cryo-EM analysis


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### Recommended Citation

Swanson, Nicholas A.; Lokareddy, Ravi K; Li, Fenglin; Hou, Chun-Feng; Pavlenok, Mikhail; Niederweis, Michael; and Cingolani, Gino, "Expression and purification of phage T7 ejection proteins for cryo-EM analysis" (2021). *Department of Biochemistry and Molecular Biology Faculty Papers*. Paper 199.

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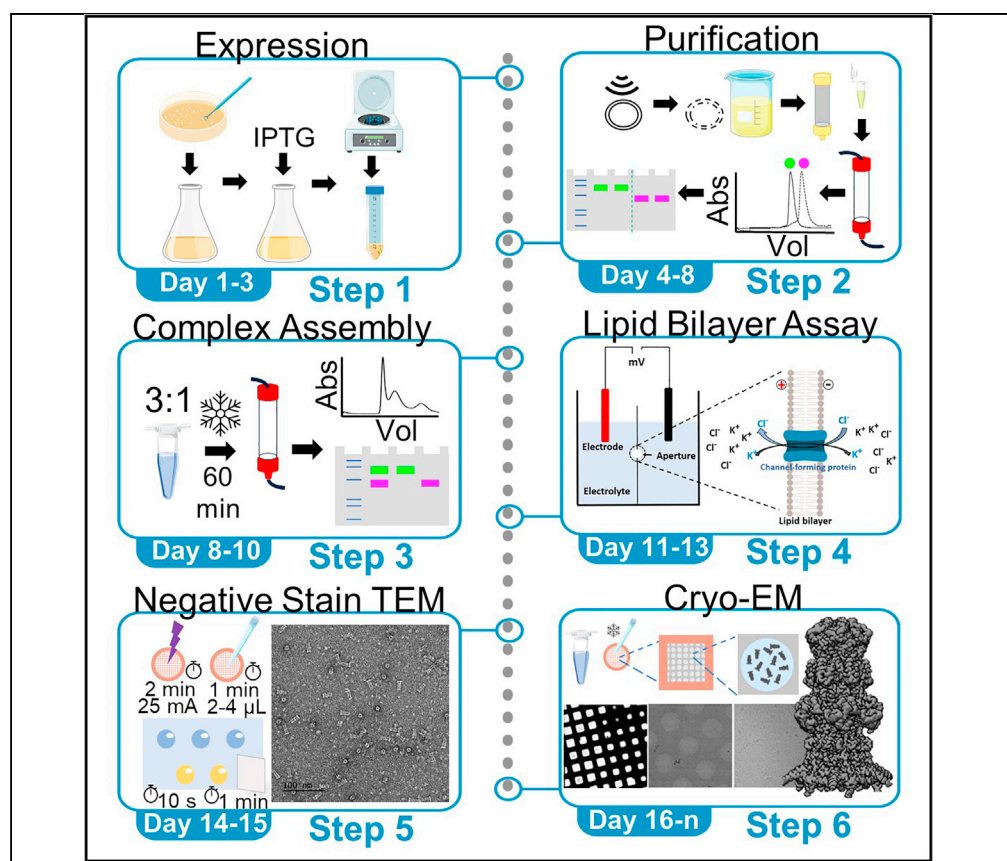
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## Authors

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## Protocol

# Expression and purification of phage T7 ejection proteins for cryo-EM analysis



Bacteriophages of the *Podoviridae* family densely package their genomes into precursor capsids alongside internal virion proteins called ejection proteins. In phage T7 these proteins (gp14, gp15, and gp16) are ejected into the host envelope forming a DNA-ejectosome for genome delivery. Here, we describe the purification and characterization of recombinant gp14, gp15, and gp16. This protocol was used for high-resolution cryo-EM structure analysis of the T7 periplasmic tunnel and can be adapted to study ejection proteins from other phages.

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### Highlights

Expression and  
purification of phage  
T7 ejection proteins  
in mg quantities

Reconstitution of  
gp15:gp16 DNA-  
ejectosome  
periplasmic tunnel *in*  
*vitro*

Pore formation assay  
reveals gp14 forms a  
constitutive pore

Vitrification of  
gp15:gp16 complex  
for cryo-EM single  
particle analysis

Swanson et al., STAR Protocols  
2, 100960  
December 17, 2021 © 2021  
The Author(s).  
<https://doi.org/10.1016/j.xpro.2021.100960>



## Protocol

## Expression and purification of phage T7 ejection proteins for cryo-EM analysis

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## SUMMARY

Bacteriophages of the *Podoviridae* family densely package their genomes into precursor capsids alongside internal virion proteins called ejection proteins. In phage T7 these proteins (gp14, gp15, and gp16) are ejected into the host envelope forming a DNA-ejectosome for genome delivery. Here, we describe the purification and characterization of recombinant gp14, gp15, and gp16. This protocol was used for high-resolution cryo-EM structure analysis of the T7 periplasmic tunnel and can be adapted to study ejection proteins from other phages.

For complete details on the use and execution of this protocol, please refer to Swanson et al. (2021).

## BEFORE YOU BEGIN

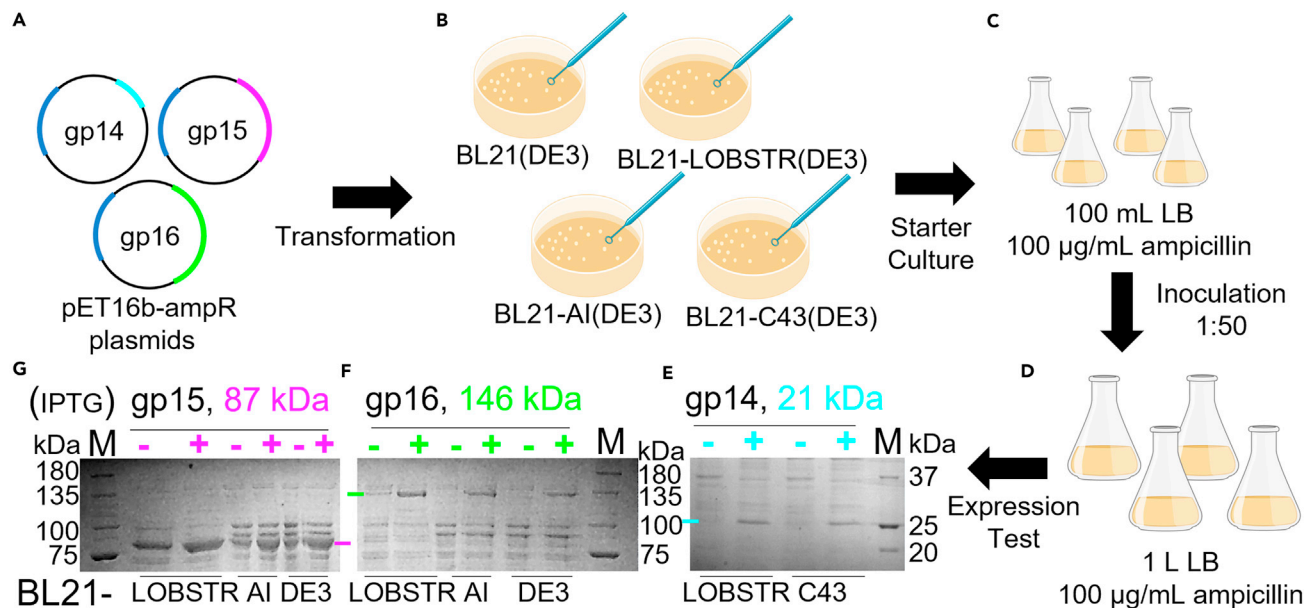
The protocol below outlines the detailed steps for biochemical and structural analysis of the internal virion proteins gp14, gp15 and gp16 from bacteriophage T7. First, we describe the specific steps used to optimize the recombinant expression of the ejection proteins in several expression hosts and relative purification strategies. We then describe biochemical reconstitution of the gp15:gp16 complex and the pore formation assay for gp14. Finally, we briefly illustrate the steps leading to single-particle cryo-EM analysis of the gp15:gp16 complex.

## Optimize expression of internal virion proteins

⌚ Timing: 3–5 days for expression tests, 3 days for full preparations

1. Verify plasmids (Figure 1A): Obtain the expression plasmids for full-length T7 gp14, gp15, and gp16 in pET-16b vector from Sebastian Leptihn (Lupo et al., 2015) and verify sequence fidelity. These plasmids produce 10× N-terminal histidine tagged (his-tagged) gp14, gp15, and gp16 with ampicillin resistance.
2. Transformation in expression strains (Figure 1B): Transform 1.2 μL of pET-16b\_gp14, pET-16b\_gp15, or pET-16b\_gp16 plasmid (~100 ng/μL) by electroporation into 90 μL BL21(DE3), BL21-LOBSTR(DE3), BL21-AI(DE3), and BL21-C43(DE3) lab-maintained *E. coli* competent cells following the manufacturers' protocols (BL21(DE3), BL21-LOBSTR(DE3), BL21-AI(DE3), and BL21-C43(DE3)) and spread around 20–100 μL cells onto an LB agar plate containing 100 ug/mL ampicillin. Incubate the plate in a 37°C incubator for 12–16 h.





**Figure 1. Expression tests for ejection proteins gp14, gp15, and gp16**

(A) Diagram of plasmids for gp14, gp15, and gp16.  
(B) Diagram of transformed colonies in different expression strains growing on agar plates treated with ampicillin (100 µg/mL).  
(C) Diagram of 100 mL starter cultures treated with ampicillin for different expression strains in 250 mL Erlenmeyer flasks.  
(D) Diagram of 1 L LB cultures treated with ampicillin and inoculated 1:50 with starter culture for different expression strains.  
(E–G) SDS-PAGE analysis for gp14, gp15, and gp16 before/after (–/+) induction with IPTG at OD<sub>600</sub> = 0.6 and expression. Expected molecular weights are gp14, 21 kDa, gp15, 87 kDa and gp16, 146 kDa as indicated on the SDS-PAGE gels in cyan, magenta, and green, respectively.

**Note:** Store plates at 4°C for less than 7 days for best growth and expression rates.

3. Starter culture (Figure 1C): Transfer a single colony from the LB agar plate to 100 mL LB with 100 µg/mL ampicillin and incubate the starter culture for 12–16 h or until optical density at 600 nm (OD<sub>600</sub>) is greater than 1 at 37°C rotating ~1 rcf.
4. Induce cell cultures (Figure 1D): Inoculate 1:50 in 1 L LB from the starter culture and incubate at 37°C rotating ~1 rcf until optical density at 600 nm (OD<sub>600</sub>) reaches ~0.6 and collect a 1 mL pre-induction sample. Induce with 0.1–0.5 mM IPTG (add 0.2% w/v arabinose for BL21-AI cells) and incubate for 2 h at 37°C rotating ~1 rcf.
5. Harvest cells: Collect 1 mL post-induction sample and measure OD<sub>600</sub>. Harvest cultures by centrifugation for 30 min at 4°C, 4,000 rcf.

**Pause point:** Cell pellets can be transferred to smaller storage vessels (ex. 15 mL or 50 mL conical tubes) by gently resuspending with a minimal amount (few mL per pellets from 1–2 L LB) of Lysis buffer followed by centrifugation 30 min at 4°C, 4,000 rcf. Flash-freeze pellets in liquid nitrogen (LN<sub>2</sub>) and store at -80°C until purification.

6. Expression levels (Figures 1E–1G): Compare internal virion protein (gp14, gp15, and gp16) expression levels by running pre-/post-induction samples normalized for equivalent cell amounts by running a lab-prepared sodium dodecyl sulfate (SDS) polyacrylamide gel with molecular weight markers. To normalize the cell amounts in 40 µL samples, use the following equation:

$$0.6_{OD600pre} \times 40 \mu L_{pre} = 1.1_{OD600post} \times X \mu L_{post}$$

Solve and pipette the X µL volume of post-induction sample into a microcentrifuge tube, then pipette LB until final volume equals 40 µL.

- a. SDS-PAGE Loading: Add 40  $\mu$ L samples to 10  $\mu$ L of 5 $\times$  loading dye and boil for 5 min at 95°C. Load 10–20  $\mu$ L of each sample and one lane of protein standards (Bio-Rad) in 10-well or 15-well 13.7% or 18% acrylamide/Tris/APS/TEMED electrophoresis gels.
- b. SDS-PAGE Running: Load gels in an electrophoresis chamber and run at 100 V at RT (4°C for higher-quality) in SDS-PAGE running buffer until dye front passes the bottom of the gel.
- c. SDS-PAGE Staining: Remove gels from casing and stain with SDS-PAGE gel stain by gentle agitation on a rocker. De-stain in SDS-PAGE gel de-stain by gentle agitation until the bands are clear and background has been removed.
- d. SDS-PAGE Analysis: Image the gels on an illuminated white background. Identify bands corresponding to proteins of interest by comparing to the standards lane and quantify band intensity. All other SDS-PAGE experiments in this protocol are completed the same way.

