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# Protein Crystallization of Two Recombinant Lpt Proteins


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## Abstract

The prerequisite for 3D structure determination of macromolecules via X-ray crystallography is well-ordered, diffracting crystals. Here, we report the recombinant production, biophysical/biochemical protein sample characterization, and vapor diffusion sitting drop crystallization protocols for two lipopolysaccharide transport proteins: LptH from *Pseudomonas aeruginosa* (*Pa*-LptH) and an inactive LptC mutant (G153R) from *Escherichia coli* (*Ec*LptC$_{24\text{-}191}$G153R).



## Key words

Protein crystallization
Vapor diffusion
Lipopolysaccharide transport protein
*E. coli*
Outer membrane biogenesis


SECTION1

## 1. Introduction

As documented by over 150 thousand crystal structures available in the protein structure repository, the Protein Data Bank (www.rcsb.org), X-ray crystallography remains to be the principal method of high-resolution 3D structure determination, despite significant progression in the single-particle cryo-electron microscopy field [ 1 ]. X-ray crystallography provides atomic-level

information that can help elucidate the molecular mechanisms of proteins and nucleic acids, providing insight into their biological roles and potential applications in numerous scientific sectors such as biomedical, biotechnology, energy, food, and agriculture sectors [ 2, 3 ].

There are several steps in 3D structure determination via X-ray crystallography. The first steps regard the preparation of milligram quantities of highly purified (>95%) protein samples of native or recombinant origin, followed by protein buffer optimization to ensure optimum protein stability and homogeneity, two factors that can directly influence successful crystallization. Once the best buffer has been selected, the protein is ready to be concentrated for crystallization trials. Despite advances in **AQ1** X-ray crystallography structure determination methods and hardware and significant evolution in beam quality at, now, fourth-generation, synchrotron light sources, the growth of well-ordered, diffraction-quality protein crystals remains the main bottleneck for high-resolution 3D structure determination. Not all proteins crystallize, and success has been reported to be as low as 5%, from estimates made by Structural Genomics programs, and the right conditions that lead to crystal formation still are largely based on trial and error [ 4 ]. The crystallization process involves bringing the protein sample to a supersaturated state that can be subdivided into three regions in the so-called solubility diagram, precipitation, labile, and metastable, relating to high, medium, and low levels of supersaturation. The labile zone promotes nucleation and crystal growth, whereas the metastable only supports crystal growth, and precipitation results in the formation of amorphous aggregates (precipitate) [ 5 ].

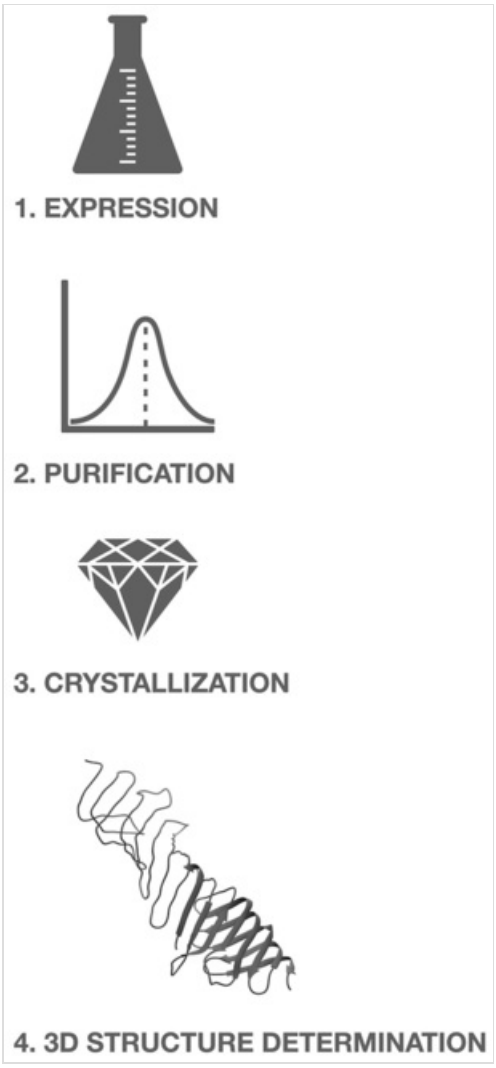
There are three main phases of crystallogenesis: (i) nucleation, whereby the most thermodynamically stable aggregates form critical nuclei that allow the second phase; (ii) crystal growth, which involves the further assembly of other protein molecules onto the nuclei surface; and (iii) termination of crystal growth that arises when all available protein molecules in solution have been contributed to crystal formation or if there is damage to the crystal surface that prevents further molecules to bind to the lattice [ 5 ].

