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Bacteriophage T4 as a Nanoparticle Platform to Display and Deliver Pathogen Antigens: Construction of an Effective Anthrax Vaccine

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Abstract

Protein-based subunit vaccines represent a safer alternative to the whole pathogen in vaccine development. However, limitations of physiological instability and low immunogenicity of such vaccines demand an efficient delivery system to stimulate robust immune responses. The bacteriophage T4 capsid-based antigen delivery system can robustly elicit both humoral and cellular immune responses without any adjuvant. Therefore, it offers a strong promise as a novel antigen delivery system. Currently *Bacillus anthracis*, the causative agent of anthrax, is a serious biothreat agent and no FDA-approved anthrax vaccine is available for mass vaccination. Here, we describe a potential anthrax vaccine using a T4 capsid platform to display and deliver the 83 kDa protective antigen, PA, a key component of the anthrax toxin. This T4 vaccine platform might serve as a universal antigen delivery system that can be adapted to develop vaccines against any infectious disease.

Keywords

Bacteriophage T4; Virus decoration proteins; Nanoparticle vaccine platform; PA; Anthrax vaccine

1 Introduction

Vaccines represent one of the most important contributions to the prevention of infectious disease in humans. Currently, there are mainly three types of licensed vaccines: (1) inactivated whole-pathogen vaccines, (2) live-attenuated whole-pathogen vaccines, and (3) protein-based subunit vaccines [1]. The majority of vaccines fall into the first two categories and are therefore considered whole-pathogen vaccines. Unfortunately, these whole pathogen vaccines pose significant safety concerns including reversion to a pathogenic form, reactions in immunocompromised hosts, and other adverse effects such as allergic and autoimmune reactions. Only a few protein-based subunit vaccines such as virus-like particles (VLPs), detoxified toxoids/toxins, and polysaccharide–protein conjugates have been approved for administration to humans. Generally these subunit vaccines have low immunogenicity and stability, thus requiring an efficient delivery system and/or an adjuvant to overcome these

[15]Each T4 capsid has 930 molecules of major protein gp23 *(“*” refers to the cleaved and matured form), whose molecular weight is 48 kDa; thus, 0.78 μg gp23* equal to 1×10^{10} phages.

[16]Proteins may nonspecifically bind to a regular Eppendorf tube. Thus, it is highly recommended to use low-binding tubes such as Protein LoBind Eppendorf tube.

limitations. If delivered efficiently, these protein-based subunit vaccines should be safer than the more traditional whole pathogen vaccines.

Recently, we have developed a novel antigen delivery system using the bacteriophage T4 capsid [2–4]. Antigens were displayed on the T4 capsid at a high density, and, upon immunization, they elicited robust humoral and cellular immune responses without any adjuvant, making T4 a robust antigen display and delivery system [2, 3, 5, 6]. The T4 capsid (head) is composed of three essential capsid proteins: gp23*, gp24*, and gp20 ("*" refers to the cleaved and matured form). There are 930 copies of the major capsid protein, gp23*, 55 copies of the vertex protein, gp24*, and 12 copies of the portal protein, gp20. In addition, the capsid also contains two nonessential proteins: *h*ighly antigenic *o*uter *c*apsid protein (Hoc) and *s*mall *o*uter *c*apsid protein (Soc). There are 870 copies of Soc (10 kDa), and 155 copies of Hoc (39 kDa) [7, 8]. Both Hoc and Soc are dispensable. Mutant phage in which both these genes are defective (amber or deletion) do not show a significant loss of infectivity or replicative capacity under laboratory conditions [9]. Furthermore, purified recombinant Soc and Hoc proteins can be assembled on $Hoc^{-}Soc^{-}$ capsids in vitro with high specificity and nanomolar affinity [10]. The phage T4 capsid nanoparticle, thus, provides an ideal platform to display foreign antigens as Soc or Hoc fusion proteins [2, 11, 12]. Large full-length proteins as well as multi-protein complexes can be displayed without compromising the ability of Soc and Hoc to bind to the capsid [6, 12]. All 1025 copies of Soc and Hoc can be replaced either with a single antigen or with multiple antigens [2, 10–12].