**Note:** Expected molecular weights for the N-terminal 10 $\times$  His-tagged T7 internal virion proteins are as follows: gp14, 21 kDa; gp15, 87 kDa; gp16, 146 kDa.

7. Strain selection: Select expression strains that show little to no expression of the desired internal virion proteins for the pre-induction sample and thick protein bands that appear in the post-induction sample.

**Note:** BL21-C43(DE3) strain, known for reducing expression-induced toxicity, was selected for gp14 which is an outer membrane protein that is insoluble without the presence of detergents or lipid membranes.

**Note:** BL21(DE3) and BL21-LOBSTR(DE3) cells produced similar expression levels and folded protein for gp15 and gp16, however BL21-LOBSTR(DE3) had reduced background expression and allowed for cleaner purifications. BL21-LOBSTR(DE3) is ideal for proteins with poor expression levels and reduces contamination of *E. coli* SlyD and ArnA.

**Note:** BL21-AI(DE3) cells had reduced, but more reproducible expression than BL21(DE3) and BL21-LOBSTR(DE3) for gp15 and gp16. We recommend BL21-AI(DE3) as an alternative if expression is lost in BL21(DE3) and BL21-LOBSTR(DE3).

8. Full-Preparations: Complete steps 3–5 with 6 L LB each with from single colonies of the optimized expression strain. For gp15 and gp16, proceed to *before you begin*: [purification of soluble ejection proteins T7 gp15 and gp16](#). For gp14, proceed to *before you begin*: [purification of insoluble ejection protein T7 gp14](#).

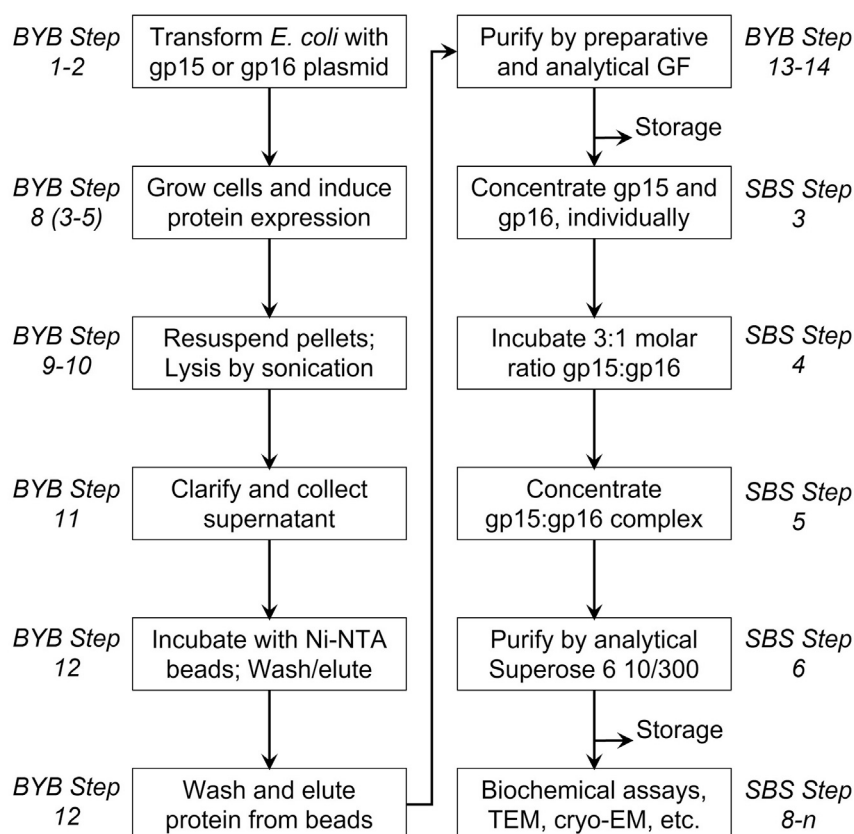
**Note:** Glycerol stocks from the initial expression tests may be a valid approach to reduce prep time, though we have not tested this for each ejection protein and prefer fresh transformations.

### Purification of soluble ejection proteins gp15 and gp16

⌚ Timing: 2–3 days each for large-scale purification

**Note:** Refer to the workflow in [Figure 2](#) for an overview connecting the following sections: *before you begin*: [optimize expression of internal virion proteins](#), *before you begin*: [purification of soluble ejection proteins gp15 and gp16](#), *step-by-step*: [biochemical reconstitution of DNA ejectosome components](#), and *step-by-step*: [sample preparation and screening for Cryo-EM studies](#).

9. Resuspension: Thaw cell pellets for the selected expression strains (BL21(DE3) and BL21-LOBSTR(DE3) are optimal for gp15 and gp16 expression) on ice and resuspend in 25 mL of chilled



**Figure 2. Workflow for the reconstitution of gp15:gp16 periplasmic tunnel of the T7 DNA-ejectosome.**

Lysis buffer-I per 1 L LB culture pellet (ex. If the pellet was originally from 2 L LB, resuspend the pellet in 50 mL buffer).

**Note:** Use gentle agitation and swirling motions to resuspend larger cell clumps.

10. Cell lysis (Figure 3A): Lyse the resuspended cells by sonication at RT using a standard  $\frac{1}{2}$ " horn (recommend: 35% amplitude, process time 1:15 min:sec, pulse-on time 25 s, and pulse-off time 25 s).

**Note:** Follow the manufacturer's protocol for the volume of cells that can be sonicated at a time.

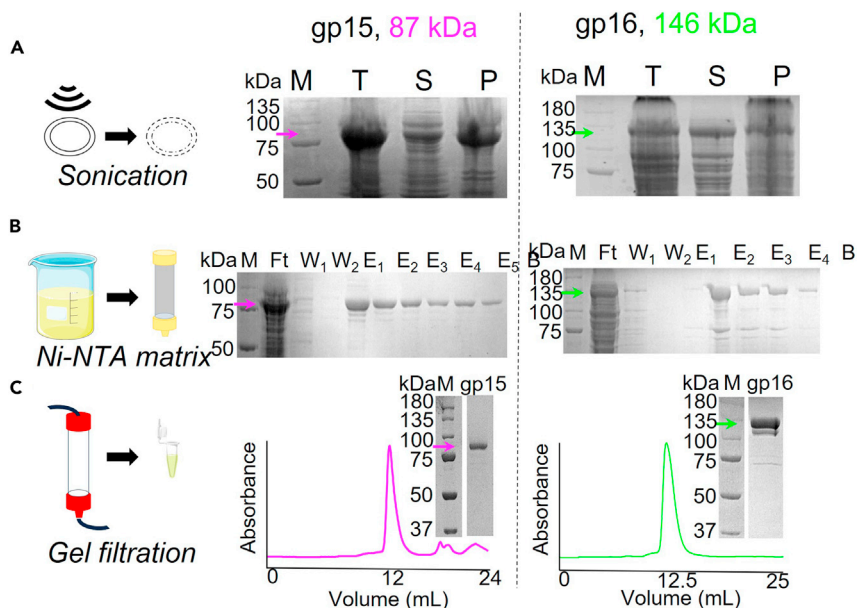
**Alternatives:** A French pressure cell press can be used in replacement of a sonicator.

11. Clarification (Figure 3A): Immediately following lysis, incubate on ice for 1 h with 10  $\mu$ L PMSF stock (100 mM), 1  $\mu$ L DNase I (1 mg/mL), 5  $\mu$ L  $MgCl_2$  (1 M), and 5  $\mu$ L RNase A (10 mg/mL) per 1 mL of cell suspension. Clarify the cell suspension by centrifugation at 4°C, 38,465 rcf for 30 min and collect the supernatant.

**Note:** During this step collect a "total" lysate sample post-sonication and "supernatant" and "pellet" samples post-centrifugation to be analyzed by SDS-PAGE to determine protein localization and recovery.

12. Ni-NTA Purification (Figure 3B): Incubate the recovered supernatant with Ni-NTA agarose low density beads (binding capacity >12 mg/mL for ~60 kDa proteins) for 2 h rotating at 4°C.





**Figure 3. Purification of T7 ejection proteins gp15 and gp16**

(A) SDS-PAGE results for sonicated samples for gp15 and gp16. Abbreviations: M = Markers for molecular weight, T = Total sample post-sonication, S = Supernatant sample, and P = Pellet sample after centrifugation. (B) SDS-PAGE results for Ni-NTA purification for gp15 and gp16. Abbreviations: Ft = Flow-through sample that passed through the gravity-flow column without binding, W<sub>1</sub> and W<sub>2</sub> = Wash 1 and 2 eluent samples, E<sub>n</sub> = Elution samples, and B = Beads sample representing protein still bound to the Ni beads. (C) Chromatograms and SDS-PAGE results for gel filtration purified gp15 and gp16 on a calibrated analytical Superdex200 10/300 column. Only relevant lanes from the same gel are shown. Parts of this figure have been reprinted from Molecular Cell, Vol 81, Issue 15, Swanson, et al., Cryo-EM structure of the periplasmic tunnel of T7 DNA-ejectosome at 2.7 Å resolution, Pages 3145-3159.e7, Copyright (2021), with permission from Elsevier.

Load the incubated beads on a gravity flow column and wash with 10 column volumes (CV) of Ni-wash buffer-I with 10 mM imidazole (wash-I) and 10 CV of Ni-wash-I with 50 mM imidazole (wash-II). Elute protein from Ni beads with 10–20 mL Ni-elution buffer-I in 1–2 mL increments with 1 min incubation periods between successive elution steps.

**Note:** Collect samples and run an SDS-PAGE for the “flow-through,” “wash-I,” “wash-II,” “elution” fractions, and “beads” to check eluted protein purity and recovery.

**Note:** For problems that may arise during this procedure please refer to troubleshooting [problems 1](#) and [2](#) below.

13. Preparative gel filtration ([Figure 3C](#)): Select, pool, and centrifuge the protein-containing fractions for 10 min at 4°C, 10,000 rcf. Inject up to 5 mL on a calibrated preparative Superdex 200 16/60 size exclusion column with GF buffer-I.

**Note:** Collect samples and run an SDS-PAGE for the “injection” and eluant peak fractions to check protein quantity, purity, and approximate size in kDa.

**Note:** Recommended calibration markers are MWGF1000 Gel Filtration Markers Kit (Sigma-Aldrich) for protein molecular weights 29,000–700,000 Da. The kit’s components are Carbonic anhydrase 0 bovine erythrocytes (29 kDa), Albumin - bovine serum (66 kDa), Alcohol dehydrogenase – yeast (150 kDa), Beta-Amylase – sweet potato (200 kDa), Apoferritin – horse spleen (443 kDa), Thyroglobulin – bovine (669 kDa), and Blue dextran (2 MDa).



14. Analytical gel filtration ([Figure 3C](#)): Select, pool, and centrifuge the gel-filtrated protein-containing fraction for 10 min at 4°C, 10,000 rcf and further polish by injecting up to 0.5 mL on a calibrated analytical Superdex 200 10/300.

**Note:** Collect samples and run an SDS-PAGE for the “injection” and eluant peak fractions to check protein quantity, purity, and approximate size in kDa. Expected quantities of gp15 and gp16 per L of LB are ~0.5–2.5 mg purified protein.

**Note:** Recommended calibration markers are MWGF1000 Gel Filtration Markers Kit (Sigma-Aldrich) for protein molecular weights 29,000–700,000 Da. The kit’s components are Carbonic anhydrase 0 bovine erythrocytes (29 kDa), Albumin - bovine serum (66 kDa), Alcohol dehydrogenase – yeast (150 kDa), Beta-Amylase – sweet potato (200 kDa), Apoferritin – horse spleen (443 kDa), Thyroglobulin – bovine (669 kDa), and Blue dextran (2 MDa).

15. Storage: T7 gp15, gp16, and other soluble ejection proteins are often prone to degradation. They should be used for biochemical analyses within five days from purification and always kept at 4°C or on ice.

**Note:** For problems that may arise during this procedure please refer to troubleshooting [problem 3](#) below.

**Alternatives:** Flash freezing in liquid nitrogen and storage at -80°C for soluble ejection proteins is possible but we do not recommend it for T7 gp15 and gp16.

### Purification of insoluble ejection protein T7 gp14

⌚ **Timing:** 2 days for detergent screening, 3–4 days for large-scale purification

**Note:** Refer to the workflow in [Figure 5](#) or an overview connecting the following sections: *before you begin:* [optimize expression of internal virion proteins](#), *before you begin:* [purification of insoluble ejection protein T7 gp14](#), *step-by-step:* [lipid bilayer experiments](#), and *step-by-step:* [sample preparation and screening for CryoEM studies](#).