Neither the optimum protein concentration nor the ideal crystallization conditions can be known a priori, and the process remains largely empirical; however, ensuring optimum protein construct design, eliminating flexible regions that can hinder lattice formation, and optimum sample preparation can increase the probability of success [ 6 ]. Moreover, the availability of high-throughput automated screening methods using crystallization robots, allowing thousands of crystallization conditions to be screened with minimal sample requirements, can also improve the odds of successful crystal growth.

Here, we report the recombinant expression and purification protocols for two lipopolysaccharide transport proteins, LptH from *Pseudomonas aeruginosa* (*Pa*-LptH; PDB entry 4UU4 [ 7 ]) and an inactive LptC mutant from *Escherichia coli* (*Ec*LptC$_{24\text{-}191}$G153R; PDB entry 4B54 [ 8 ]), followed by the biophysical analyses as preparatory steps, prior to protein crystallization, that led to their successful 3D structure determination via X-ray crystallography (*see* Fig. 1).

**Fig. 1**

Illustration of experimental workflow, from recombinant Lpt expression in bacterial cells, to purification via affinity chromatography and characterization, to protein crystallization and 3D structure determination via standard molecular replacement crystallographic methods




An image of four objects, 1 is a conical flask with scales mentioned on it and is named expression, 2 is a bell-shaped graph with its highest peak at the middle marked with a dotted line and is named purification, 3 is in the shape of a diamond and is named crystallization and 4 is a braided 3 D model named as 3 D structure determination.

SECTION1

# 2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical-grade reagents; all crystallization solutions should be filtered-sterilized with a syringe filter with a pore size of 0.2 μm. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials. Do not add sodium azide to reagents.

SECTION2

## 2.1. ~~Pa~~*Pa*-LptH and ~~Ec~~*Ec*LptC$_{24\text{-}191}$G153R)

1. Expression plasmid: pQE-30 bacterial vector (ampicillin resistance) (Qiagen) for the expression of *Pa*-LptH and *Ec*LptC$_{24\text{-}191}$G153R, fused to a N-terminal hexahistidine tag (*see* **Note 1**).
2. Cells: M15/pREP4 chemically *E. coli* Express competent cells with kanamycin resistance (Qiagen) (*see* **Note 2**).

3. Luria-Bertani broth Miller (LB): Weigh 1 g of tryptone, 1 g of NaCl, and 0.5 g of yeast extract, and transfer all to a 100 mL graduated beaker. Add 80 mL water and, using a magnetic stirrer, mix until the solutes have dissolved. Adjust the final volume to 100 mL. Transfer to a 500 mL Pyrex conical flask for sterilization. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm$^2$) on liquid cycle before usage.

4. Terrific broth (TB): Add approximately 100 mL of water to a 2 L graduated cylinder or a glass beaker (*see* **Note 3**). Weigh 18 g of tryptone, 36 g of yeast extract, 3.46 g of potassium phosphate monobasic ($KH_2PO_4$), and 18.88 g of potassium phosphate dibasic ($K_2HPO_4$), and add them to the cylinder. Add 30 mL of glycerol and water to a volume of 1.5 L. Transfer 800 mL of TB in two 2 L flasks. Cap the flasks with tin foil, apply autoclave tape, and sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm$^2$) on liquid cycle before usage. After sterilization, allow the solutions to cool to about 40 °C before adding antibiotics.

5. Luria-Bertani broth Lennox (LD) [ 9 ]: Weigh 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl in 1 L beaker. Add approximately 800 mL water, and dissolve using a magnetic stirrer. Transfer to a 1 L cylinder, and make up to 1 L with water. Transfer 500 mL to a 2 L glass culture flask. Cap the flasks with tin foil, apply autoclave tape, and sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm$^2$) on liquid cycle before usage. After sterilization, allow the solutions to cool to about 40 °C before adding antibiotics.

6. LB agar: Add 80 mL water to a sterile media bottle. Weigh 1 g of tryptone, 1 g of NaCl, 0.5 g of yeast extract, and 1.5 g of Bacto agar. Adjust the volume to 100 mL and shake (*see* **Note 4**). Transfer the solution to a 250 mL bottle. Apply autoclave tape to the bottles and flasks, and sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm$^2$) on liquid cycle before usage. After sterilization, allow the solutions to cool to below 40 °C before adding antibiotics. Add 100 µl of ampicillin and 100 µl of kanamycin solutions, and pour approximately 10 mL into labeled, sterile vented Petri dishes (diameter 10 cm). Allow to cool to room temperature. Once the media has solidified, seal the dishes with thermoplastic film (i.e., Parafilm), and store them upside down at 4 °C.

