Chapter 16

Purification of Histone Lysine Methyltransferase SMYD2 and Co-Crystallization with a Target Peptide from Estrogen Receptor α

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Abstract

Methylation of estrogen receptor α by the histone lysine methyltransferase SMYD2 regulates ERα chromatin recruitment and its target gene expression. This protocol describes SMYD2 purification and crystallization of SMYD2 in complex with an ERα peptide. Recombinant SMYD2 is overexpressed in *Escherichia coli* cells. After release from the cells by French Press, SMYD2 is purified to apparent homogeneity with multiple chromatography methods. Nickel affinity column purifies SMYD2 based on specific interaction of its 6×His tag with the bead-immobilized nickel ions. Desalting column is used for protein buffer exchange. Gel filtration column purifies SMYD2 based on molecular size. The entire purification process is monitored and analyzed by SDS-polyacrylamide gel electrophoresis. Crystallization of SMYD2 is performed with the hanging drop vapor diffusion method. Crystals of the SMYD2–ERα peptide complex are obtained by microseeding using seeding bead. This method can give rise to large size of crystals which are suitable for X-ray diffraction data collection. X-ray crystallographic study of the SMYD2–ERα complex can provide structural insight into posttranslational regulation of ERα signaling.

Key words Proteinpurification, Chromatography, Nickel affinity, Gel filtration, Crystallization, Seeding, X-ray crystallography

1 Introduction

X-ray crystallography is the most powerful method for characterizing the three-dimensional structure of biological molecules, such as proteins, to atomic resolution. X-ray crystal structures not only allow for a deeper understanding of protein function, but also can serve as the basis for structure-aided drug discovery against disease. However, structure determination by X-ray crystallography requires milligram quantities of highly purified protein and a crystal formed from the protein. *Escherichia coli* (*E. coli*) is one of the most popular hosts for recombinant protein production since it provides high-level expression and is rapid and easy to use. Expressed proteins are often purified from *E. coli* with multiple chromatography steps in

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order to achieve adequate purity. In this protocol, nickel affinity and gel filtration chromatography are employed to purify the histone lysine methyltransferase SMYD2 into apparent homogeneity. Nickel affinity chromatography separates proteins based on a specific interaction between poly-histidine-tagged proteins and the nickel ions immobilized to a chromatography matrix. Gel filtration, which is also called size exclusion, separates proteins based on the size of the molecules. Crystallization of purified proteins is performed with the hanging-drop vapor diffusion method at room temperature. Vapor diffusion allows for a gradual and gentle increase in protein and precipitant concentration, which slowly creates supersaturation and facilitates the growth of large and well-ordered crystals. However, crystallization remains as the main bottleneck of X-ray crystallography due to the complex nature of proteins. Protein crystallization is essentially a trial and error process that requires large-scale screening of hundreds or even thousands of crystallization conditions. One of the important techniques to facilitating crystallization is crystal seeding, which separates nucleation from growth by introducing crystal seeds to remove the need for spontaneous nucleation [1]. This technique is employed to obtain crystals of SMYD2 in complex with an estrogen receptor α (ERα) peptide.

SMYD2 methylates ERα at lysine 266 [2]. This methylation prevents ERα from binding to the chromatin, thereby negatively affecting ERα target gene expression [2]. In order to understand the molecular basis of this methylation, a viable approach is to determine the structure of the SMYD2–ERα peptide complex. By analyzing this structure, the binding specificity of the SMYD2–ERα interaction can be elucidated at the atomic level [3]. This protocol will describe SMYD2 cocrystallization with an ERα peptide, which is a prerequisite step for X-ray structure determination.