16. Resuspension: Thaw cell pellets for the selected expression strains (BL21-C43(DE3) is optimal for gp14) on ice and resuspend in 25 mL of chilled [Lysis buffer-I](#) per 1 L LB culture pellet (ex. If the pellet was originally from 2 L LB, resuspend the pellet in 50 mL buffer).

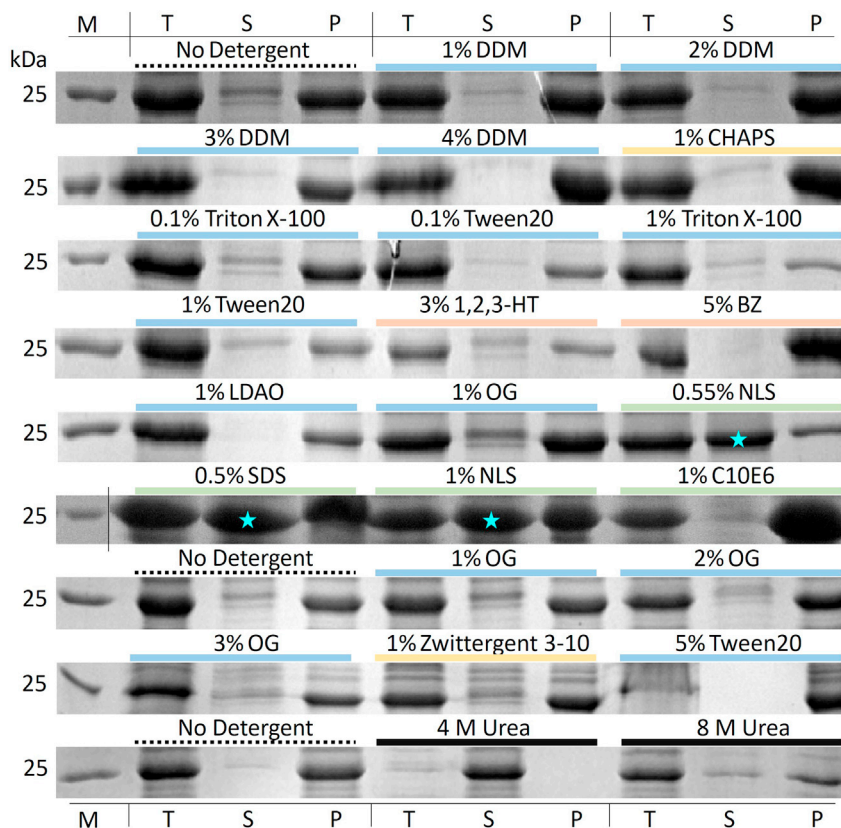
**Note:** Use gentle agitation and swirling motions to resuspend larger cell clumps.

17. Cell lysis ([Figure 6A](#)): Lyse the resuspended cells by sonication at RT using a standard 1/2” horn (recommend: 35% amplitude, process time 1:15 min:sec, pulse-on time 25 s, and pulse-off time 25 s).

**Note:** Follow the manufacturer’s protocol for the volume of cells that can be sonicated at a time.

**Alternatives:** A French pressure cell press can be used in replacement of a sonicator to achieve gentler cell lysis.

18. Clarification ([Figure 6A](#)): Immediately following lysis, incubate on ice for 1 h with 10 µL PMSF stock (100 mM), 1 µL DNase I (1 mg/mL), 5 µL MgCl<sub>2</sub> (1 M), and 5 µL RNase A (10 mg/mL) per 1 mL of cell suspension. The cell suspension was clarified by ultracentrifugation at 4°C, 200,000 rcf for 45 min.

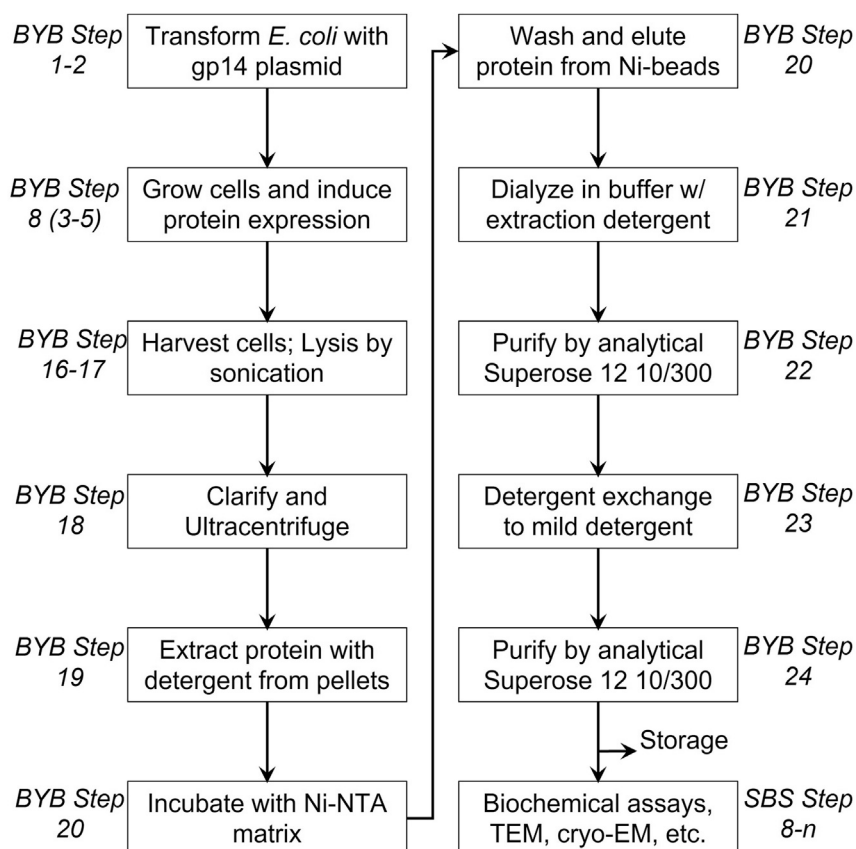


**Figure 4. Detergent screen for insoluble ejection protein gp14**

Dashed underlines: Samples treated without detergent (No Detergent). Blue underlines: samples treated with non-ionic detergents including n-Dodecyl  $\beta$ -D-maltoside (DDM), Octyl- $\beta$ -D-Glucopyranoside (OG), Lauryldimethylamine oxide (LDAO), Triton X-100, and Tween-20. Yellow underlines: samples treated with zwitterionic detergents including CHAPS and Zwittergent 3-10. Green underlines: samples treated with anionic detergents including sodium dodecyl sulfate (SDS) and N-Lauroylsulfate (NLS), and Hexaethylene glycol monododecyl ether (C10E6). Orange underlines: samples treated with amphiphiles including 1,2,3-Heptanetriol (1,2,3-HT) and Benzamidinium-HCl (BZ). Black underlines: samples treated with Urea. Abbreviations: M = Markers for molecular weight, T = Total lysate sample post-sonication, S = Supernatant sample post-centrifugation, P = Pellet sample post-centrifugation. Blue Stars: represent solubilized gp14 protein in NLS or SDS. Each row represents one SDS-PAGE gel displaying only the relevant gp14 bands. Row 6 was manipulated to remove the irrelevant lanes between the marker and samples. Parts of this figure have been reprinted from Molecular Cell, Vol 81, Issue 15, Swanson, et al., Cryo-EM structure of the periplasmic tunnel of T7 DNA-ejectosome at 2.7 Å resolution, Pages 3145-3159.e7, Copyright (2021), with permission from Elsevier.

**Note:** During this step collect a “total” lysate sample post-sonication and “supernatant” and “pellet” samples post-centrifugation to analyzed by SDS-PAGE to determine protein localization and recovery.

19. Extraction (Figure 6A): Resuspend the pellets post-ultracentrifugation in 10 mL Extraction buffer-I containing the optimal extraction detergent from screening (see sub-steps below; we used 0.8 % w/v N-Lauroylsarcosine (NLS)). Allow solubilization by rotating samples overnight (12–16 h) at 4°C, then centrifuge at 4°C, 38,465 rcf for 30 min to collect detergent-solubilized protein in the supernatant. Check extraction efficiency on SDS-PAGE with “total”, “supernatant”, and “pellet” fractions.
  - a. Extraction detergent screen (Figure 4): Treat pellet fractions containing T7 gp14 (or other insoluble ejection proteins) with a screen of non-ionic, zwitterionic, amphiphiles, and anionic surfactants above the critical micelle concentration (CMC) dissolved in 10 mL Extraction



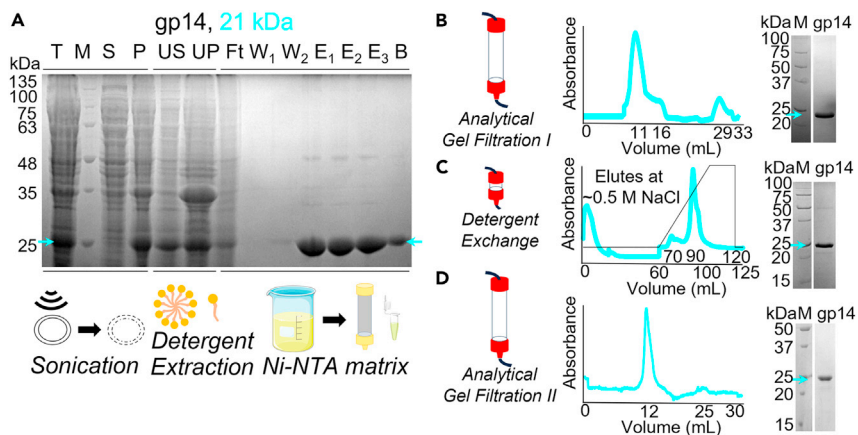
**Figure 5. Workflow for purifying gp14 that forms the outer membrane pore of the T7 DNA-ejectosome.**

buffer-I. Include a “no detergent” control sample as well. Allow solubilization by rotating samples overnight (12–16 h) at 4°C. Recommended detergents:

- i. Non-ionic detergents: n-Dodecyl  $\beta$ -D-maltoside (DDM), Octyl- $\beta$ -D-Glucopyranoside (OG), Lauryldimethylamine oxide (LDAO), Triton X-100, Tween-20
  - ii. Zwitterionic detergents: CHAPS, Zwittergent 3–10
  - iii. Amphiphiles: 1,2,3-Heptanetriol (1,2,3-HT), Benzamidinium-HCl (BZ)
  - iv. Anionic detergents: sodium dodecyl sulfate (SDS), N-Lauroylsarcosine (NLS), hexaethylene glycol monodecyl ether (C10E6)
  - v. Other solubilization reagents: Urea
- b. Extraction detergent selection: Take a “total” solubilized sample, then centrifuge remaining samples at 4°C, 38,465 rcf for 30 min to remove membrane debris in the pellet and retain detergent-solubilized protein in the supernatant. Collect “supernatant” and “pellet” samples after centrifugation and run an SDS-PAGE gel, alongside a molecular weight marker, comparing “total”, “supernatant”, and “pellet” samples for each detergent and the no detergent control.

**Note:** Protein bands that appear in the supernatant fraction for your protein of interest have been successfully solubilized by the detergent. Always compare to the “No Detergent” control for background bands from *E. coli* proteins.

**Note:** Solubilization efficiency can be estimated by dividing the band intensities for the detergent solubilized lane by the total sample lane.



**Figure 6. Purification of T7 ejection protein gp14**

(A) SDS-PAGE results for sonicated, NLS detergent extracted, and Ni-NTA purified samples of gp14. Abbreviations: M = Markers for molecular weight, T = Total sample post-sonication, S = Supernatant sample, and P = Pellet sample after centrifugation, US = Ultracentrifuged Supernatant sample, UP = Ultracentrifuged Pellet sample, Ft = Flow-through sample that passed through the gravity-flow column without binding, W<sub>1</sub> and W<sub>2</sub> = Wash 1 and 2 eluent samples, E<sub>n</sub> = Elution samples, and B = Beads sample representing protein still bound to the Ni beads. (B) Chromatogram and SDS-PAGE results for gel filtration purified gp14 solubilized in NLS (extraction) detergent. (C) Chromatogram and SDS-PAGE results for gp14 detergent exchange from NLS to DDM detergent using an anion exchange column. (D) Chromatogram and SDS-PAGE results for gel filtration purified gp15 solubilized in DDM (mild) detergent. Only relevant lanes from the same gel are shown in (B–D). Parts of this figure have been reprinted from Molecular Cell, Vol 81, Issue 15, Swanson, et al., Cryo-EM structure of the periplasmic tunnel of T7 DNA-ejectosome at 2.7 Å resolution, Pages 3145-3159.e7, Copyright (2021), with permission from Elsevier.