7. Antibiotics and isopropyl β-d-thiogalactopyranoside (IPTG): 50 mg/mL ampicillin stock solution: Add 0.5 g of ampicillin sodium salt to 10 mL water. 50 mg/mL kanamycin stock solution: Add 0.5 g of kanamycin sodium salt to 10 mL water. 0.5 M IPTG stock solution: Add 1.19 g IPTG to 10 mL water. Vortex to dissolve the solutes. Filter-sterilize the solutions (0.22 µm PVDF membrane), make aliquots, and store at 4 °C.

8. Sterile cell spreader.

9. Shaking incubator at 37 °C.

10. Stationary incubator at 37 °C.

11. Water bath or thermomixer set at 42 °C.

12. Ice bucket filled with ice.

13. Optical density (OD) cell reader at 600 nm wavelength.

14. Refrigerated, large-capacity centrifuge, rotor, and 500 mL centrifuge tubes.

SECTION2

## 2.2. Recombinant Lpt* Protein Purification

1. 1 M sodium potassium phosphate pH 8.0 stock solution: Prepare 500 mL of sodium phosphate monobasic ($NaH_2PO_4$) (weigh and dissolve 60 g of powder in 500 mL water) and 1 M sodium phosphate dibasic ($Na_2HPO_4$) (weigh and dissolve 70.98 g of powder in 500 mL of water). Add the appropriate volume of $NaH_2PO_4$ to $Na_2HPO_4$ until it reaches ~~the~~ pH ~~of~~ 8.0.

2. Buffer A (or lysis buffer). For *Pa*-LptH: 50 mM Tris-HCl pH 8.0, 0.3 M NaCl. Add about 100 mL of water to a 1 L glass beaker. Weigh 6.1 g of Tris-HCl and 17.53 g NaCl, and transfer to the beaker. Add water to a volume of 900 mL. Mix and adjust pH with HCl. Make up to 1 L with water. Store at 4 °C. For *Ec*LptC$_{24\text{-}191}$G153R: 50 mM sodium phosphate buffer pH 8.0, 0.3 M NaCl, 10 mM imidazole, and 10% *(v/v)* glycerol. Weigh 17.53 g of NaCl and 0.34 g of imidazole, and transfer to a 500 mL beaker. Dissolve in 400 mL of water, add 50 mL of glycerol and 25 mL of 1 M sodium potassium phosphate pH 8.0 stock solution, and make up to 500 mL.

3. 2 M magnesium sulfate ($MgSO_4$): Weigh 2.41 g of $MgSO_4$ in a 15 mL polypropylene tube. Dissolve in water and make up to 10 mL.

4. 2 mg/mL DNase: Weigh 0.02 g of deoxyribonuclease I from bovine pancreas in a 15 mL polypropylene tube. Dissolve in water and make up to 10 mL.

5. 50 mg/mL lysozyme solution: Weigh 0.5 g of lysozyme from chicken egg white, dissolve in water, and make up to 10 mL. Aliquot and store at −20 °C.

6. Protease and phosphatase inhibitor cocktails (Roche Complete EDTA free tablets): Dissolve one tablet per 100 mL of lysis buffer.

7. 200 mM phenylmethylsulfonyl fluoride (PMSF) solution. Dissolve 0.34 g of PMSF in 10 mL of isopropanol, and store at −20 °C (*see* **Note 5**).

8. Buffer B. For *Pa*-LptH: 50 mM Tris-HCl pH 8.0, 0.3 M NaCl, 500 mM imidazole. Add about 100 mL of water to a 1 L glass beaker. Weigh 6.1 g Tris-HCl, 17.53 g NaCl, and 34.04 g of imidazole, and transfer to the beaker. Add water to a volume of 900 mL. Mix and adjust pH with HCl. Make up to 1 L with water. Store at 4 °C. For *Ec*LptC$_{24\text{-}191}$G153R: 50 mM sodium potassium phosphate pH 8.0, 300 mM NaCl, 0.5 M imidazole, 10% *(v/v)* glycerol. Weigh 17.53 g NaCl and 17.02 g imidazole, and transfer to a 500 mL beaker. Dissolve in 400 mL water, add 50 mL glycerol and 25 mL 1 M sodium potassium phosphate pH 8.0 stock solution (*see* **Item 1**), and make up to 500 mL.

9. 5 M sodium hydroxide (NaOH) solution: For 100 mL, weigh and dissolve 20 g NaOH pellets in water and make up to 100 mL (*see* **Note 6**).