2 Materials

2.1 Stock Solutions

1. 2000× Streptomycin solution (100 mg/mL): Dissolve 1 g streptomycin in 10 mL deionized water. Filter-sterilize with 0.22 μm filter. Store at −20 °C (*see* **Note 1**).
2. 1 M Tris–HCl, pH 8.0 solution (1 L): Dissolve 121.1 g of Tris base in 800 mL deionized water. Adjust the pH to 8.0 with concentrated HCl. Add deionized water to 1 L. Autoclave.
3. 5 M NaCl (1 L): Dissolve 292.2 g NaCl in 800 mL deionized water. Add deionized water to 1 L. Autoclave.
4. 40 % glycerol solution (1 L): Mix 400 mL glycerol with 600 mL deionized water. Autoclave and store at room temperature.
5. 10× ST buffer: 100 mM Tris–HCl, pH 8.0, and 1 M NaCl. Store at room temperature or 4 °C.

6. 0.4 M Isopropyl β-d-1-thiogalactopyranoside (IPTG) (1 mL): Dissolve 953.2 mg IPTG in 1 mL deionized water. Filter-sterilize with 0.22 μm filter. Store at −20 °C.
7. 100 mM Phenylmethylsulfonyl fluoride (PMSF) (100×): Dissolve 17.4 mg PMSF in 1 mL methanol. Store at −20 °C.
8. DNase I (2 mg/mL): Dissolve 5 mg DNase I in a ice-cold solution made of 312.5 μL 80 % glycerol, 18.75 μL 5 M NaCl, and 2.169 mL deionized water. Store at −20 °C.
9. 0.5 M $MgCl_2$ (500 mL): Dissolve 23.8 g $MgCl_2$ in 400 mL deionized water. Add deionized water to 1 L. Filter-sterilize with 0.22 μm filter. Store at room temperature.
10. 0.4 M NaH_2PO_4 stock (1 L): Dissolve 55.2 g NaH_2PO_4 in 1 L deionized water. Autoclave and store at room temperature.
11. 0.4 M Na_2HPO_4 stock (1 L): Dissolve 56.9 g Na_2HPO_4 in 1 L deionized water. Autoclave and store at room temperature.
12. 2 M Imidazole, pH 7.4 (1 L): Dissolve 130.6 g imidazole in 800 mL deionized water. Adjust the pH to 7.4 with concentrated HCl. Add deionized water to 1 L. Filter-sterilize with 0.22 μm filter. Store at room temperature.
13. 0.2 M sodium phosphate buffer, pH 7.4 (500 mL): Mix 95 mL of 0.4 M NaH_2PO_4 with 405 mL of 0.4 M Na_2HPO_4. Add deionized water to 500 mL. Adjust pH if necessary. Filter-sterilize with 0.22 μm filter. Store at room temperature.
14. Luria Broth (LB) medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1 L deionized water. Sterilize by autoclaving.
15. 1 M Dithiothreitol (DTT) stock: Dissolve 1.5 g DTT in 8 mL deionized water. Adjust the volume to 10 mL with deionized water. Store at −20 °C.
16. 5 mM ERα peptide stock: GGRMLKHKRQR (11 aa, residues 261–271). Dissolve 1.367 mg ERα peptide in 200 μL deionized water. Store at −20 °C (*see* **Note 2**).

2.2 Protein Expression

1. Plasmid carrying SMYD2 with N-terminal 6×His-SUMO tag [3].
2. Competent *E. coli* BL21 Codon Plus (DE3) cells.
3. Cell culture dishes.
4. 2.8 L-Fernbatch flasks.
5. Refrigerated incubator shaker.
6. UV/Vis spectrophotometer.
7. Centrifuges.
8. Vortex mixer.
9. French Press (Thermo Scientific) or sonicator.

10. 250 mL centrifuge tubes.
11. 50 mL sterile centrifuge tubes.
12. 0.2 μm filter.