20. Ni-NTA Purification (Figure 6A): Incubate the recovered supernatant with Ni-NTA agarose low density beads (binding capacity >12 mg/mL for ~60 kDa proteins) for 2 h rotating at 4°C. Load the incubated beads on a gravity flow column and wash with 10 column volumes (CV) of Extraction buffer-II with 20 mM imidazole, followed by another 10 CV of Extraction buffer-II with 50 mM imidazole. Elute protein from Ni beads with 10–20 mL Ni-elution buffer-II in 1–2 mL increments with 1 min incubation periods between successive elution steps.

**Note:** Collect samples and run an SDS-PAGE for the “flow-through,” “wash-I,” “wash-II,” “elution” fractions, and “beads” to check eluted protein purity and recovery.

**Note:** For problems that may arise during this procedure please refer to troubleshooting [problems 1](#) and [2](#) below.

21. Dialysis: Pool and transfer eluted protein-containing fractions in dialysis tubing with a molecular weight cut-off (MWCO) value smaller than the MW of the protein of interest (ex. 10k MWCO for 21 kDa T7 gp14). Gently agitate overnight (12–16 h) at 4°C in 3 L Dialysis buffer containing the same extraction detergent used above at CMC (ex. 0.8 % NLS).
22. Analytical gel filtration I (Figure 6B): Collect dialyzed sample and inject on a calibrated analytical Superose12 10/300 column containing fresh Dialysis buffer.

**Note:** Check eluted fractions on SDS-PAGE.

**Note:** Recommended calibration markers are MWGF1000 Gel Filtration Markers Kit (Sigma-Aldrich) for protein molecular weights 29,000–700,000 Da. The kit’s components are Carbonic anhydrase 0 bovine erythrocytes (29 kDa), Albumin - bovine serum (66 kDa), Alcohol

dehydrogenase – yeast (150 kDa), Beta-Amylase – sweet potato (200 kDa), Apoferritin – horse spleen (443 kDa), Thyroglobulin – bovine (669 kDa), and Blue dextran (2 MDa).

23. Detergent exchange (Figure 6B): Pool eluted NLS-solubilized gp14 (or other anionic extraction detergent-solubilized protein) and exchange to a mild detergent at or above CMC for biochemical assays and reconstitution (ex. 0.25% N-Dodecyl  $\beta$ -D-maltoside (DDM)). Using ion-exchange affinity chromatography on a 5 mL Mono Q 5/50 GL column, inject and wash with at least 50 mL ion-exchange buffer-I containing 25 mM NaCl and elute with a gradient across 40 mL to ion-exchange buffer-II containing 1 M NaCl and 0.25% DDM (instead of NLS).

**Note:** For problems that may arise during this procedure please refer to troubleshooting problems 4, 5, and 6 below.

⚠ **CRITICAL:** NLS extraction detergent needs to be thoroughly removed for the following biochemical assays and structural studies. (Ex. After insufficient detergent exchange, residual NLS has contaminated our crystallization efforts. Additionally, NLS is incompatible with the Lipid Bilayer Measurements described below).

24. Analytical gel filtration II (Figure 6C): Select, pool, and centrifuge the protein-containing fractions for 10 min at 4°C, 10,000 rcf. Inject on a calibrated analytical Superose12 10/300 size exclusion column with DDM buffer.

**Note:** Collect samples and run an SDS-PAGE for the “injection” and eluant peak fractions to check protein quantity, purity, and approximate size in kDa.

**Note:** Recommended calibration markers are MWGF1000 Gel Filtration Markers Kit (Sigma-Aldrich) for protein molecular weights 29,000–700,000 Da. The kit’s components are Carbonic anhydrase 0 bovine erythrocytes (29 kDa), Albumin - bovine serum (66 kDa), Alcohol dehydrogenase – yeast (150 kDa), Beta-Amylase – sweet potato (200 kDa), Apoferritin – horse spleen (443 kDa), Thyroglobulin – bovine (669 kDa), and Blue dextran (2 MDa).

25. Storage: T7 gp14, and other detergent-solubilized ejection proteins, in our hands, are less prone to degradation due to the detergent micelles. However, we recommend they should be used for biochemical analyses within ten days from purification and kept at 4°C or on ice.

### Prepare lipid solution and measurement cuvette for lipid bilayer experiments

⌚ **Timing:** 1–2 h for lipid solutions preparations, 1–2 days for cleaning the cuvette

26. Prepare lipid solutions: For most measurements we use 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DphPC) available in powder form (Avanti Polar Lipids).

**Note:** Lipids in chloroform are available from Avanti as well. Calculate the concentrations accordingly.

- a. Priming lipid solution is used to pre-treat the custom-made Teflon cuvette before membrane painting. Make 2% DphPC (w/v) in chloroform: weigh out 8 mg of DphPC and dissolve in 400  $\mu$ L of chloroform.
- b. Painting lipid solution: 1% DphPC (w/v) in n-decane. Weigh out 4 mg of DphPC and dissolve in 200  $\mu$ L of chloroform. Use argon to evaporate the chloroform. Once lipid film is formed dissolve it in 400  $\mu$ L of n-decane.

**Note:** To avoid chloroform toxicity work in the fume hood when preparing lipid solutions. Always use glass vials to prepare and store lipid solutions. Aliquot both lipid solutions (100  $\mu$ l) using a Hamilton syringe in separate glass vials under the argon to prevent oxidation. Lipid solutions can be stored at -20 C for at least a month.

27. Clean the measuring cuvette and Teflon loop: Soak the cuvette and the loop in cleaning solution (5.5% (v/v) HCl, 4.2 % (v/v) H<sub>2</sub>O<sub>2</sub>) overnight (12–16 h). Rinse with water, ethanol, and water. Routinely, when not in use, the cuvette and the loop are stored in ethanol (~90% v/v).

**Note:** The measuring cuvette is custom-made from a Teflon block. The measuring cuvette has a glass pane at the front (cis side) for observing the membrane and a transverse wall in the middle, which separates two compartments of the same size from one another. The partition wall is slightly inclined so that when the membrane is illuminated, the light is not reflected back frontally. In the middle of the partition is a very thin-edged aperture. We use apertures with a diameter of 0.8 mm and 1.1 mm with the area of the opening of 0.5 mm<sup>2</sup> and 0.95 mm<sup>2</sup>, respectively. The Teflon loop is used to paint the lipid membrane across the cuvette's aperture. It consists of a 10 cm long, triangularly shaped Teflon-coated copper wire at the lower end, and an additional Teflon tube which serves as a handle. Outside and during the measurement, the Teflon loop can be stored in a beaker with ethanol.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
<i>E. coli</i> BL21 (DE3) Competent Cells	NOVAGEN	CAT#69450-3
<i>E. coli</i> BL21-LOBSTR(DE3) Competent Cells	Kerafast	CAT#EC1002
<i>E. coli</i> BL21-AI(DE3) Competent Cells	Invitrogen	CAT#C6070-03
<i>E. coli</i> OverExpress C43(DE3) Chemically Competent Cells	Lucigen	CAT#60446-1
<b>Chemicals, peptides, and recombinant proteins</b>		
n-Dodecyl-B-D-maltoside (DDM)	GoldBio	CAT#DDM25
N-Lauroylsarcosine sodium BioUltra, for molecular biology, $\geq 99.0\%$ (HPLC)	Sigma-Aldrich	CAT#61743
Luria Broth medium	Fisher Scientific	CAT#BP97235
Ampicillin	Inalco Pharmaceuticals	CAT#1758-9314
HEPES	Sigma-Aldrich	CAT#90909C
Acetic acid	Fisher Scientific	CAT#64-19-7
Methanol	Sigma-Aldrich	CAT#322415
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	CAT#11667289001
$\beta$ -mercaptoethanol (BME)	Sigma-Aldrich	CAT#M6250
Sodium cholate	Sigma-Aldrich	CAT#C6445
Sodium citrate dihydrate	Sigma-Aldrich	CAT#W302600
Isopropyl b-D-1-thiogalactopyranoside (IPTG)	Fisher Scientific	CAT#BP1755-100
DNase I	Roche	CAT#10104159001
RNase A	Roche	CAT#10109169001
NDSB-201	Merck Millipore	CAT#480005
Phenylmethylsulfonyl fluoride (PMSF)	Roche	CAT#PMSF-RO
Benzamidine HCl	Sigma-Aldrich	CAT#B6506
Tris-HCl	Grainger	CAT#30UC36
Imidazole	ACROS Organics	CAT#AC39674500
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)	Sigma-Aldrich	CAT#C4706
Glycerol 99+%	Alfa Aesar	CAT#A16205
Brilliant Blue G-250	Fisher Bioreagents	CAT#BP100-50
1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) or 4ME 16:0 PC	Avanti Polar Lipids	CAT#850356P

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**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chloroform	Merck Millipore	CAT#C2432-1L
n-Decane	Sigma-Aldrich	CAT#8034050250
Carbon-coated 400-mesh copper grids	Electron Microscopy Sciences	CAT#CF400-CU
300-mesh Quantifoil R 1.2/1.3 holey carbon grids	Electron Microscopy Sciences	CAT#Q3100CR1.3
<b>Critical commercial assays</b>		
Superose 6 Increase 10/300 GL	GE Healthcare Life Sciences	CAT#29091596
Nickel Agarose Beads (Low Density)	GoldBio	CAT#H-321-100
Superdex 200 Increase 10/300 GL size exclusion column	GE Healthcare Life Sciences	CAT#28990944
Superose 12 10/300 GL	GE Healthcare Life Sciences	CAT#29036225
Gel Filtration Markers Kit for Protein Molecular Weights 29,000-700,000 Da	Sigma-Aldrich	CAT#MWGF1000
SnakeSkin™ Dialysis Tubing, 10K MWCO	Thermo Fisher Scientific	CAT#68100
Precision Plus Protein Unstained Standards	Bio-Rad Laboratories	CAT#1610396
Mono Q 5/50 GL	Sigma-Aldrich	CAT#54807
Vivaspin® 20, 100 kDa MWCO Polyethersulfone	Millipore-Sigma	CAT#GE28-9323-63
NativePAGE™ 4 to 16%, Bis-Tris, 1.0 mm, Mini Protein Gel, 10-well	Invitrogen	CAT#BN1002BOX
NativePAGE™ Running Buffer Kit	Invitrogen	CAT#BN2007
NativePAGE™ Sample Buffer (4×)	Invitrogen	CAT#BN2003
<b>Deposited data</b>		
Coordinates of the gp15:gp16-N Periplasmic Tunnel	(Swanson et al., 2021)	PDB:7K5C
Map of the gp15:gp16-N Periplasmic Tunnel	(Swanson et al., 2021)	EMD-22680
Original/source data for figures in the paper	(Swanson et al., 2021)	Mendeley Data: <a href="https://doi.org/10.17632/xf2w3yczz2.1">https://doi.org/10.17632/xf2w3yczz2.1</a>
<b>Recombinant DNA</b>		
pET-16b vector	Sigma-Aldrich	CAT#69662
pET-16b_gp14	(Lupo et al., 2015)	N/C
pET-16b_gp15	(Lupo et al., 2015)	N/C
pET-16b_gp16	(Lupo et al., 2015)	N/C
<b>Software and algorithms</b>		
Relion 3.1	(Zivanov et al., 2018); (Scheres, 2012)	<a href="http://www2.mrc-lmb.cam.ac.uk/relion">http://www2.mrc-lmb.cam.ac.uk/relion</a>
GCTF	(Zhang, 2016)	<a href="https://www2.mrc-lmb.cam.ac.uk/research/locally-developed-software/zhang-software/#gctf">https://www2.mrc-lmb.cam.ac.uk/research/locally-developed-software/zhang-software/#gctf</a>
MotionCorr2	(Zheng et al., 2017)	<a href="https://emcore.ucsf.edu/ucsf-software">https://emcore.ucsf.edu/ucsf-software</a>
ImageJ	(Schneider et al., 2012)	<a href="https://imagej.net/">https://imagej.net/</a>
IGOR Pro 5.03	WaveMetrics	<a href="https://www.wavemetrics.com/support/versions">https://www.wavemetrics.com/support/versions</a>
SigmaPlot 11.0	Systat Software	<a href="https://systatsoftware.com/products/sigmaplot/">https://systatsoftware.com/products/sigmaplot/</a>
<b>Other</b>		
MicroPulser Electroporator	Bio-Rad Laboratories	CAT#1652100
Incubator shaker	New Brunswick Scientific	CAT#M1282-0000
4 L Pyrex glass Erlenmeyer flasks	Fisher Scientific	CAT#10-040P
CO8000 Personal Cell Density Meter	Denville	CAT#1159Q31
Sorvall BP 8 centrifuge equipped with 8 × 1000 mL swinging bucket rotor	Thermo Fisher Scientific	CAT#75007681
S-4000 Ultrasonic Liquid Processor with a standard 1/2" horn	Misonix	Discontinued; Replaced by Q700 Sonicator CAT#Q700-110
Sorvall Lynx 4000 centrifuge	Thermo Fisher Scientific	CAT#75006580
F21-8x50y FiberLite rotor (r = 10.6 cm)	Thermo Fisher Scientific	CAT#096-084275
Eppendorf centrifuge 5415 R	Eppendorf	CAT#Z605212
F-45-24-11 rotor (r = 8.4 cm)	Millipore Sigma	CAT#RFA452411-EP
NGC 10 Medium-Pressure Chromatography System	Bio-Rad Laboratories	<a href="https://www.bio-rad.com/en-us/category/ngc-medium-pressure-liquid-chromatography-systems?ID=MFCV7415">https://www.bio-rad.com/en-us/category/ngc-medium-pressure-liquid-chromatography-systems?ID=MFCV7415</a>
Mini-PROTEAN Tetra Vertical Electrophoresis Cell	Bio-Rad Laboratories	CAT#1658004
Sorvall wX+ Ultra Series centrifuge	Thermo Fisher Scientific	CAT#75000100