10. Buffer C (or crystallization buffer). For *Pa*-LptH: 25 mM Tris-HCl pH 7, 10 mM NaCl. Add about 100 mL water to a 1 L glass beaker. Weigh 6.1 g Tris-HCl and 8.77 g NaCl, and transfer to the beaker. Add water to a volume of 900 mL. Mix and adjust pH with 37% fuming HCl. Make up to 1 L with water. Store at 4 °C. For *Ec*LptC$_{24\text{-}191}$G153R: 10 mM HEPES pH 7.0, 50 mM NaCl. For 1 L, weigh 2.60 g HEPES sodium salt and 2.92 g NaCl, and dissolve in 800 mL water. Adjust pH to 7.0 with 5 M NaOH solution.

11. Vortex.

12. French pressure cell press or cell disrupter.

13. 50 mL syringes equipped with 0.45 µm syringe filters.

14. Refrigerated, large-capacity centrifuge and 40 mL centrifuge tubes.

15. Single-use 10 mL GraviTrap column (Cytiva). Column preparation: cut off the bottom tip, remove the top cap, add the provided filter, and place the column in a column stand.

16. Nickel Sepharose Fast Flow resin (Cytiva) (for *Pa*-LptH) and nickel-nitrilotriacetic acid (Ni-NTA) agarose *Ec*LptC$_{24\text{-}191}$G153R (Qiagen). Add 1 mL of resin into the GraviTrap column. Allow the 20% *(v/v)* ethanol, where the resin is stored, to drip. Wash with 10 mL of water, and equilibrate the resin with 10 mL protein-specific Buffer A.

17. Dialysis membrane: Use a Spectrum™ Spectra/Por™ 3 RC Cellulose Dialysis Membrane Tubing 3500 Dalton MWCO (*see* **Note 7**).

18. Magnetic stirrer and a stirring bar.

19. 1.5 mL microcentrifuge tubes.

20. Eppendorf® Centrifuge 5702 or any standard, refrigerated benchtop centrifuge, suitable for 15 ml tubes.

21. Amicon® Ultra 15 mL filter device with a MW cut-off (MWCO) of 3000.

22. NanoDrop Spectrophotometer Instrument.

SECTION2

## 2.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 4× NuPAGE LDS Sample Buffer (Invitrogen) supplemented with 0.4 M 2-mercaptoethanol: Add 6 µl of 14.3 M 2-mercaptoethanol to 200 µl of LDS Sample Buffer.
2. 1.5 mL microcentrifuge tubes.
3. Thermomixer.
4. Precast 12% ExpressPlus™ PAGE Gels (GenScript).
5. Novex XCell SureLock™ Gel Tank for precast gel.
6. Ready-to-use StoS prestained protein ladder 10–245 ~~K~~kDa (Genespin).
7. 1× running buffer: Dissolve one pack of Tris-MOPS-SDS Running Buffer Powder (GenScript) in 1 L deionized water.
8. Stain solution: 0.01% *(w/v)* Coomassie Brilliant Blue, 40% *(v/v)* ethanol, 10% *(v/v)* acetic acid. Add 100 mL of acetic acid to 450 mL of water. Dissolve 3 g of Coomassie R-250 dye in 450 mL ethanol. Mix the acetic acid and ethanol solutions. Filter the solution before use (optional).
9. Destain solution: 30% *(v/v)* ethanol, 10% *(v/v)* acetic acid. Add 300 mL of ethanol and 100 mL of acetic acid to 600 mL of water and mix.
10. Power supply.
11. Orbital shaker.

SECTION2

## 2.4. Dynamic Light Scattering (DLS)

1. Quartz cuvettes. Cuvette preparation: Rinse cuvette first with filtered water (at least three times), and then dry it gently blowing into it using compressed air. Wipe the cuvette from the outside with lens paper to remove any dust. Avoid scratching the quartz sides of the cuvette.
2. Protein Solutions DynaPro 99 instrument with a DynaProMSTC200 microsampler (Protein Solutions, Charlottesville, VA, USA).
3. Eppendorf Centrifuge 5424 R or any standard benchtop microcentrifuge.

SECTION2

## 2.5. Crystallization

1. Oryx-8 nanodispenser crystallization robot (Douglas Instruments, East Garston, UK).
2. Commercial crystal screens (Hampton crystal screen, Hampton Research, and Wizard™, Emerald BioSystems, Stura Footprint Screen) (Molecular Dimensions).
3. CrystalQuick™ 96-well 3-drop, flat-bottomed sitting drop crystallization plates (Greiner).
4. ClearVue Sealing Sheets (Molecular Dimensions).
5. Stereo microscope.
6. Eppendorf Centrifuge 5424 Refrigerated.