Bacillus anthracis is the etiological agent of anthrax, a highly lethal infection and also a serious biothreat as evident from the 2001 anthrax attacks in the USA [13, 14]. An alum-adsorbed anthrax vaccine (AVA) was approved in the 1970s for military use, and a reformulated version has recently been approved for civilian adults. This vaccine is based on crude bacterial culture supernatant containing secreted protective antigen (PA). PA is one of the components of the anthrax toxin that consists, in addition, of lethal factor (LF) and edema factor (EF) [15, 16]. Previous studies demonstrated that PA alone is sufficient to provide complete protection against anthrax [16–18]. AVA requires a long immunization regimen and causes significant side reactions in vaccinated individuals [19]. Therefore, recent efforts have been directed to developing a safer next generation subunit vaccine using recombinant PA (rPA) (*see* reviews [15, 16, 19–21]).

Results from our laboratory and others have demonstrated that soluble rPA adjuvanted with alum, as well as bacteriophage T4-displayed rPA can protect macaques against aerosol challenge with Ames *Bacillus anthracis* spores [5, 17, 18, 21–24]. In this chapter, we describe the methods to generate T4 nanoparticle rPA conjugate that might serve as a candidate for the next generation anthrax vaccine.

2 Materials

2.1 Construction of Plasmids

1. Vector, gene DNA, and cells: *E. coli* expression vector pET28b (Novagen, MA), bacteriophage RB69 Soc gene DNA and *Bacillus anthracis* PA gene DNA

(prepared in our lab), competent *E. coli* DH5α cells (New England Biolabs, MA).

2. Growth media: SOC medium (Quality Biologicals, MD) and Luria–Bertani (LB) medium (Quality Biological, MD).
3. 1000× kanamycin (50 mg/mL): Add 0.5 g kanamycin (Gold Biotechnology, MD) to 10 mL Milli-Q water.
4. Kanamycin LB plates: Add 2 g LB powder (Affymetrix, OH) and 1.5 g agar to 100 mL Milli-Q water, autoclave for 15 min at 121 °C. When cooled to about 50 °C, add 0.1 mL 1000× kanamycin. Mix and pour into sterile petri plates (Akro-Mils, OH).
5. Enzymes: 2× Phusion High-Fidelity PCR Master Mix (Thermo Scientific), FastDigest *Nhe*I (Thermo Scientific), FastDigest *Hind*III (Thermo Scientific), FastDigest *Xho*I (Thermo Scientific), FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific), and T4 DNA Ligase (Thermo Scientific).
6. Agarose gel running buffer: add 100 mL 10× AccuGENE™ Tris–borate–EDTA (TBE) agarose gel running buffer (Lonza Chemicals Company, Switzerland) to 900 mL Milli-Q water to make 1× agarose gel running buffer.
7. Kits: GeneJET Gel Extraction Kit (Thermo Scientific) and GeneJET Plasmid Miniprep Kit (Thermo Scientific).

2.2 Protein Purification

1. Expression cell: Competent *E. coli* BL21-CodonPlus (DE3)-RIPL cells (Agilent Technologies, CA).
2. Growth media: SOC medium (Quality Biologicals, MD) and Moore's medium (1 L medium contains 20 g tryptone, 15 g yeast extract, 8 g NaCl, 2 g dextrose, 2 g Na_2HPO_4 and 1 g KH_2PO_4; add 1 mL 1000× (50 mg/mL) kanamycin and 1 mL 1000× (50 mg/mL) chloramphenicol before use. The final concentration of each antibiotic is 50 μg/mL.
3. Antibiotics: 1000× kanamycin (50 mg/mL): Add 0.5 g kanamycin (Gold Biotechnology, MD) to 10 mL Milli-Q water; 1000× chloramphenicol (50 mg/mL): Add 0.5 g chloramphenicol (Amresco) to 10 mL ethanol.
4. Kanamycin–chloramphenicol LB plates: Add 2 g LB powder (Affymetrix, OH) and 1.5 g agar to 100 mL Milli-Q water, autoclave for 15 min at 121 °C. When cooled to about 50 °C, add 0.1 mL 1000× kanamycin and 0.1 mL 1000× chloramphenicol. Mix and pour into sterile petri plates (Akro-Mils, OH).
5. Chemical reagents: 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) (add 238 mg IPTG [Gold Biotechnology, MO] to 1 mL Milli-Q water), Coomassie blue R-250 staining solution (Teknova, CA), complete proteinase inhibitor cocktail (Roche), and acetylated bovine serum albumin (BSA) standard (Affymetrix, OH).