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### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TH-641 swinging bucket rotor package	Thermo Fisher Scientific	CAT#54295
Keithley 428 current amplifier	Axiom Test Equipment	CAT#428-PROG
Autogrid C-clip rings	FEI	CAT#1036173
Autogrid C-clips	FEI	CAT#1036171
Custom Teflon cuvette	(Heinz and Niederweis, 2000)	See <a href="#">before you begin</a> step 27 Note, materials and equipment: Description of lipid bilayer apparatus, and <a href="#">Figure 8A</a> .
Hamilton syringe	Hamilton Company	CAT# 81217
Ag. AgCl/ 3M KCl Electrodes	Metrohm AG	CAT#6.0733.100
Glacios Cryo-TEM	Thermo Fisher Scientific	<a href="https://www.thermofisher.com/us/en/home/electron-microscopy/products/transmission-electron-microscopes/glacios-cryo-tem.html?SID=srch-srp-GLACIOSTEM">https://www.thermofisher.com/us/en/home/electron-microscopy/products/transmission-electron-microscopes/glacios-cryo-tem.html?SID=srch-srp-GLACIOSTEM</a>
FEI Titan Krios equipped with a Gatan K3 Camera	Thermo Fisher Scientific	Model not available; Replaced by Krios G4 Cryo-TEM <a href="https://www.thermofisher.com/us/en/home/electron-microscopy/products/transmission-electron-microscopes/krios-g4-cryo-tem.html">https://www.thermofisher.com/us/en/home/electron-microscopy/products/transmission-electron-microscopes/krios-g4-cryo-tem.html</a>
FEI Vitrobot Mark IV	Thermo Fisher Scientific	<a href="https://www.thermofisher.com/us/en/home/electron-microscopy/products/sample-preparation-equipment-em/vitrobot-system.html?SID=srch-srp-VITROBOT">https://www.thermofisher.com/us/en/home/electron-microscopy/products/sample-preparation-equipment-em/vitrobot-system.html?SID=srch-srp-VITROBOT</a>
FEI Tecnai T12 electron microscope equipped with a 4k x 4k OneView camera	FEI	Discontinued

## MATERIALS AND EQUIPMENT

### PMSF stock (100 mM) - Protease Inhibitor Cocktail

Reagent	Final concentration	Amount
Benzamidinium HCl (156 g/mol)	100 mM	3.12 g
Phenylmethylsulfonyl fluoride (PMSF) (174 g/mol)	100 mM	3.48 g
Absolute anhydrous ethanol	≥ 99.5% (200 proof)	Fill to 200 mL
<b>Total</b>	<b>n/a</b>	<b>200 mL</b>

Dissolve Benzamidinium HCl and PMSF into the absolute anhydrous ethanol. Label and store at -20°C for up to 6 months.

### Lysis Buffer-I

Reagent	Final concentration	Amount
HEPES pH 8.0 (1 M)	50 mM	50 mL
NaCl (5 M)	300 mM	60 mL
NDSB-201 (201.24 g/mol)	100 mM	20.13 g
Glycerol (100 %)	10 % (v/v)	100 mL
PMSF stock* (100 mM)	1 mM	10 mL
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

\* Add PMSF stock within 30 min of use due to quick degradation. Store at 4°C for 1 week.

### Lysis Buffer-II

Reagent	Final concentration	Amount
HEPES pH 7.0 (1 M)	20 mM	50 mL
NaCl (5 M)	200 mM	40 mL
MgSO <sub>4</sub> (1 M)	20 mM	20 mL

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**Continued**

Reagent	Final concentration	Amount
Tris(2-carboxyethyl) phosphine (TCEP) (0.5 M)	0.5 mM	1 mL
PMSF* (100 mM)	1 mM	10 mL
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

\* Add PMSF stock within 30 min of use due to quick degradation. Store at 4°C for 1 week.

**Extraction buffer-I**

Reagent (stock or MW)	Final concentration	Amount
Tris-HCl pH 8.0 (1 M)	20 mM	10 mL
NaCl (5 M)	60 mM	6 mL
β-mercaptoethanol (BME) (14.3 M)	3 mM	105 μL
Extraction detergent (see <a href="#">before you begin</a> , Step 18: Detergent Screen) (ex. NLS (293.38 g/mol))	CMC of detergent (ex. 0.8 % (w/v))	To CMC (ex. 4 g)
PMSF* (100 mM)	1 mM	5 mL
ddH <sub>2</sub> O	n/a	Fill to 500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

\* Add PMSF stock within 30 min of use due to quick degradation. Store at 4°C for 1 week.

**Extraction buffer-II**

Reagent	Final concentration	Amount
Tris-HCl pH 8.0	20 mM	10 mL
NaCl (5 M)	60 mM	6 mL
β-mercaptoethanol (BME) (14.3 M)	3 mM	105 μL
PMSF* (100 mM)	1 mM	5 mL
Extraction detergent (see <a href="#">before you begin</a> , Step 18: Detergent Screen) (ex. NLS (293.38 g/mol))	CMC of detergent (ex. 0.8 % (w/v))	To CMC (ex. 4 g)
Glycerol (100 %)	10 % (v/v)	50 mL
Imidazole (5 M)	20 mM or 50 mM**	2 mL or 5 mL
ddH <sub>2</sub> O	n/a	Fill to 500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

\* Add PMSF stock within 30 min of use due to quick degradation. \*\*20 mM imidazole is used for the first 10 column volumes (CV) and 50 mM imidazole is used for the second 10 CV. Store at 4°C for 1 week.

**Ni-wash buffer-I**

Reagent	Final concentration	Amount
HEPES pH 8.0 (1 M)	50 mM	25 mL
NaCl (5 M)	300 mM	30 mL
NDSB-201 (201.24 g/mol)	20 mM	2.01 g
Glycerol (100 %)	5 % (v/v)	25 mL
PMSF* (100 mM)	1 mM	5 mL
Imidazole (5 M)	10 mM or 50 mM**	1 mL or 5 mL
ddH <sub>2</sub> O	n/a	Fill to 500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

\* Add PMSF stock within 30 min of use due to quick degradation. \*\*10 mM imidazole is used for first 10 column volumes (CV) and 50 mM imidazole is used for the second 10 CV. Store at 4°C for 1 week.

### Ni-wash buffer-II

Reagent	Final concentration	Amount
HEPES pH 7.5 (1 M)	20 mM	10 mL
NaCl (5 M)	200 mM	20 mL
Imidazole (5 M)	40 mM	4 mL
ddH <sub>2</sub> O	n/a	Fill to 500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

Store at 4°C for 1 week.

### Ni-elution buffer-I

Reagent	Final concentration	Amount
HEPES pH 7.5 (1 M)	20 mM	400 µL
NaCl (5 M)	200 mM	800 µL
Sodium cholate (430.6 g/mol)	25 mM	215.3 mg
Imidazole (5 M)	400 mM	1.6 mL
ddH <sub>2</sub> O	n/a	Fill to 20 mL
<b>Total</b>	<b>n/a</b>	<b>20 mL</b>

Store at 4°C for 1 week.

### Ni-elution buffer-II

Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (1 M)	20 mM	400 µL
NaCl (5 M)	150 mM	600 µL
β-mercaptoethanol (BME) (14.3 M)	3 mM	4.2 µL
N-Lauroylsarcosine sodium (NLS) (293.38 g/mol)	0.8 % (w/v)	160 mg
Imidazole (5 M)	400 mM	1.6 mL
ddH <sub>2</sub> O	n/a	Fill to 20 mL
<b>Total</b>	<b>n/a</b>	<b>20 mL</b>

Store at 4°C for 1 week.

### Elution buffer

Reagent	Final concentration	Amount
HEPES pH 8.0 (1 M)	50 mM	1 mL
NaCl (5 M)	300 mM	1.2 mL
NDSB-201 (201.24 g/mol)	20 mM	80.5 mg
CaCl <sub>2</sub> (1 M)	10 mM	200 µL
MgCl <sub>2</sub> (1 M)	10 mM	200 µL
Glycerol (100 %)	5 % (v/v)	1 mL
PMSF* (100 mM)	1 mM	200 µL
Imidazole (5 M)	800 mM	3.2 mL
ddH <sub>2</sub> O	n/a	Fill to 20 mL
<b>Total</b>	<b>n/a</b>	<b>20 mL</b>

\* Add PMSF stock within 30 min of use due to quick degradation. Store at 4°C for 1 week.

### Gel Filtration (GF) buffer-I

Reagent	Final concentration	Amount
HEPES pH 8.0 (1 M)	50 mM	50 mL
Sodium citrate (258.07 g/mol)	100 mM	25.807 g
MgCl <sub>2</sub> (1 M)	10 mM	10 mL
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

Store at 4°C for 1 week.

#### Dialysis buffer

Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (1 M)	20 mM	60 mL
NaCl (5 M)	25 mM	15 mL
Extraction detergent (see <a href="#">before you begin</a> , Step 18: Detergent Screen) (ex. NLS (293.38 g/mol))	CMC of detergent (ex. 0.8 % (w/v))	To CMC (ex. 24 g)
ddH <sub>2</sub> O	n/a	Fill to 3 L
<b>Total</b>	<b>n/a</b>	<b>3 L</b>

Store at 4°C for 1 week.

#### Ion-exchange buffer-I

Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (1 M)	20 mM	10 mL
NaCl (5 M)	25 mM	2.5 mL
Extraction detergent (see <a href="#">before you begin</a> , Step 18: Detergent Screen) (ex. NLS (293.38 g/mol))	CMC of detergent (ex. 0.8 % (w/v))	To CMC (ex. 4 g)
ddH <sub>2</sub> O	n/a	Fill to 500 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

Store at 4°C for 1 week.

#### Ion-exchange buffer-II

Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (1 M)	20 mM	20 mL
NaCl (5 M)	1 M	200 mL
n-Dodecyl-B-D-maltoside (DDM) (510.62 g/mol)	0.25 % (w/v)	2.5 g
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

Store at 4°C for 1 week.

#### DDM buffer

Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (1 M)	20 mM	60 mL
NaCl (5 M)	150 mM	90 mL
n-Dodecyl-B-D-maltoside (DDM) (510.62 g/mol)	0.25 % (w/v)	2.5 g
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

Store at 4°C for 1 week.

#### 5× loading dye

Reagent	Final concentration	Amount
Brilliant Blue G-250	0.02 % (w/v)	2 mg
BME (14.3 M)	5 % (v/v)	0.5 mL
Sodium dodecyl sulfate (SDS) (288.37 g/mol)	10 % (w/v)	1 g
Tris-HCl pH 6.8 (1 M)	250 mM	2.5 mL
Glycerol (100%)	30 % (v/v)	3 mL
ddH <sub>2</sub> O	n/a	Fill to 10 mL
<b>Total</b>	<b>n/a</b>	<b>10 mL</b>

Store at RT for up to 3 months.

### SDS-PAGE running buffer

Reagent	Final concentration	Amount
Tris base (121.14 g/mol)	0.302 % (w/v)	30.2 g
Glycine (75.07 g/mol)	1.44 % (w/v)	144 g
Sodium dodecyl sulfate (SDS) (288.37 g/mol)	0.10 % (w/v)	10 g
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>10 L</b>

Store at RT for up to 3 months.

### SDS-PAGE gel stain

Reagent	Final concentration	Amount
Brilliant Blue G-250	0.2 % (w/v)	2 g
Methanol (100 %)	45 % (v/v)	450 mL
Acetic acid	10 % (v/v)	100 mL
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

Store at RT for up to 3 months.

### 2 × SDS-PAGE gel destain

Reagent	Final concentration	Amount
Methanol (14.3 M)	45 % (v/v)	450 mL
Acetic acid	10 % (v/v)	100 mL
ddH <sub>2</sub> O	n/a	Fill to 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

Store at RT for up to 3 months.