SECTION1

# 3. Methods

Carry out all procedures at room temperature, unless otherwise specified. Keep protein solutions on ice.

SECTION2

## 3.1. Recombinant Lpt* Protein Production in *E. coli* Cells

1. Transform the chemically competent M14/pREP4 cells using the heat-shock method. Add 1–5 µl pQE-30 plasmid containing *Pa*-LptH or *Ec*LptC$_{24\text{-}191}$G153R gene (usually 1–100 ng) to 50 µl M15/pREP4 competent cells thawed on ice (*see* **Note 8**). Incubate the competent cell/DNA mixture on ice for 30 s. Heat shock by placing the tubes into a 42 °C water bath for 90 s. Put the tubes back on ice for 3 min. Add 800 µl of LB (without antibiotic) to the bacteria, and grow in a 37 °C shaking incubator for 1 h. Plate 100 µl of the transformation reaction using a sterile spreading device onto a 10 cm LB agar Petri dish containing 50 µg/mL of ampicillin and 50 µg/mL of kanamycin, and leave to dry (*see* **Note 9**). Label the plates with the date and name of the protein. Invert the plates and incubate it at 37 °C overnight in a stationary incubator.

2. Prepare the starter culture: Add 100 µl of 50 mg/mL ampicillin and 100 µl of 50 mg/mL kanamycin solutions to 100 mL LB in a 500 mL Pyrex conical flask. Pick a single colony of the transformed M15/pREP4 from a freshly streaked transformation plate (from **step 1**), and transfer to 100 mL LB. Grow the starter culture at 37 °C with shaking at 220 rpm, overnight. The procedure is identical for *Ec*LptC$_{24\text{-}191}$G153R except for LD broth instead of LB (*see* **Note 10**).

3. Add 800 µl of 50 mg/mL ampicillin and 800 µl of 50 mg/mL kanamycin solutions to 800 mL TB (or LD for *Ec*LptC$_{24\text{-}191}$G153R) in a 2 L glass, conical flask (*see* **Note 11**). Inoculate the TB with 10 mL starter culture. Incubate at 37 °C with shaking (*see* **Note 12**). Measure optical density (OD) of the culture every hour with an optical density (OD) cell reader at a wavelength of 600 nm until $OD_{600}$ reaches 0.6–0.8. Then cool the cultures to 25 °C, and induce the expression of the recombinant protein by the addition of 0.5 mM IPTG. Incubate with shaking at 220 rpm overnight.

4. Transfer the cell culture to clean 500 mL centrifuge tubes, and harvest the cells at 6000 × *g* for 15 min at 4 °C using a large-capacity centrifuge (*see* **Note 13**).

5. Discard the supernatant, use bacterial pellets immediately, or store them at −80 °C for long-term storage avoiding freeze-thaw cycles. For *Ec*LptC$_{24\text{-}191}$G153R, resuspend the cell pellet from 500 mL culture in 50 mL lysis buffer and transfer to a 50 mL polypropylene tube and store at −80 °C. Thaw overnight at 4 °C before use (*see* **Note 14**).

SECTION2

## 3.2. Recombinant Lpt* Protein Purification

1. Add 500 µL of DNAse solution and 1 mL of 2 M $MgSO_4$ solution to 100 mL lysis buffer. Dissolve one tablet of protease and phosphatase inhibitor cocktail (*see* **Note 15**). Add the final lysis buffer to the pelleted cells from **step 5** in Subheading 3.1 using vortex or manual pipetting until the cells are completely resuspended. Store on ice. For *Ec*LptC$_{24\text{-}191}$G153R, add 250 µL of PMSF solution (1 mM final concentration) to 50 mL thawed pellet resuspension.

2. Lyse cells using a French press cell disruptor at 25 kpsi of pressure. Incubate 30 min on ice.

3. Clarify the lysate by centrifugation at 30,000 × *g* for 30 min. Filter the supernatant through a 0.45 µm syringe filter, and discard the pellet (*see* **Note 16**).