6. Buffers (*see* Note 1): HisTrap binding buffer (50 mM Tris– HCl pH 8.0, 300 mM NaCl, and 20 mM imidazole); HisTrap washing buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, and 50 mM imidazole); HisTrap elution buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, and 400 mM imidazole); Gel filtration buffer (20 mM Tris–HCl, pH 8.0 and 100 mM NaCl); SDS-loading buffer (20 mM Tris–HCl pH 6.8, 100 mM dithiothreitol, 2.5% β-mercaptoethanol, 1% SDS (w/v), 0.1% bromophenol blue, and 10% glycerol); Tris–glycine running buffer (add 100 mL 10× Tris–glycine running buffer (Bio-Rad) to 900 mL Milli-Q water to make 1× Tris–glycine running buffer).
7. Destaining solution: Add 100 mL methanol and 100 mL acetic acid to 800 mL Milli-Q water.
8. Columns: 1 mL HisTrap HP Nickel affinity chromatography column (GE Healthcare) and Hi-load 16/60 Superdex 200 gel filtration chromatography column (GE Healthcare).
9. Amicon Ultra-4 centrifugal filter units (Millipore, MA).
10. 4–20% (w/v) polyacrylamide gel (Life Technologies).

2.3 T4 Phage Purification

1. Phage and *E. coli*: $Hoc^{-}Soc^{-}$ phage T4 mutant (constructed in our laboratory) and *E. coli* P301.
2. Growth media: LB medium (Quality Biological, MD) and M9CA medium (to 800 mL Milli-Q water, add 12.5 g M9CA medium powder [Amresco, OH], adjust to 1 L with Milli-Q water, and autoclave for 15 min at 121 °C).
3. LB plates: Add 2 g LB powder (Affymetrix, OH) and 1.5 g agar to 100 mL Milli-Q water, and autoclave for 15 min at 121 °C. Mix and pour into sterile petri plates.
4. Top-Agar: Add 2 g LB powder (Affymetrix, OH) and 0.75 g agar to 100 mL Milli-Q water, sterilize, and keep it at 42 °C.
5. Chemical reagents: Deoxyribonuclease I (DNase I) (Sigma-Aldrich) and HPLC-grade chloroform (Thermo Fisher Scientific).
6. Buffers: Pi-Mg buffer (26 mM Na_2HPO_4, 22 mM KH_2PO_4, 70 mM NaCl, and 1 mM $MgSO_4$); Dialysis buffer I (10 mM Tris–HCl pH 7.5, 200 mM NaCl, and 5 mM $MgCl_2$); Dialysis buffer II (10 mM Tris–HCl pH 7.5, 50 mM NaCl, and 5 mM $MgCl_2$).
7. Cesium chloride (CsCl) stock solution: 8 M CsCl, 100 mM Tris–HCl, pH 7.5, 85 mM NaCl, and 20 mM NH_4Cl.
8. Slide-A-lyzer dialysis cassette with molecular weight cut-off (MWCO) of 10 K (3–12 mL capacity; Thermo Scientific™ Pierce™ Protein Biology, IL).

[1]All buffers used for protein purification have to pass through 0.22 μm filter in order to avoid clogging the column.

2.4 Antigen Preparation

1. 1× PBS pH 7.4: Add 100 mL 10× PBS pH 7.4 (Quality Biological, MD) to 900 mL Milli-Q water to make 1× PBS buffer.
2. 5 M NaCl (Quality Biological, MD).
3. Protein LoBind Eppendorf tube 1.5 mL (Hamburg, Germany).

3 Methods

Both Hoc and Soc can be used to display antigens. Here we use Soc to display PA, because it has 5.6 times more binding sites (870 per capsid) compared with Hoc (155 per capsid). Thus, through Soc, antigens can be displayed on T4 capsid at a higher density.