### Laboratory Equipment (recommended)

Instrument type	Recommended	Use
Electroporator	MicroPulser Electroporator (Bio-Rad)	Electroporation transformation
Incubator	Incubator shaker (New Brunswick Scientific)	Cell culture shaker
Erlenmeyer flask	4 L glass Erlenmeyer flasks (Pyrex)	Cell culture vessel
Cell density meter	CO8000 Personal Cell Density Meter (Denville)	OD <sub>600</sub> cell density reading
Centrifuge (large volume, refrigerated)	Sorvall BP 8 centrifuge equipped with 8 × 1000 mL swinging bucket rotor (Thermo Scientific)	Cell harvesting (1 L bottles)
Sonicator	S-4000 Ultrasonic Liquid Processor (Misonix) using a standard 1/2" horn	Cell lysis
Centrifuge (medium volume, refrigerated)	Sorvall Lynx 4000 centrifuge (Thermo Scientific) equipped with a F21-8x50y FiberLite rotor (r = 10.6 cm; Thermo Scientific)	Lysate clarification (50 mL bottles, soluble proteins)
Centrifuge (small volume, refrigerated)	Eppendorf centrifuge 5415 R equipped with a F-45-24-11 rotor (r = 8.4 cm; Millipore Sigma)	Small sample spin down (1–2 mL tubes)
Fast protein liquid chromatography (FPLC) system	NGC 10 Medium-Pressure Chromatography System (Bio-Rad)	Protein purification
Electrophoresis chamber	Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad)	Protein gel electrophoresis
Ultracentrifuge (refrigerated)	Sorvall wx+ Ultra Series centrifuge equipped with TH-641 swinging bucket rotor (Thermo Scientific)	Lysate clarification (membrane proteins)
Amplifier	Keithley 428 current amplifier	Lipid bilayer assay
Cryo-TEM Screening/Data Collection Microscope	Glacios Cryo-TEM for Life Sciences (Thermo Scientific)	Screening Cryo-EM samples or Data Collection for large proteins/complexes
Cryo-TEM Data Collection Microscope	FEI Titan Krios equipped with a Gatan K3 Camera	High-resolution data collection for SPA

### Brief description of lipid bilayer apparatus

The lipid bilayer apparatus (LBA) consists of a number of individual devices and accessories (Heinz and Niederweis, 2000). The central unit of the LBA is a measuring cuvette with two compartments, which are connected via an aperture in the middle of the separating wall. A lipid membrane is formed or “painted” over this aperture with the help of a triangular-bent Teflon loop. Two electrodes (Ag. AgCl/ 3M KCl) are bathed in the cuvette, through which a voltage is applied using a voltage source. To avoid electrostatic interference the electrodes and the measuring cuvettes are located in a grounded aluminum housing (Faraday cage). To dampen vibrations, the Faraday cage is located on a solid stone slab sitting on a vibration damper. The electrodes are either connected to an electrometer to measure the applied voltage, or to a current amplifier for the actual measurement, which amplifies the very small currents in the picoampere range and passes the amplified currents to a strip chart recorder and to an acquisition board. The acquisition board digitizes the signal, which is then recorded by a computer. The formation of a membrane is observed visually: the aperture is illuminated with light through an opening in the housing which is aligned with a glass window in the cuvette. Upon membrane formation the color changes from transparent to black. Additionally, membrane formation is indicated by the chart recorder when the ink needle moves to the zero current position. The measuring cuvette is in a hollow aluminum block with an inlet and an outlet. If necessary, this can be connected to a cryostat and serve as a temperature control chamber.

⚠ **CRITICAL:**  $\beta$ -mercaptoethanol (BME) can be toxic if ingested, and fatal if absorbed through the skin or inhaled. BME should be pipetted under a fume hood while always wearing gloves.

## STEP-BY-STEP METHOD DETAILS

### Biochemical reconstitution of DNA ejectosome components

⌚ **Timing:** 8–10 days

**Note:** Refer to the workflow in Figure 2 for an overview connecting the following sections: *before you begin:* optimize expression of internal virion proteins, *before you begin:* purification of soluble ejection proteins gp15 and gp16, *step-by-step:* biochemical reconstitution of DNA ejectosome components, and *step-by-step:* sample preparation and screening for CryoEM studies.

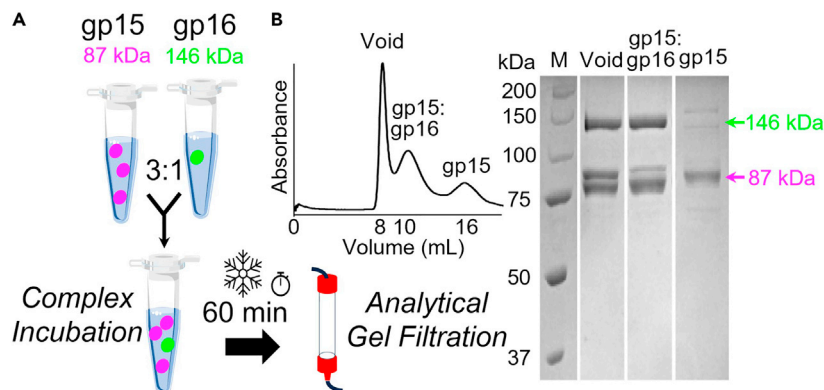
The DNA-ejectosome is formed from highly dynamic soluble and insoluble proteins that assemble a complex spanning the bacterial envelope. While gp14 is embedded in the outer membrane, gp15 and gp16 interact to form the periplasmic tunnel (PT) of the T7 DNA ejectosome that spans the periplasm through the peptidoglycan layer, across the inner membrane, and into the cytoplasm of the host *E. coli* cell. Here we describe the *in vitro* biochemical reconstitution of the gp15:gp16 complex (final stoichiometry 6:6) that was formed using a 3:1 molar ratio during incubation steps.

1. Expression (Figure 1): Follow the *before you begin* protocol for optimize expression of internal virion proteins for full preparations of gp15 and gp16 starting from fresh transformations of pET16-b\_gp15 and pET16-b\_gp16 in BL21-LOBSTR(DE3).

**Note:** Expected molecular weights are gp15, 87 kDa and gp16, 146 kDa.

2. Purification (Figure 3): Complete the *before you begin* protocol purification of soluble ejection proteins T7 gp15 and gp16 for 6 L LB each of gp15 and gp16.

**Note:** For the next steps, we recommend  $\geq 2$  mg of gp15 and  $\geq 1$  mg of gp16.



**Figure 7. In vitro assembly of the T7 periplasmic tunnel from purified gp15 and gp16**

(A) Diagram of complex components incubated in a 3:1 molar ratio on ice for 60 min

(B) Chromatogram and SDS-PAGE analysis of gel filtrated gp15:gp16 sample. Magenta arrow represents gp15 expected molecular weight at 87 kDa. Green arrow represents gp16 expected molecular weight at 146 kDa. Only relevant lanes from the same gel are shown. Parts of this figure have been reprinted from Molecular Cell, Vol 81, Issue 15, Swanson, et al., Cryo-EM structure of the periplasmic tunnel of T7 DNA-ejectosome at 2.7 Å resolution, Pages 3145-3159.e7, Copyright (2021), with permission from Elsevier.

3. Component concentration (Figure 7A): Separately, pool fractions of purified gp15 and gp16. Concentrate gp15 using a 30 kDa MWCO centrifugal filter concentrator to ~1–2 mg/mL. Concentrate gp16 using a 50 kDa MWCO centrifugal filter concentrator to ~5 mg/mL. Check the protein absorbance at 280 nm throughout the concentration process to monitor protein loss.

**Note:** Concentrate samples slowly in short 2–5 min intervals at ~1,000 rcf (or follow recommendations from manufacturer).

**Note:** Between intervals, mix gently with pipette and be careful not to disrupt the filter membrane.

**Note:** For problems that may arise during this procedure please refer to troubleshooting [problem 7](#) below.

4. Assembly (Figure 7A): Mix gp15 and gp16 in a 3:1 molar ratio in GF buffer-I. Our best results were observed using 12.75 mM gp15 (1.44 mg) with 4.5 mM gp16 (0.856 mg) in a total volume of 1.3 mL. Mix by gently pipetting up and down a few times. Incubate on ice for 1 h.

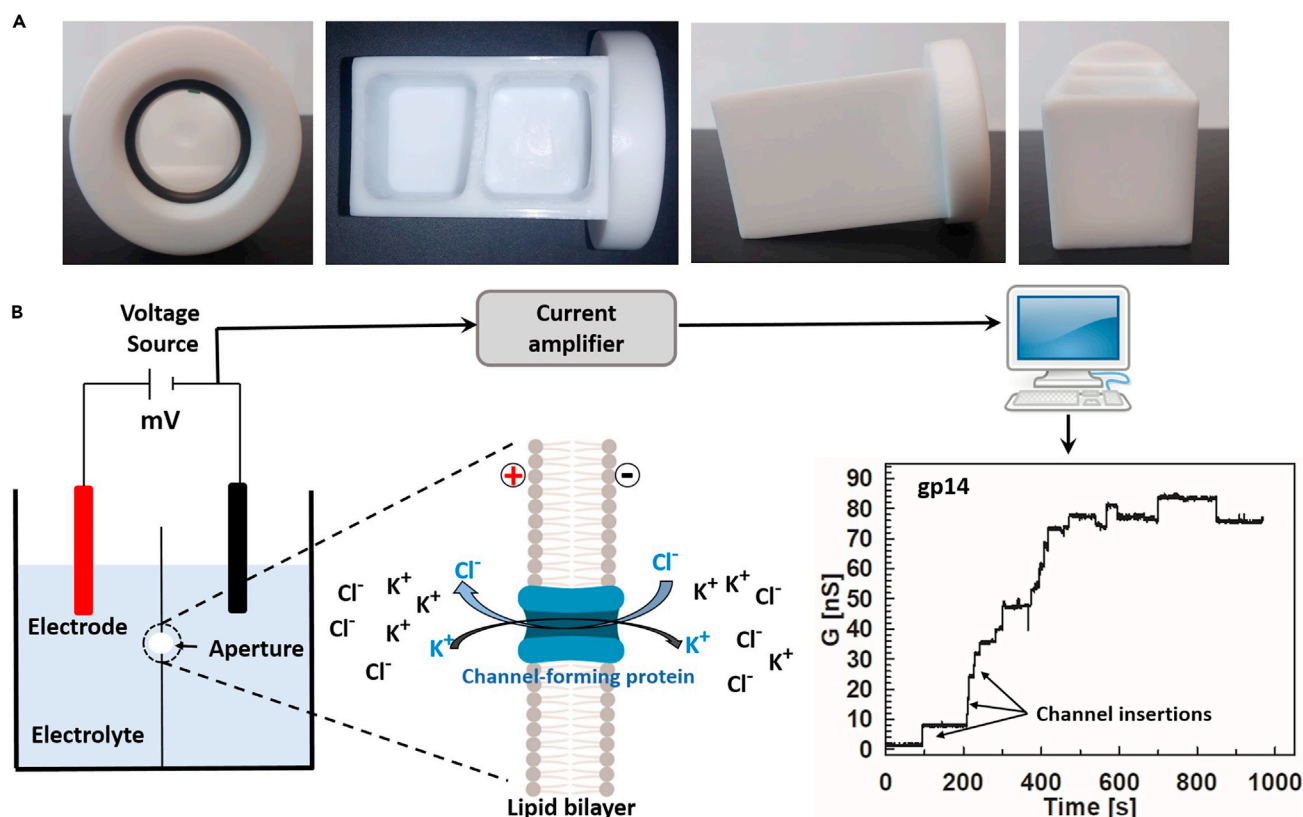
**Note:** For problems that may arise during this procedure please refer to troubleshooting [problem 8](#) below.

5. Complex concentration: Concentrate the sample to one-third of the total volume using a 100 kDa MWCO centrifugal filter concentrator. Our best resulting sample was concentrated from 1.3 mL to 400 µL containing ~2 mg. Concentrate in short intervals and mix by pipetting. Some precipitation may be observed.

**Note:** For problems that may arise during this procedure please refer to troubleshooting [problems 7](#) and [8](#) below.

6. Gel Filtration (Figure 7B): Inject the complex sample on a calibrated analytical Superose 6 10/300 GL column equilibrated in GF buffer-I. Estimate the molecular weight of the resulting peaks and observe a shift for larger MW species compared to individual components. Run an SDS-PAGE gel





**Figure 8. Lipid bilayer apparatus set-up**

(A) Front, top, side and back views of custom-made Teflon measuring cuvette. It has a rectangular shape with the following dimensions: 49 mm length, 29 mm height, 21 mm width of the back (trans) side. The front (cis) side has a circular shape with a 40 mm diameter that has a glass wall insulated by an o-ring for viewing of the bilayer formation. The cuvette is partitioned into two equal compartments by a slightly inclined wall to prevent direct back light reflection. In the middle of the partition wall there is a thin-edged aperture with a diameter of 0.8 mm or 1.1 mm. The area of the opening is 0.5 mm<sup>2</sup> or 0.95 mm<sup>2</sup>, accordingly.