4. Add the resulting supernatant to 1 mL Nickel Sepharose Fast Flow resin prepared as described in **Item 16** of Subheading 2.2 and allow the column to drain and wash the resin with 10 mL of Buffer A. Collect the resulting flow-through in a clean beaker or cylinder and all the following purification fractions in clean tubes. Elute *Pa*-LptH with 10 mL of 50% Buffer B (mixing in a clean tube 5 mL of Buffer A and 5 mL of Buffer B; the final elution buffer composition is 50 mM Tris-HCl pH 8, 300 mM NaCl, 250 mM imidazole). Wash the resin with 10 mL of Buffer B. Elute *Ec*LptC$_{24\text{-}191}$G153R using a stepwise imidazole gradient made by mixing Buffer B with Buffer A in five distinct steps (10, 20, 50, 70, and 100% Buffer B). At each step, 1 column volume is passed down the column. Collect 1 mL fractions in 1.5 mL microcentrifuge tubes, and place on ice.

5. Dialyze the fractions containing pure Lpt* proteins (as judged by SDS-PAGE; *see* Subheading 3.3) overnight into Buffer C using a dialysis tubing membrane (*see* **Note 17**). Cut the membrane at the required length adding extra length to be able to tie a knot at either end (*see* **Note 18**). Soak cleaved membrane in water for 10 min. Close the bottom of the tube with a knot, then gently open the top of the membrane with gloved fingers, and insert the pipette tip. Slowly dispense the fractions containing the Lpt* proteins in the dialysis tube, and close the top with a second knot. Transfer the dialysis tube into a beaker or cylinder containing 1 L of Buffer C. Put a stirring bar in the beaker, and gently stir the solution overnight at 4 °C.

6. Concentrate *Pa*-LptH to 4 mg/mL and *Ec*LptC$_{24\text{-}191}$G153R to 22.3 mg/mL using an Amicon Ultra centrifugal filter device with 3000 MW cut-off (*see* **Notes 19** and **20**). Fill the filter device with eluted protein sample, and place it capped into a centrifuge (swinging bucket is preferred). Spin the device at 4000 rpm at 4 °C until the protein sample reaches the desired concentration.

7. Measure protein concentration using a NanoDrop instrument pipetting 2 µl of Lpt* protein solution directly onto the pedestal. Calculate protein concentration using the theoretical protein extinction coefficient (molar absorption) at 280 nm, calculated from the protein sequence using Expasy Protparam (https://web.expasy.org/protparam/) and the Beer-Lambert law (*see* **Note 21**).

8. Check the purity of the protein preparation by SDS-PAGE as described in Subheading 3.2.

SECTION2

## 3.3. SDS-PAGE

1. Sample preparation: add 4 µL of 4× NuPAGE Sample Buffer supplemented with 2-mercaptoethanol to 10 µL Lpt protein samples collected during the purification steps (*see* **step 4** in Subheading 3.2) in clean 1.5 mL microcentrifuge tubes. Denature samples for 10 min at 95 °C in a thermomixer. Centrifuge the tubes for 30 s at 10,000 × *g*.

2. Gel preparation: remove the plastic package, remove the sealing tape at the bottom, and then gently remove the comb. Place the gel in the Novex XCell SureLockTM Gel Tank. Pour 30 mL 1× running buffer into the inner chamber until the wells are filled with running buffer, and check for possible leaks. Fill the external chamber with buffer.

3. Load 12 µL protein samples in the different wells of the gels leaving a well for the 10 µL StoS prestained protein marker.

4. Connect the electrophoresis cell to the power supply, and perform electrophoresis according to the following conditions: run condition 160–180 V (current per gel should be lower than 100 mA) until the bromophenol blue present in the sample buffer reaches the lower limit of the gel (*see* **Note 22**).

5. After electrophoresis is complete, turn the power supply off, and disconnect the electrical leads. Open the gel cassette by prising apart the plastic cast with force and gently remove the gel and transfer to a plastic container containing water and a resealable lid.

6. Wash the gel with water. Then transfer to approximately 25 mL of stain solution, and incubate at room temperature with shaking for 20 min. Discard the staining solution (reusable) and place the gel in the destain solution and incubate with shaking until the protein bands are clearly visible (*see* **Note 23**). Store the gel in water.

7. Determine the purity of the protein sample by visual inspection. Carry on the characterization of the protein solution if the sample is at least 90–95% pure.

SECTION2

## 3.4. Dynamic Light Scattering (DLS)

1. Centrifuge purified Lpt protein solutions from **step 6** in Subheading 3.2, at 13,000 × *g* for 10 min in a refrigerated benchtop microcentrifuge prior to DLS analysis (*see* **Note 24**). After centrifugation, carefully remove the solution, taking care not to displace any potential pelleted debris (*see* **Note 25**).

2. Switch on the DynaPro 99 instrument and the temperature-controlled sample unit. Set the desired temperature (10 °C). Give the laser at least 20 min to warm up.