3.1 Construction of pET-Soc-PA

A universal vector, pET-Soc-N, is first constructed such that it contains a multiple cloning site (MCS) at the COOH-terminus of Soc by inserting Soc (*see* Note 2) into pET28b expression vector with *Nhe*I/*Hind*III. PA or any other antigen genes can be amplified by PCR and cloned into pET-Soc-N to generate an in-frame fusion with the C-terminal end of Soc (*see* Note 3).

1. 1. Use Thermo 2X Phusion High-Fidelity PCR Master Mix to amplify RB69 Soc gene DNA from RB69 phage genome DNA, using the following primers, where the underlined sequences correspond to the recognition sequences for the respective restriction enzymes:

   Soc *Nhe*I Forward:

   5′ - G C ATCCGCTAGCGGTGGTTATGTA A A CATCAAA-3′.

   Soc *Hind*III Reverse:

   5′ - G C A G A A G C T T C A C C A C T T A C T G G T GTAGGGGTAAAC-3′.
2. Add 10 μl 10× DNA loading buffer to 90 μl PCR product, load onto 2% agarose gel, and separate by agarose gel electrophoresis.
3. Cut out the expected DNA band and extract DNA using Thermo gel extraction kit according to the manufacturer's instructions.
4. Cut 1 μg purified PCR product with *Nhe*I and *Hind*III at 37 °C for 2 h. At the same time, cut 1 μg pET28b vector plasmid DNA using the same restriction enzymes at 37 °C for 2 h.

[2]We used RB69 Soc instead of T4 Soc to construct Soc fusion. RB69 phage is a relative of T4 and previous studies found that recombinant RB69 Soc is more soluble than T4 Soc and binds to T4 capsid at nearly the same affinity as recombinant T4 Soc [10].
[3]We had also constructed a universal vector, pET-Soc-C, which contains a multiple cloning site (MCS) at NH2-terminus of Soc [4]. Any other antigen gene can be amplified by PCR and cloned into pET-Soc-C to generate an in-frame fusion with the NH2-terminal end of Soc.

5. Directly add 1 μl FastAP thermosensitive alkaline phosphatase into *Nhe*I- and *Hind*III-digested pET28b vector without changing the restriction enzyme buffer and incubate at 37 °C for 30 min.

6. Load the digested insert and vector onto 2% and 1% agarose gel respectively and separate by agarose gel electrophoresis.

7. Cut out the expected DNA bands and extract DNA using Thermo gel extraction kit according to the manufacturer's instructions.

8. Ligate insert and vector at a molar ratio of 3:1 using T4 DNA ligase for 1 h at 22 °C.

9. Transform *E. coli* DH5α with the ligation product according to the manufacturer's instructions. Incubate the LB-kanamycin plate overnight at 37 °C.

10. Pick a single colony and inoculate into a 125 mL flask containing 10 mL LB medium containing 50 μg/mL of kanamycin. Incubate the flask in a shaking incubator overnight at 220 rpm and 37 °C.

11. Isolate the plasmid DNA using Thermo GeneJET plasmid miniprep kit according to the manufacturer's instructions. The thus generated plasmid was named pET-Soc-N, which contained a hexa-histidine tag at the NH2-terminus and a MCS at the COOH-terminus of Soc.

12. Use Thermo 2× Phusion High-Fidelity PCR Master Mix to amplify the PA DNA from the template (pET-F1mutV-PA) using the following primers, where the underlined sequences correspond to the recognition sequences for the respective enzymes:

    *Hind*III Forward: 5′-ACCCAAGCTT CTGCTGAAGTTAA ACAGGAGAACCGGTTATT-3′.

    *Xho*I Reverse: 5′-GCCCTCGAGTTATCCTATCTCATAGCC TTTTTTAG-3′.

13. Repeat steps **2–3**.

14. Digest 1 μg purified PCR product with *Hind*III and *Xho*I at 37 °C for 2 h. At the same time, digest 1 μg pET-Soc-N plasmid DNA using the same restriction enzymes at 37 °C for 2 h.

15. Directly add 1 μl FastAP Thermosensitive Alkaline Phosphatase into *Hind*III- and *Xho*I-digested pET-Soc-N without changing the restriction enzyme buffer and incubate at 37 °C for 30 min.

16. Repeat steps **6**–**10**.

17. Isolate the plasmid DNA using Thermo GeneJET plasmid miniprep kit according to the manufacturer's instructions. The resulting clone, pET-Soc-PA, contains PA fused in-frame to the COOH-terminus of RB69 Soc.