(B) Schematic of lipid bilayer apparatus experiments. Passage of ions across the bilayer can be detected, measured, and interpreted in resulting channel activity plots.

for “inject” and peak fractions alongside a molecular weight standard to determine complex formation for fractions containing both components.

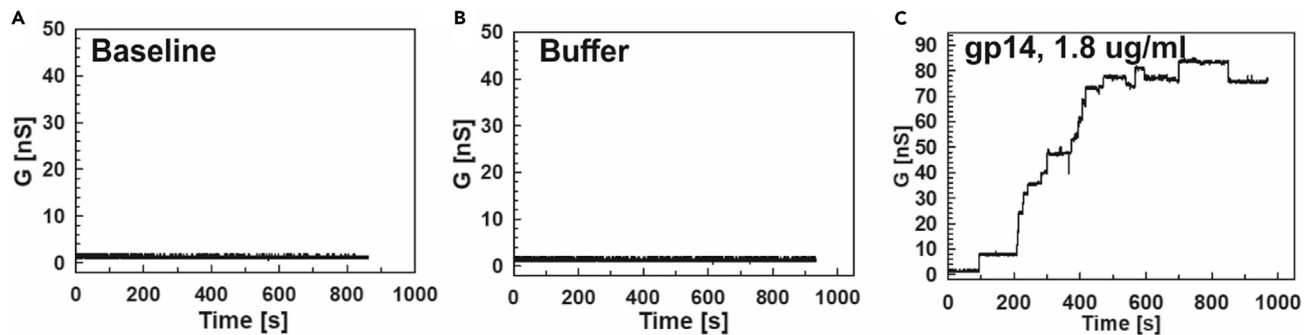
**Optional:** For further evidence of interaction between DNA-ejectosome components gp15 and gp16, run a NativePAGE (Invitrogen) with individual components versus the complex and observe shifts in band migration. Follow manufacturer’s protocol for gel running conditions.

7. Storage: The T7 periplasmic tunnel complex degrades over time and tends to aggregate if concentrated. Use within 1–3 days post-complex formation to avoid loss due to aggregation. Store at 4°C or on ice until use. Not recommended to flash freeze and store at –80°C.

### Lipid bilayer experiments

⌚ Timing: 8 h per protein sample

**Note:** Refer to the workflow in [Figure 5](#) for an overview connecting the following sections: *before you begin*: [optimize expression of internal virion proteins](#), *before you begin*:



**Figure 9. Lipid bilayer experiments with T7 DNA-ejectosome outer-membrane protein gp14**

(A) Baseline current trace.

(B) Current trace when DDM-containing buffer is present in the cuvette (1.2 % buffer).

(C) Current trace of the gp14 protein sample (1.8 µg/mL in the cuvette). Upward stepwise insertions are visible after protein addition indicating pore formation by gp14. Parts of this figure have been reprinted from Molecular Cell, Vol 81, Issue 15, Swanson, et al., Cryo-EM structure of the periplasmic tunnel of T7 DNA-ejectosome at 2.7 Å resolution, Pages 3145-3159.e7, Copyright (2021), with permission from Elsevier.

purification of insoluble ejection protein T7 gp14, [step-by-step: lipid bilayer experiments](#), and [step-by-step: sample preparation and screening for CryoEM studies](#).

Lipid bilayer experiments are performed to determine if a protein has channel-forming properties (Figure 8). Detergent-solubilized channel-forming proteins insert into artificial lipid bilayers resulting in increase of measured conductance (Figure 9).

8. Turn on current amplifier (Keithley 428) one hour before the measurements.
9. Have the lipid solution ready before the experiments: Follow the *before you begin* protocol [prepare lipid solution and measurement cuvette for lipid bilayer experiments](#) – Step 26.
10. Clean measuring cuvette and Teflon loop: Follow the *before you begin* protocol [prepare lipid solution and measurement cuvette for lipid bilayer experiments](#) – Step 27 (Figure 8).
11. Prime the measuring cuvette:
  - a. Take 3 µL of priming lipid solution and apply around aperture at the back (trans) side. Let the chloroform evaporate for 15 min.
  - b. Take 2 µL of priming lipid solution and apply around aperture at the front (cis) side. Let the chloroform evaporate for 15 min.
12. Clean Ag/AgCl electrodes with water, then ethanol, then water, and dry by blotting with absorbent paper (Figure 8).

**Note:** Electrodes are stored in 3M KCl solution between uses. When switching from one protein to another, it is recommended to clean electrodes by bathing them in pepsin solution (5% pepsin in 0.1 M HCl) for 2 h and then rinse well with water.

13. Fill the measuring cuvette with 10 mL of electrolyte solution (1 M KCl, 10 mM HEPES, pH 7.4) (Figure 8).

**Note:** Each side of the aperture has 5 ml volume. Start with the trans side. If solution does not come out to the cis side the aperture is clogged by the lipids. Use pipette tip to de-clog. The electrolyte solution should already have the measuring temperature at the beginning of the measurement, so that its specific conductivity does not increase due to an increase in temperature. It should therefore not be stored in the refrigerator, but at room temperature (20°C–22°C) or brought to room temperature beforehand.

14. Place electrodes into the measuring cuvette (Figure 8).

15. Perform preliminary quality checks:
  - a. Voltage source test: Set power source to 10 mV. Using a voltmeter connected to a trans side measure the voltage output, it should be  $10 \pm 0.5$  mV.
  - b. Zero voltage test: Without applying voltage and bridged electrodes the reading should be  $0 \text{ mV} \pm 0.03$  mV.
  - c. Electrode asymmetry test: In the case of differences in the concentration of the electrode filling solution, the electrodes themselves build up a voltage which is referred to as electrode asymmetry. If the bridging cable is attached, the electrode asymmetry will even out itself over time. Electrode asymmetry is measured without applied voltage and without bridging. The electrode asymmetry should not be more than 1 mV.
16. Set voltage to -10 mV.
17. Set up Keithley 428 current amplifier:
  - a. Gain:  $10^6$  V/A for membrane painting;  $10^9$  V/A for experiment recording.
  - b. Zero check: off position
  - c. Filter rise time: 30 ms
18. Make a membrane: The membrane is “painted” on the trans side of the cuvette. Apply 3  $\mu\text{L}$  of the painting lipid solution on the triangular-shaped Teflon loop. Slowly and gently move the loop up and down across the aperture on the back side of the cuvette. When the membrane is formed the ink needle of a chart recorder will go to zero position. Observe the appearance of black color in the aperture using light source and a scope.

**Note:** Painting the membrane is one of the most difficult tasks of this method. It takes a certain amount of patience, and practice to master this step. One should therefore take their time, especially at the beginning.

19. Assess the membrane stability: Before buffer or sample addition produce three separate membranes and record them for at least 15 min. These membranes are your baseline. This step ensures that priming of the cuvette went well, that all the components of the system are clean, and that there are no contaminations in the electrolyte solution (Figure 9A).

**Note:** The first membrane will usually rupture by itself within first few minutes. This is normal. If subsequent membranes keep breaking it may indicate that the priming was inefficient or the painting solution is old. Replace the cuvette, painting solution, or repeat the cuvette priming process.

20. Test the protein buffer: Once a stable baseline is recorded, add protein sample buffer to both sides of the cuvette and record three membranes for at least 15 min each. This step is used to exclude buffer components interference with the membrane stability. There should be no insertion-like events when only buffer is present in the cuvette (Figure 9B).

**Note:** It is recommended to start with 5  $\mu\text{L}$  of buffer in the cuvette and end with 15  $\mu\text{L}$  (0.05%–0.15% (v/v)). If later more protein sample (volume-wise) was added to the cuvette then repeat the protein buffer test with the same volume as in the added protein sample.

21. Test the protein sample: Add protein of interest (ex. gp14) to both sides of the cuvette. When insertions occur, one will see a “jump” in the current. Record at least 100 insertions from several membranes (Figure 9C).

**Note:** Start with 100 ng/ml of the protein (ex. gp14) in the cuvette. If no insertions are observed, paint new membrane and increase protein concentration in the cuvette. Determine optimal concentration of the protein for further experiments.

22. Analyze the recorded data using Igor Pro 5.03 (WaveMetrics), make graphs with the SigmaPlot 11.0 (Systat Software).

**Note:** IGOR software uses a macro written by Dr. Harald Engelhardt (Max-Planck Institute for Biochemistry, Munich, Germany) and converts the recorded voltage to the conductance using the formula:  $G = \frac{V_a}{V_e + f_v}$ ,

where G – conductance,  $V_a$  – output voltage,  $V_e$  – applied voltage,  $f_v$  – gain.

### Sample preparation and screening for CryoEM studies

#### ⌚ Timing: 2–4 days

**Note:** Both workflows in [Figures 2](#) and [5](#) lead to this section. Refer to those workflows for previous steps.

The successful assembly of T7 PT from purified gp15 and gp16 is validated by direct visualization using negative stain electron microscopy. After the PT is observed on a grid, several parameters should be screened for cryo-EM analysis, such as concentration, orientation, ice thickness, aggregation, and localization into holey carbon, all variables to optimize for high-resolution data collection. Here, we describe the steps taken to adjust these parameters.

23. Negative stain sample preparation ([Figure 10A](#)): With purified individual components or gp15:gp16 complex, dilute to 0.01–0.05 mg/mL in the final gel filtration buffer or distilled water.

- a. Physical charging: Glow discharge 2–4 nm carbon-coated 300 or 400-mesh copper grids (EMS) for 2 min at 25 mA in a vacuum chamber with the carbon-side facing up.

**Note:** The carbon-side of a copper grid is usually darker and duller than the copper-side. Typically, sample is applied to the near-transparent carbon layer.

- b. Adsorption: While holding the glow-discharged grid by the edge with tweezers, pipette 2–4  $\mu$ L onto the carbon side and allow adsorption for 1 min.

**Note:** Reverse action tweezers are recommended for holding grids for longer periods of time and repetitive tasks.

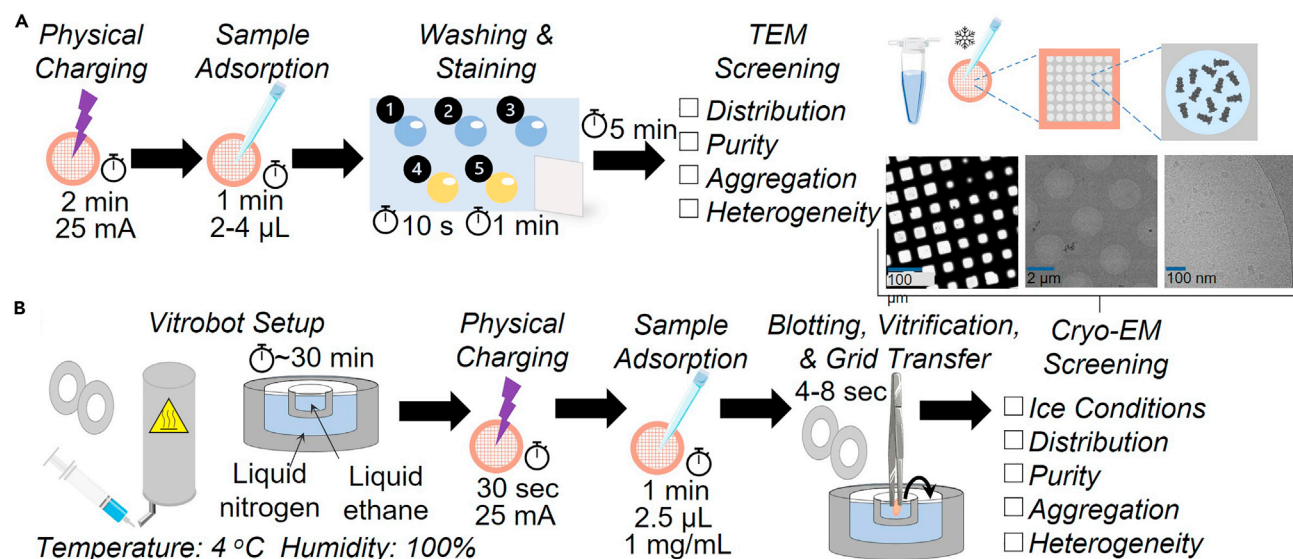
- c. Washing: On a clean sheet of parafilm, pipette three 25  $\mu$ L droplets of distilled water and two 25  $\mu$ L droplets of 2 % uranyl acetate. Blot the excess sample from the grid with filter paper, then quickly wash the carbon-side of the grid by dabbing on the three water droplets.