3. Add 60 µL Lpt protein sample (corresponding approximately to a concentration higher to 1 mg/mL) to a clean cuvette, and insert the cuvette into the sample holder of the DLS instrument, positioning it with the frosted side in the suggested orientation (depending on the instrument). Equilibrate the sample to experiment temperature for at least 5 min.

4. Start data acquisition with 20 scans, 30 s/scan, depending on the instrument.

SECTION2

## 3.5. Protein Crystallization

1. Centrifuge purified Lpt* protein solution from **step 6** in Subheading 3.2 at 13,000 × *g* for 10 min at 4 °C in a refrigerated benchtop microcentrifuge (*see* **Note 26**).

2. Test several commercial crystal screen solutions that screen a variety of pH, precipitant types and concentrations, cations, anions, and additives (*see* **Note 27**). Fill reservoirs of the 96-well sitting drop plates with 100 μL each crystallization solution (*see* **Notes 28** and **29**).

3. Sitting drop vapor diffusion crystallization trials are performed using an Oryx-8 nanodispenser robot at 20 °C. Dispense three, 0.5 μL drops at three different *Pa*-LptH/precipitant ratios (25%–50%–75% of protein): in the first drop, mix 0.125 μL of protein and 0.375 μL of reservoir solution (provided by the kit); in the second drop, mix 0.25 μL of protein and 0.25 μL of reservoir solution; and in the last drop, mix 0.375 μL of *Pa*-LptH and 0.125 μL of reservoir solution. Analogous drops are prepared for *Ec*LptC$_{24\text{-}191}$G153R but at protein concentrations of 30, 50, and 70%.

4. Seal plates with optically transparent ClearVue Sealing Sheets, and incubate at 20 °C. Monitor crystal~~s'~~s growth after 2, 5, and 10 days using a stereo microscope. Score observations (*see* **Note 30**).

5. Prior to X-ray diffraction tests at a synchrotron light source, cryoprotect crystals before flash freezing in liquid nitrogen (*see* **Note 31**). Cryoprotect *Pa*-LptH and *Ec*LptC$_{24\text{-}191}$G153R crystals in ~~a~~ reservoir solution, supplemented with 25% *(v/v)* and 30% *(v/v)* glycerol, respectively. It may be necessary to optimize crystal growth for poorly diffracting crystals by refining precipitant concentration and/or pH (*see* **Note 32**).

SECTION1

# 4. Notes

1. For a new protein, we routinely carry out small-scale (10 mL) expression at different induction temperatures, expression strains, and growth medium. After testing different plasmids/strains, we found that the pQE30 plasmid in M15/pREP4 is the best for the expression of periplasmic Lpt proteins. In this protocol, the periplasmic domains of the *Pa*-LptH and *Ec*LptC$_{24\text{-}191}$(G153R) proteins, devoid of the native signal sequence and transmembrane domain, respectively, are expressed in the cytoplasm of *E. coli*. It is worth mentioning that, in the case of *Ec*LptC$_{24\text{-}191}$(G153R), the use of an inactive mutant of LptC [ 10 ] resulted in increased crystallization success over the wild-type version of the protein, likely as a consequence of lowered flexibility of the protein structure.

2. pQE vectors and constructs can be maintained in *E. coli* strains that carry the pREP4 repressor plasmid. This plasmid allows tightly regulated gene expression from pQE vectors.

3. Having water at the bottom of the cylinder allows the magnetic stirring bar to work immediately, accelerating the dissolution of the added powder.

4. Bacto agar dissolves during the sterilization process.

5. PMSF is an inhibitor of serine proteases including trypsin, chymotrypsin, and thrombin that can degrade the target protein after cell lysis. PMSF is toxic. Wear a mask to prevent inhalation and wear gloves.

6. Dissolving NaOH in water is highly exothermic; therefore, use safety goggles and gloves to avoid burns.

7. Dialysis membrane is susceptible to many cellulolytic microorganisms; therefore, it is recommended to use always gloves when handling the membrane.

8. Gently flick the bottom of the tube with the fingers; do not vortex or pipette the solution up and down.

9. Remove agar plates from storage at 4 °C at the beginning of the procedure, and let them warm up to room temperature. Do not leave too much liquid medium on the agar plates: if the culture volume is too big, gently collect the cells by centrifugation, or remove the exceeding volume with a pipette.

10. We found that growing the cells in lower salt medium (LD) improved *Ec*LptC$_{24\text{-}191}$G153R protein expression.