3.2 Purification of Recombinant Soc-PA from E. coli BL21-CodonPlus (DE3)-RIPL

1. Transform 10 ng of pET-Soc-PA into 25 μl BL21-CodonPlus (DE3)-RIPL competent cells according to the manufacturer's instructions. Incubate on LB-kanamycin/chloramphenicol plates overnight at 37 °C.
2. Pick a single colony and inoculate into 30 mL Moore's medium with 50 μg/mL kanamycin and 50 μg/mL chloramphenicol. Incubate the flask overnight in a shaking incubator at 220 rpm and 37 °C.
3. Inoculate 20 mL of overnight cultures into a 2 L flask containing 1 L of Moore's medium supplemented with 50 μg/mL kanamycin and 50 μg/mL chloramphenicol. Incubate the flask in a shaking incubator at 220 rpm and 37 °C until the cell density reaches 1.5–2.0×10^8 cells/mL.
4. Change the temperature of the shaking incubator to 28 °C and keep shaking for 30 min at 220 rpm before adding IPTG (*see* Note 4).
5. Add 1 mL IPTG (isopropyl β-D-1 thiogalactopyranoside) (1 M) to the culture and induce protein expression for 2 h at 28 °C.
6. Distribute the culture into 500 mL centrifuge bottles and collect the cells by centrifugation at 7000 rpm (8288 × *g*) for 10 min at 4 °C with GS3 rotor in Sorvall RC-5C plus centrifuge or equivalent.
7. Discard the supernatant and resuspend the pellet with 40 mL HisTrap binding buffer supplemented with one pill of complete proteinase inhibitor cocktail (*see* Note 5).
8. Set up French press (Thermo Scientific) and lyse the cells at 12,000 psi twice.
9. Distribute the cell lysate into 30 mL centrifuge tubes and centrifuge at 17000 rpm (34,572 × *g*) for 22 min at 4 °C with SS34 rotor in Sorvall RC-5C plus centrifuge or equivalent.
10. Collect the supernatant which contains soluble Soc-PA protein, and filter it through 0.22 μm filters before loading onto the HisTrap column (*see* Note 6).
11. Set up 1 mL HisTrap HP column on AKTA-prime system. First, wash the column with 20 mL of water, and then equilibrate the column with 20 mL of HisTrap binding buffer.
12. Load the supernatant collected in step **10** onto the HisTrap HP column at a loading speed of 1 mL/min.
13. Wash the column with 20 mL HisTrap washing buffer (*see* Note 7).

[4]The purpose of this step was to cool down the *E. coli* to 28 °C before IPTG induction. Higher induction temperature may increase the chance of partitioning the overexpressed Soc-PA into the inclusion bodies, thus reducing the yield of soluble protein.
[5]Add proteinase inhibitor cocktail to binding buffer right before use. Proteinase inhibitors are necessary during purification to prevent protein degradation.
[6]All samples to be loaded onto HisTrap column or Hi-load 16/60 Superdex 200 column have to go through 0.22 μm filter to prevent clogging the columns.
[7]After washing with 20 mL HisTrap washing buffer, the A280 reading of the HisTrap HP column flow through should be stable with minute variations. If not, keep washing with HisTrap washing buffer until the A280 reading becomes stable.

14. Elute the protein with 20–400 mM linear imidazole gradient with the HisTrap binding buffer as buffer A and the HisTrap elution buffer as buffer B. AKTA-prime was set as follows:

    Concentration (% Buffer B): 0; Gradient Length: 40; Target (% Buffer B): 100; Flow Rate: 1 mL/min; Fraction Base: mL; Fraction Size: 1; Pressure Limit: 0.3.

15. Collect and pool the peak fractions (*see* Note 8).

16. Wash the Hi-load 16/60 Superdex 200 column with 150 mL Gel filtration buffer. Load the HisTrap peak fractions onto the Hi-load 16/60 Superdex 200 column with a flow rate of 1.0 mL/min.

17. Collect and pool the peak fractions from gel filtration elution, and concentrate using Amicon Ultra-4 centrifugal filtration (10 kDa cut-off).