2.3 Protein Purification

1. HisTrap HP column: A ready-to-use Ni^{2+} affinity column for preparative purification of a His-tagged recombinant protein.
2. HiPrep 26/10 Desalting column: For protein buffer exchange.
3. HiLoad 16/60 Superdex 200 prep grade column: Gel filtration (or size exclusion) column designed for preparative protein purification.
4. ÄKTA FPLC Protein Purification System (GE Healthcare) or equivalent.
5. HisTrap column-binding buffer (1 L): 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole, 5 % glycerol, 1 mM BME (*see* **Notes 3** and **4**).
6. HisTrap column-elution buffer (1 L): 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 500 mM imidazole, 5 % glycerol, 1 mM BME.
7. Gel filtration buffer (1 L): 10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 5 % glycerol, 1 mM BME.
8. Fraction collector.
9. 10 mL Test tubes.
10. Gel electrophoresis system.
11. 13 % SDS polyacrylamide gels.
12. Yeast SUMO protease 1 (ULP1): 1 mg/mL.
13. Microcentrifuge tubes.
14. 30K-molecular weight cutoff concentrators.

2.4 Crystallization

1. Protein sample buffer: 10 mM HEPES, pH 7.5, 50 mM NaCl, 5 % glycerol, 2 mM DTT. Store at 4 °C.
2. Well solutions or precipitant solutions: *S1*: 15 % polyethylene glycol (PEG) 8000, 50 mM NaCl, 0.1 M Tris–HCl, pH 8.5 for crystallization of the SMYD2–AdoHcy binary complex (AdoHcy, S-adenosyl-L-homocysteine, cofactor product). *S2*: 0.1 M Tris–HCl, pH 7.5, 20 % PEG3350, 5 % ethanol for cross-seeding of the SMYD2–AdoHcy–ERα ternary complex.
3. Buffer exchange columns such as the Micro Bio-Spin Chromatography Columns (Bio-Rad).
4. 10 mM S-adenosyl-L-homocysteine (AdoHcy) stock.
5. 24-Well crystallization plate.
6. Dow Corning vacuum grease.
7. Siliconized glass cover slides.
8. Cooled crystallization incubator.

9. Seed Bead (Hampton Research).
10. Cat whisker or MicroGripper (MiTeGen).
11. Stereomicroscope (magnification: 5–80×).

3 Methods

3.1 Large-Scale Protein Production (*See* Note 5)

1. Transform recombinant SMYD2, which consists of an N-terminal 6×His-SUMO tag (~12 kDa), into *E. coli* BL21 Codon Plus (DE3) cells [3–6].
2. Prepare a starter cell culture: Take a single colony from a freshly streaked plate or use a freezer glycerol stock. Inoculate in 5 mL LB media supplemented with 50 μg/mL streptomycin. Shake at 250 rpm, 37 °C, overnight.
3. Prepare 500 mL LB media in a 2.8 L-Fernbatch flask. Add streptomycin to a final concentration of 50 μg/mL. Inoculate 5 mL overnight starter culture (from **step 2**, Subheading 3.1) into the media.
4. Shake at 280 rpm and at 37 °C until the optical density at 600 nm (OD600) reaches between 0.4 and 0.6.
5. Pause the shaker and gradually lower the temperature to 15 °C by 5 °C intervals and hold for 10 min each. Hold at 15 °C for 30 min. Add IPTG to a final concentration of 0.1 mM. Shake at 250 rpm, 15 °C, overnight or 18–20 h (*see* **Note 6**).

3.2 Harvest Cells

1. Transfer the cell culture to 250 mL centrifuge tubes. Centrifuge at 4500 × *g*, 4 °C, for 30 min. Collect cell pellet and discard supernatant.
2. Add 20–30 mL ice-cold ST buffer to the cell pellet. Vortex until the cells are completely resuspended.
3. Transfer the cell suspension to 50 mL sterile centrifuge tubes. Centrifuge at 3200 × *g*, 4 °C, for 20 min. Collect cell pellet and discard supernatant.
4. Proceed to the next step for cell lysis or store the cell pellet at −20 °C for later use.