**Note:** When blotting, carefully touch the edges of the grid with the filter paper at a 90-degree angle. Do not use the torn edges of filter paper to avoid debris.

- d. Staining: Blot the excess water with filter paper and then hold the carbon-side of the grid on the first 2% uranyl acetate stain droplet for 10 s. Blot the excess stain with filter paper, and then hold on the second stain droplet for 1 min. Blot the excess.

**Note:** Allow the grid to air-dry for at least 5 minutes before loading into a transmission electron microscope.

**⚠ CRITICAL:** Uranyl acetate is toxic and poses acute and chronic health hazards. Work with uranyl acetate in a fume hood, always wear gloves, and avoid inhalation or contact with skin.



**Figure 10. Negative stain TEM and Cryo-EM screening**

(A) Diagram of negative stain TEM sample preparations.

(B) Diagram of CryoEM sample preparations and representative screening of a vitrified grid.

**Alternatives:** The choice of stain is sample dependent. Other staining reagents include uranyl formate, lanthanide acetate, ammonium molybdate, sodium phosphotungstate, and Uranyl-Less (EMS).

24. Negative stain TEM screening (Figure 10A): Load grids into a transmission electron microscope operated at 100–200 kV and observe sample particle distribution, purity, aggregation levels, conformational flexibility and heterogeneity of shape and size. Record micrographs for particle size analysis using ImageJ or processing with Relion for particle picking and 2D class average generation. Proceed to cryo-EM if sample quality is good (i.e., homogeneous particles, evenly distributed, with little to no aggregation).

25. Vitrobot Mark IV (Thermo Fisher Scientific) Set-Up (Figure 10B):

- Turn on the vitrobot and attach blotting paper to the blotting pads inside the climate chamber.

**Note:** The blotting paper needs to be replaced after a maximum of 16 blots.

- Attach the humidifier below the climate chamber and fill with 60 mL distilled water with a syringe through the tubing.
- On the control screen, set the temperature and humidity of the climate chamber to 4°C and 100 percent humidity, respectively.
- Set blotting parameters on the control screen. These can be modified for each sample and grid.
- Fill the coolant container peripheral ring with liquid nitrogen and allow all metal components to equilibrate.

**△ CRITICAL:** Liquid nitrogen may cause cryogenic burns or injury. Wear safety goggles or a face shield and cryogenic gloves when handling liquid nitrogen.

- Fill the central cup of the coolant container with liquid ethane and allow the ethane to cool until cloudy. Remove the metal spindle once the ethane is chilled.

**Note:** Remove the metal spindle before the ethane solidifies. Solid ethane will bend your grids and could damage the tweezer and plunging apparatus.

**△ CRITICAL:** Liquid ethane is an extremely flammable gas and may cause cryogenic burns or injury. Avoid heat sources and wear safety goggles or a face shield and cryogenic gloves when handling liquid ethane.

g. Load coolant container onto the platform ring and top off with liquid nitrogen in the peripheral ring during use.

26. Vitrification ([Figure 10B](#)): With purified individual components or gp15:gp16 complex, dilute to 1 mg/mL in the final gel filtration buffer or distilled water.
  - a. Physical charging: Glow discharge 300-mesh Quantifoil 1.2/1.3 holey carbon grids for 30 s at 25 mA in a vacuum chamber with the carbon-side facing up.
  - b. Adsorption: Load grid on tweezers and raise the tweezer arm and coolant chamber using the control screen or foot pedal. Pipette 2.5  $\mu$ L of 1 mg/mL sample through the sample port onto the grid. Allow adsorption for 10–60 s.
  - c. Blotting and plunging: Adjust blotting parameters on the control screen for each grid/sample. Initiate the blotting/plunging procedure from the control screen or use the foot pedal.

**Note:** We typically use 4–8 sec blot times with blot force 0–4. Wait and drain times are usually set to 0 sec. Blotting conditions for each sample were determined empirically.

d. Grid transfer: Carefully disconnect the tweezers from the central axis without letting the grid touch the sides of the central cup. Quickly transfer the grid from the central cup to the peripheral ring filled with liquid nitrogen then carefully drop the grid into a slot of a grid storage box.

**Note:** Any bent grids should be discarded.

e. Repeat to prepare grids for each sample and conditions of interest.

27. Cryo-EM screening ([Figure 10B](#)): Load grids into cryo-electron microscope operated at 200 kV–300 kV and observe ice conditions, sample particle distribution, purity, aggregation levels, conformational flexibility and heterogeneity of shape and size. Record micrographs for particle size analysis using ImageJ or processing with Relion for particle picking and 2D class average generation. Proceed to cryo-EM data collection if grid and sample quality is good (i.e., homogeneous particles, evenly distributed, with little to no aggregation).

**Optional:** If screening or loading into cryo-electron microscopes with an autoloader, use Autogrid C-clip rings and Autogrid C-clips for clipping and docking grids.

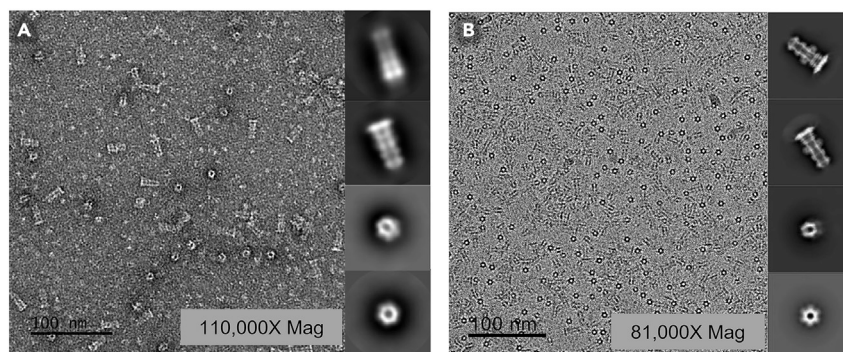
## EXPECTED OUTCOMES

Ejection proteins are known for their biochemical fragility and conformational flexibility. In this protocol, we stabilized ejection proteins gp15 and gp16 by forming a complex *in vitro*, limiting the conformational flexibility and heterogeneity. Below in [Figure 11](#), we show typical negative stain and cryo-EM micrographs for gp15:gp16 complexes.

## LIMITATIONS

This protocol is optimized and worked reproducibly for T7 DNA-ejectosome components gp14, gp15, and gp16. Each DNA-ejectosome assembly for different systems will present unique challenges not encountered in our studies due to differences in the protein biochemistry of each component. However, we hope that the general procedures of optimizing expression strains, detergent





**Figure 11. Expected results for negative stain TEM and cryo-EM screening**

(A and B) Representative micrographs of (A) negatively stained and (B) vitrified gp15:gp16 complex. On the right-hand side are 2D-class averages. Scale bars, 100 nm. Reprinted from Molecular Cell, Vol 81, Issue 15, Swanson, et al., Cryo-EM structure of the periplasmic tunnel of T7 DNA-ejectosome at 2.7 Å resolution, Pages 3145-3159.e7, Copyright (2021), with permission from Elsevier.

screening for insoluble proteins, and exploring molar ratios for complex assembly will help users. The T7 DNA-ejectosome was stable on the scale of 1–2 days in our hands at 4°C, while other systems may be limited by a more transient existence or form only in the presence of the whole virus tail.

## TROUBLESHOOTING

### Problem 1

Significant amount of protein still bound to Ni beads after washes with Ni-elution buffer. (Step 12 - [purification of soluble ejection proteins T7 gp15 and gp16](#); Step 19 - [purification of insoluble ejection protein T7 gp14](#)).

#### Potential solution

If there is a significant amount of protein still bound to beads, first try further additions of Ni-elution buffer to the beads to elute off more protein. Secondly, try increasing the imidazole concentration up to 1 M if protein remain bound after additional washes with Ni-elution buffer. If protein remain bound, then they are likely aggregated on the beads and can be cleared with a Ni-bead regeneration protocol.

### Problem 2

Most of the protein elutes in the “flow-through” of the gravity column after incubation with Ni beads. (Step 12 - [purification of soluble ejection proteins T7 gp15 and gp16](#); Step 19 - [purification of insoluble ejection protein T7 gp14](#))

#### Potential solution

If most of the protein is in the “flow-through,” try increasing the amount of Ni beads for the incubation step, switch to high density Ni beads, or consider Cobalt beads for this affinity step. Increasing the bead incubation time can also help. However, if there is no binding to the beads, the His-tags may be inaccessible or cleaved off.

### Problem 3

Gp16 experiences proteolytic degradation in solution. (Step 15 - [purification of soluble ejection proteins T7 gp15 and gp16](#)).

#### Potential solution

Purifying gp16 without degradation requires special care. We found shorter expressions at 37°C and adding 1 mM PMSF stock to all buffers before each step reduced proteolytic cleavage. Also consider the addition of protease inhibitor cocktail to the final assembled complex.



### Problem 4

Majority of the protein elutes after injection during the initial wash with [Dialysis buffer-II](#). This stage is sometimes referred to as the “binding” phase. (Step 23 - [purification of insoluble ejection protein T7 gp14](#))

### Potential solution

The protein either could have exceeded the binding capacity of the column or had no binding. In either case, the collected flow-through can be salvaged and re-run on the same column or another to optimize binding (see [problem 5](#) for more info on alternative columns).

### Problem 5

Bound protein elutes too early in the salt gradient during ion exchange chromatography. (Step 23 - [purification of insoluble ejection protein T7 gp14](#)).

### Potential solution

If the protein elutes off the column too early in the gradient, the initial salt concentration can be lowered, and the gradient can be spread across more volume.

### Problem 6

Weak or no binding causing the protein of interest to be in the flowthrough or too strong binding causing the protein of interest to be crashed out on column. (Step 23 - [purification of insoluble ejection protein T7 gp14](#))

### Potential solution

Depending on the extraction detergent used and charges of the insoluble protein of interest, different ion-exchange columns may be more suitable. We recommend testing Q, S, Heparin, and other exchange columns to select the most efficient binding for detergent exchange.

### Problem 7

Most of the protein is in the flow-through of the concentrator. (Steps 3 and 5 - [biochemical reconstitution of DNA ejectosome components](#))

### Potential solution

The concentrator filter membrane may be damaged, or the protein is too small for the concentrator MWCO. Switching to a new concentrator often fixes this problem and consider using concentrators with lower MWCO values. We suggest short intervals with plenty of mixing by pipette in between cycles to reduce the loss by crashing out on the filter membrane.

### Problem 8

Precipitation is observed upon mixing the two proteins together. (Step 4 - [biochemical reconstitution of DNA ejectosome components](#)).

### Potential solution

Make sure that the two buffers match. It may help to dialyze in the same buffer to ensure equal conditions. Reducing the concentration slightly may reduce the amount of aggregation but could limit the rate at which complex forms. There is a tradeoff between concentration induced precipitation and amount of complex formed. Testing ratios is recommended for different ejection proteins. ~3:1 gp15:gp16 works best in our hands, however alternative ratios may be preferred for different systems.

### Problem 9

Gp15 and gp16 fail to form a complex. (Step 4 - [biochemical reconstitution of DNA ejectosome components](#)).

### Potential solution

The initial concentrations of gp15 may be too high and the protein has formed a hexamer on its own. The complex is best formed from dilute solutions of both factors. In our hands, keeping the concentration of gp15 below 5 mg/mL helps reduce oligomerization.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gino Cingolani, PhD ([gino.cingolani@jefferson.edu](mailto:gino.cingolani@jefferson.edu)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

Original/source data for figures in the paper is available [Mendeley Data: <https://doi.org/10.17632/xf2w3yccz2.1>].

### ACKNOWLEDGMENTS

This work was supported by NIH grants R01 GM100888 and R35 GM140733 to G.C. and R01 AI137338 to M.N. Research in this publication includes work carried out at the Sidney Kimmel Cancer Center X-ray Crystallography and Molecular Interaction Facility at Thomas Jefferson University, which is supported in part by National Cancer Institute Cancer Center Support Grant P30 CA56036. This research was, in part, supported by the National Cancer Institute's National Cryo-EM Facility at the Frederick National Laboratory for Cancer Research under contract HSSN261200800001E. Atomic coordinates for the T7 DNA-Ejectosome components gp15:gp16-N have been deposited in the Protein Data Bank with accession code 7K5C, and the cryo-EM density map has been deposited in the Electron Microscopy Data Bank with accession code EMD-22680.

### AUTHOR CONTRIBUTIONS

N.S., R.L., F.L., C-F.H., and M.P., performed and validated the experiments. M.N., and G.C. helped conceptualize the experiments. All authors analyzed the data, provided expertise and feedback. N.S., G.C., M.P., and M.N. wrote the manuscript with help from all other authors.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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