11. Terrific broth (TB) is a nutritionally rich medium with increased concentration of peptone and yeast extract which provides glycerol as carbon source. The presence of potassium phosphate in the medium ensures balancing of the pH preventing cell death due to drop in the pH, whereas glycerol is added instead of glucose to avoid acetic fermentation. TB supports extended growth phase improving recombinant protein production.

12. Pay attention to the turbidity of the culture and starter culture. If the culture is too clear, cells are likely dead or contaminated. Start again from a single colony from a freshly streaked plate.

13. After centrifugation, if the cells are not completely pelleted at the bottom of the tube and cell clumps are floating or attached to the tube walls, they are likely dead or contaminated. Start again from a colony from a freshly streaked plate.

14. If the bacterial pellet is not required immediately, it may be stored at -80 °C. On the day of purification, thaw the pellet on ice before proceeding with the purification protocol.

15. If the cells resuspended in the lysis buffer are viscous and look like a “blob”: (i) add more DNAse solution (250 µL); (ii) you may have forgotten $MgSO_4$, which is crucial for DNase activity. Leave on ice for at least 30 min. You can add DNAse after mechanical lysis, to avoid foaming during lysis. Leave on ice for at least 30 min after cell disruption.

16. Sometimes, filtering the supernatant is difficult if the solution is too viscous, usually because of present DNA and RNA. You can add more DNAse and $MgSO_4$ or sonicate for a few minutes before filtering.

17. It is recommended to avoid combining different purification batches when performing crystallization trials as purification conditions are never identical and this can negatively affect the reproducibility. Screen each protein batches individually.

18. The minimal length of dialysis membrane to be used for 10 mL of solution is about 20 cm, but this calculation may vary depending on the dialysis membrane used.

19. Before use, to remove any preservatives that may damage the protein, wash the membrane of the centrifugal concentrator by adding 10 mL of water and spinning at 4000 rpm for 15 min.

20. Protein concentration recommended for initial characterization is approximately 10 mg/mL. This concentration may vary, depending on the solubility of the protein.

21. The expression of the Beer-Lambert law is $A = \varepsilon l c$, where ε is the molar absorptivity of the protein; l is the path length of the cuvette (1 cm); and $c$ is the protein concentration. When calculating molar absorptivity, remember to include vector regions in the sequence as some vector regions can add a significant number of amino acids to the polypeptide; therefore, they should be considered.

22. Do not let bromophenol blue escape completely from gel. Low molecular weight proteins or peptides may be lost.

23. Destain solution can be reused by passage through a filter lined with filter paper filled with activated charcoal.

24. To increase the rate of success of a crystallization experiment, the protein should be as pure, homogeneous, and monodisperse as possible. The presence of heterogeneity in protein solutions can impair crystal growth or result in crystals unsuitable for X-ray structural analysis.

25. Samples must be free of any particulates like dust, unwanted aggregates, and bubbles that will affect light scattering.

26. Sometimes, not centrifuging the sample before crystallization trials is another variable that should be assessed.

27. To avoid repeatedly opening commercial screen tubes, thus increasing evaporation of conditions that contain volatile solvent, prepare 2 mL screens by dispensing 20~~1~~00 µL of solution in the wells of 96-deep-well plates, sufficient for 20 screens.

28. Equilibrate the sitting drop plate containing the crystallization screen conditions prior to dispensing drops, to avoid condensation forming once the plate is sealed and placed at 20 °C.

29. Crystals of *Pa*-LptH grow in different conditions of Hampton crystal screen, Hampton Research, and Wizard™, Emerald BioSystems. *Ec*$LptC_{24\text{-}191}$G153R crystals grow after 1 week in the Stura Footprint Screen 3.1.

30. The purpose of crystallization trials is identifying a condition that brings a protein to its saturation limit that support intermolecular interactions that lead to crystal formation. The outcomes of the screens performed should carefully be recorded and rationalized to identify any variable that alters protein solubility.

31. As soon as the wells are opened, add 2 µL of reservoir solution to the drops containing crystals to ensure that they do not dry out, inducing salt formation if present in the crystallization solution. During cryoprotection, transfer a 10 µL-drop of reservoir solution onto a glass coverslip, and transfer the crystals into the drop. Remove 2.5 µL and add 2.5 µL of reservoir solution containing glycerol. Leave for a few minutes. Repeat this process. After the third time, transfer the crystal to another drop of cryoprotectant solution. This avoids potential damage to the crystal upon repeated transfer to serial drops.

32. If crystal optimizations are unsuccessful using homemade stock solutions, it may be necessary to purchase stock solutions from the manufacturer of the screen in which crystal growth is observed.

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