3.3 Lyse Cells

1. Thaw a frozen cell pellet on ice or use the freshly prepared cell pellet (**step 4**, Subheading 3.2).
2. Add 30 mL lysis buffer to the cell pellet.
3. Then add 300 μL 100× PMSF (protease inhibitor) to a final concentration of 100 μg/mL. The volume of the lysis buffer depends on the capacity of the French Press pressure cells (*see* **Note 7**).
4. Vortex to resuspend the cells. *From this step forward, the sample MUST be kept on ice or at 4 °C to avoid protein degradation.*

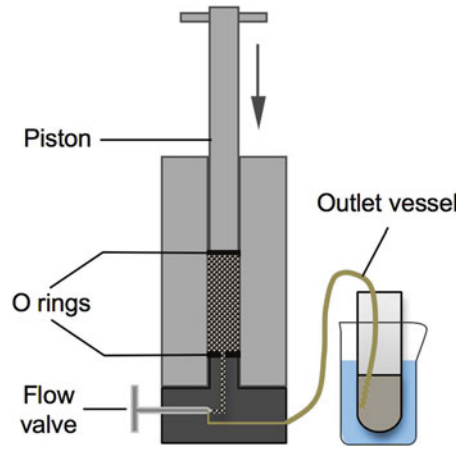

Fig. 1 French Press pressure cell

5. Lyse the cells with the French Press which breaks the plasma membrane with high mechanical pressure (Fig. 1). Collect the cell lysate (*see* **Notes 8** and **9**).
6. Add 150 µL 2 mg/mL DNase I and 300 µL 0.5 M $MgCl_2$ per 30 mL cell lysate. Incubate on ice for 15–20 min (This step can be skipped if sonication is used to lyse the cells) (*see* **Note 9**).
7. Centrifuge at 21,000 × *g*, 4 °C, for 20 min to remove insoluble cell debris.
8. Transfer the supernatant into a clean beaker.
9. Filter the supernatant with a 0.2 µm filter.

3.4 Protein Purification

1. Equilibrate a HisTrap HP column with the column-binding buffer (*see* **Note 10**) (Fig. 2).
2. Load the filtered sample (from **step 9**, Subheading 3.3) onto the column.
3. Wash with the buffer until the A_{280} value reaches a steady baseline.
4. Elute the column with the elution buffer using a linear gradient. Five column volumes are usually sufficient for elution, but a shallow gradient, such as a gradient over 20 column volumes or more, may increase resolution and separation. Collect elution fractions into test tubes (Fig. 3a).
5. Analyze the elution using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
6. Pool the fractions containing a high amount of SMYD2 (Fig. 3a).
7. Add yeast SUMO protease 1 (1 µg per 10 mg of substrate) to the pooled fractions. Incubate at 4 °C overnight. This step is used to cleave off the 6×His-SUMO tag from the protein.