18. Quantify concentration of the Soc-PA protein using Nanodrop (Thermo Scientific) (*see* Note 9).

19. Aliquot the concentrated Soc-PA protein and store at −80 °C for future use.

3.3 Purification of Hoc⁻Soc⁻ Phage T4

1. Use sterilized plain wood applicator (Fisher Scientific) to streak the glycerol stock of *E. coli* P301 cells on an LB plate. Incubate the plate at 37 °C overnight (*see* Note 10).

2. Pick a single colony, inoculate into 20 mL LB medium, and incubate the flask in a shaking incubator at 220 rpm and 37 °C for 8 h. Store the culture at 4 °C cold room for use the following day.

3. Inoculate 10 mL of cultures into a 2 L flask containing 500 mL of LB and M9CA medium (250 mL LB + 250 mL M9CA). Incubate the flask in a shaking incubator at 220 rpm and 37 °C until the cell density reaches 2.0×10^8 cells/mL.

4. Infect *E. coli* P301 with Hoc⁻Soc⁻ phage T4 at multiplicity of infection (MOI) of 0.2 by adding 2×10^{10} plaque forming units (PFU) of Hoc⁻Soc⁻ phage T4 (*see* Note 11), and keep the flask shaking in a 37 °C incubator at 200 rpm for 2–3 h.

5. Observe phage growth (*see* Note 12) during incubation. After confirmation of phage growth, add 20 mL chloroform into the flask, and keep it shaking at 200 rpm for 10 min at 37 °C.

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[8]The maximum loading volume of Hi-load 16/60 Superdex 200 column is 5 mL. If the volume of the pooled peak fractions is more than 5 mL, concentrate them to 5 mL so that the pooled peak fractions can be loaded onto the Hi-load 16/60 Superdex 200 column.

[9]Each protein has its own molar extinction coefficient, which is 91,680 M^{-1} cm^{-1} in the case of Soc-PA. The default extinction coefficient of NanoDrop is based on BSA. Remember to change the extinction coefficient and molecular weight when using NanoDrop.

[10]Hoc⁻Soc⁻ phage T4 is an amber mutant, not a gene deletion mutant. In order to produce Hoc⁻Soc⁻ capsid, only non-suppressor *E. coli*, such as P301, can be used as the host cell to propagate Hoc⁻Soc⁻ phage T4.

[11]Mix it immediately after adding the phage so as to distribute the phage uniformly.

[12]The growth of phage can be assessed by (1) looking for turbidity and floating cell debris in the culture flask, (2) chloroform treatment, or (3) observing under light microscope.

6. Collect the phages by centrifuging the culture for 45 min at 12,000 rpm (23,440 × *g*) at 4 °C with GSA rotor in Sorvall RC-5C plus centrifuge or equivalent.

7. Resuspend the pellet in 30 mL Pi-Mg buffer, add 500 μl chloroform and 43 μl of 7 mg/mL DNase I (final concentration of 10 μg/mL), and keep shaking at 220 rpm in 37 °C for 30 min.

8. Transfer the phage suspension to a 30 mL centrifuge tube, and centrifuge at 6000 rpm (4300 × *g*) for 10 min at 4 °C with SS34 rotor in Sorvall RC-5C plus centrifuge or equivalent to remove any cell debris.

9. Transfer the supernatant containing the phages to a new 30 mL centrifuge tube, and centrifuge at 16,000 rpm (30,624 × *g*) for 45 min at 4 °C with SS34 rotor in Sorvall RC-5C plus centrifuge or equivalent to pellet the phages.

10. Discard the supernatant and resuspend the phage pellet in 2 mL Pi-Mg buffer.

11. Prepare CsCl gradient for phage purification. First, prepare layer buffer according to the table below (Table 1). Then, from the bottom to the top, sequentially add 750 μl of layer buffer No.6, No.5, No.4, No.3, No.2, and No.1 to a 5 mL Beckman centrifuge tube.

12. For each Beckman centrifuge tube, load 0.5 mL resuspended phage sample from step **10** onto the top of the CsCl gradient solution, centrifuge at 35,000 rpm (148,596 × *g*) for 1 h at 4 °C using SW55 Ti rotor in Beckman L-60 Ultracentrifuge or equivalent.

13. Fasten the Beckman centrifuge tube to a vertical holder after centrifugation. Pierce the wall of centrifuge tube at the bottom of the turbid phage band using a 5 mL syringe needle. Aspirate the phage band into the syringe.

14. Transfer the phage sample into a 3–12 mL Slide-A-lyzer dialysis cassette and dialyze first against dialysis buffer I for 5 h at 4 °C and then against dialysis buffer II overnight at 4 °C.

15. Collect the phage sample, quantify the concentration of phages, and store at 4 °C for future use. The following steps (steps **16–21**) will determine the concentration of the phage by SDS-PAGE with BSA (1 mg/mL) as a standard.

16. Mix equal volume of $Hoc^{-}Soc^{-}$ T4 phage or BSA with 2× SDS loading buffer and boil for 5 min.

17. Load 1, 2, 3, and 4 μl of $Hoc^{-}Soc^{-}$ phage, as well as 1, 2, 4, and 8 μg of BSA to different wells of an SDS-PAGE gel (4–20% Tris-Gly gel), and electrophorese according to the manufacturer's instructions.

18. Disassemble the gel and transfer it into a clean tray. Add Coomassie blue R-250 staining solution to the tray after rinsing with water, microwave for 1 min, and keep shaking gently at room temperature for 15 min.

19. Discard the Coomassie blue R-250 staining solution, add destain solution to the tray, microwave for 1 min, and keep shaking gently at room temperature until the background becomes clean.

20. Scan the gel with laser densitometry (PDSI, GE Healthcare) and quantify the protein bands with ImageQuant 5.2 software (GE Healthcare) according to the manufacturer's instructions.

21. Generate a BSA standard curve using Microsoft Excel with the numbers calculated in step **20** and calculate the concentration of Hoc^{-}Soc^{-} T4 phage based on the BSA standard curve (*see* Note 13).

3.4 Preparation of Antigen for Immunizations

The exact amount of Hoc^{-}Soc^{-} T4 phages and protein depends on how many animals will be used. The dose we mention here is for one animal (10 μg antigen/animal).

1. Take about 6.0×10^{11} phage particles and centrifuge at 15,000 rpm (21,130 × *g*) in 1.5 mL LoBind Eppendorf tubes for 45 min at 4 °C using AM 2.18 rotor in Jouan MR-23i centrifuge or equivalent (*see* Note 14).

2. Discard the supernatant and wash the pellet by adding 1.0 mL PBS and one more round of centrifugation as in step **1**.

3. Discard the supernatant, add 200 μl PBS to the tube, and leave it at 4 °C overnight to completely resuspend the phage pellet.

4. Add 1.16 mg Soc-PA to the resuspended phage, adjust the volume to 800 μl with PBS, gently vortex to mix, and incubate at 4 °C for 45 min.

5. Sediment the phage particles with Soc-PA bound at 15,000 rpm (21,130 × *g*) for 45 min at 4 °C using AM 2.18 rotor in Jouan MR-23i centrifuge or equivalent.

6. Wash the phage pellet containing the bound Soc-PA twice as in step **1**.

7. Add 50 μl PBS to the pellet, leave it at 4 °C overnight to completely resuspend the phage pellet, and analyze it by SDS-PAGE as described in procedures from steps **16** to **21** under Subheading 3.3. Determine the copy number of PA per capsid.

8. Immunize the animals by intramuscular injection with 10 μg T4-displayed PA. The immunization regimens, analyses of PA antibody and lethal toxin neutralizing antibody titers, and anthrax challenge models have been described previously [17, 18, 22].

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13 *Chloroform treatment*: Take 1 mL of culture in a test tube and add four drops of chloroform. If the cells are infected well, they lyse instantly, clearing the cell suspension, and cellular debris can be seen floating in the sample.

14 *Observation under light microscope*: Put a drop of culture on the chamber of the cell counter and cover it with a cover slip. Focus at individual *E. coli* cells by fine adjustment. The appearance of clear center and black/dark spots at the poles (ends) of the cells indicate good phage infection.

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Table 1

Prepare CsCl gradient layers for phage purification

Layer No.	Stock CsCl (mL)	H_2O (mL)	Total volume (mL)
1	1	4	5
2	1.5	3.5	5
3	2	3	5
4	2.5	2.5	5
5	3	2	5
6	3.5	1.5	5