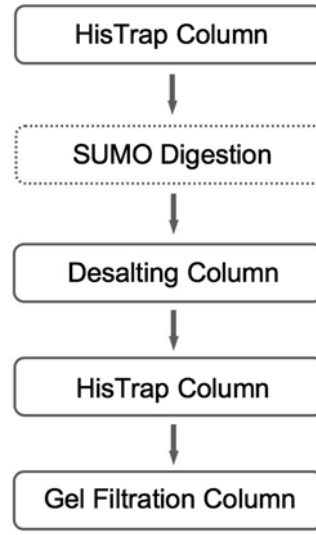

Fig. 2 General procedure of multi-step FPLC protein purification

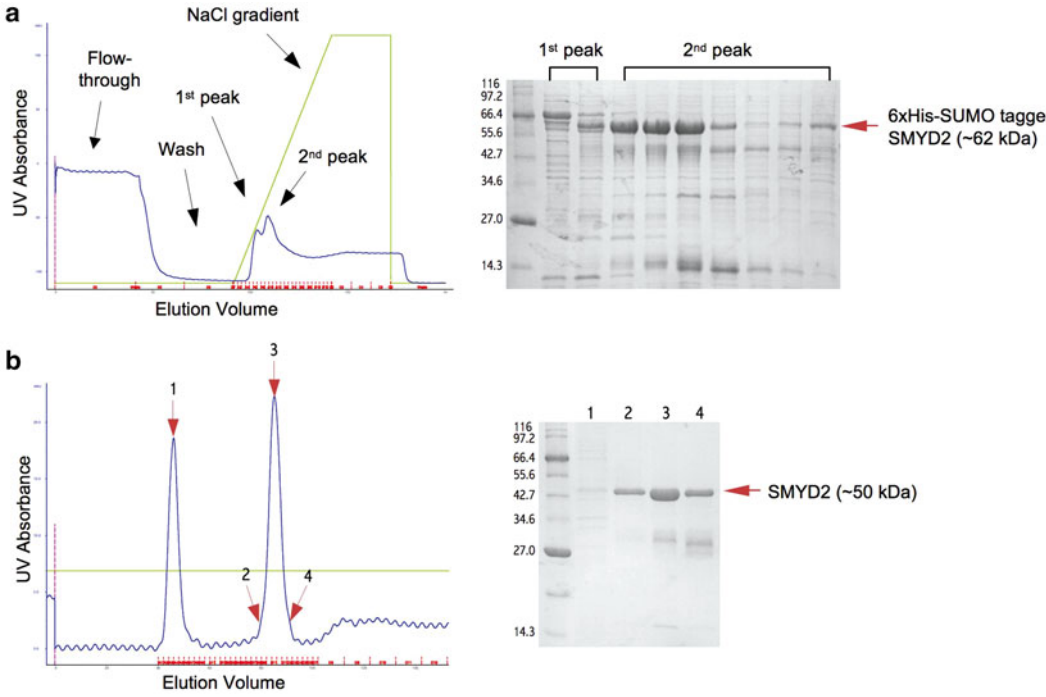

Fig. 3 Proteinpurification. (**a**) Elution profile of the first HisTrap column (*left*). Proteins are separated and eluted out using a linear gradient. SDS-PAGE analysis of elution fractions (*right*). (**b**) Elution profile of the gel filtration column (*left*). SDS-PAGE analysis of elution fractions (*right*). Lanes of SDS-PAGE correspond to the labels in the elution profile

8. Load the cleaved protein onto the desalting column, and elute with the HisTrap HP column-binding buffer. Collect elution fractions. The goal of this step is to exchange the buffers and prepare for a second Ni^{2+} affinity purification, which will separate SMYD2 from the cleaved 6×His-SUMO tag.
9. Pool the fractions containing SMYD2 according to chromatography.
10. Load the sample onto the second HisTrap HP column.
11. Wash the column with 10 column volumes of HisTrap-binding buffer.
12. Collect "flow-through" and "wash."
13. Pool the "flow-through" and "wash" from the previous step. Concentrate to ~1 mL using a 30K-MWCO (molecular weight cutoff) concentrator (*see* **Note 11**).
14. Transfer the concentrated protein to a clean pre-chilled microcentrifuge tube. Centrifuge at 13,000 × *g* for 10 min to remove any precipitate.
15. Load the supernatant onto the gel filtration column.
16. Elute with one column volume of gel filtration buffer. Proteins will be separated according to their size (Fig. 3b).
17. Analyze the peak fractions using SDS-PAGE (Fig. 3b).
18. Pool the SMYD2-containing fractions and concentrate to a final concentration of ~20 mg/mL using a 30K-MWCO concentrator.
19. Aliquot and store at −80 °C (*see* **Note 12**).

3.5 Crystallization

1. Exchange the buffers of the protein sample with Micro Bio-Spin Chromatography Columns. The new buffer is the protein sample buffer (*see* **Note 13**).
2. Measure protein concentration according to the absorbance at 280 nm.
3. Dilute the protein concentration to 10 mg/mL.
4. Incubate the protein sample with 2 mM AdoHcy at 4 °C for 2 h.
5. Centrifuge at 13,000 × *g* for 1 min to remove any precipitates; collect the supernatant.
6. Set up crystallization using the hanging drop vapor diffusion method at 20 °C. First, add 500 μL well solution *S1* (from **step 2**, Subheading 2.4) to the wells of a pre-greased 24-well plate (*see* **Notes 14** and **15**).
7. Pipette a 1 μL drop of well solution S1 onto a siliconized cover slide.
8. Add 1 μL of the protein sample (from **step 3**, Subheading 3.5) to the drop. Then, flip the cover slide, put it on top of the well, and press down gently until the grease seals the well.

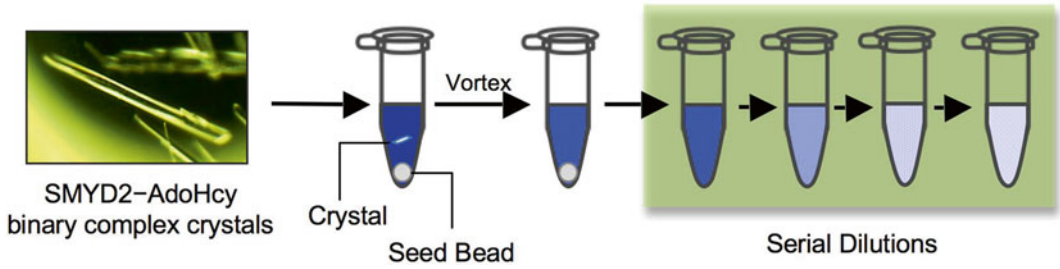

Fig. 4 Preparation of microseed solutions

9. Keep the plate in a 20 °C-crystallization incubator. Crystals typically appear overnight and achieve a full size in a week (Fig. 4).
10. Take a clean cat whisker or MicroGripper and dip into the drop to pick up seed crystals (*see* **Note 16**).
11. Transfer the seed crystals onto a clean cover slide with a fresh drop of 5 μL well solution. Soak for 30 s to wash off any amorphous precipitates.
12. Place the washed crystals in a microcentrifuge tube containing the Seed Bead and 50 μL of the well solution. Vortex for ~3 min or until the crystals are crushed into microcrystalline particles. This seed stock can be stored at 20 °C for later use (up to 2 days).
13. Perform serial dilutions of the seed stock (1:5, 1:10, and 1:100) using well solution S1. Check each dilution under the microscope. This step is useful for controlling the number of the seeds in the experiment (Fig. 4).
14. Prepare a protein sample for crystal seeding: Dilute the protein from **step 2**, Subheading 3.5 to a lower protein concentration of 3 mg/mL.
15. Incubate with 2 mM AdoHcy and 2 mM ERα peptide at 4 °C for 2 h.
16. Centrifuge at 13,000 × *g* for 1 min to remove any precipitates. Collect the supernatant.
17. Set up crystal seeding using the hanging-drop vapor diffusion method at 20 °C. First, add 500 μL well solution *S2* (from **step 2**, Subheading 2.4) to the wells of a pre-greased 24-well plate.
18. Pipette 0.7 μL of well solution S2 onto a siliconized cover slide. Add 1 μL protein sample (from **step 16**, Subheading 3.5) and 0.3 μL seed solution (from **step 13**, Subheading 3.5) to the drop.
19. Flip the cover slide, put it on top of the well, and seal.
20. Keep the plate in the 20 °C-crystallization incubator.
21. Crystals appear within a day and achieve a full size in a week. These crystals are suitable for X-ray diffraction data collection.

4 Notes

1. Prepare all buffers and solutions using deionized water and molecular biology-grade reagents.
2. Choose an appropriate solvent to dissolve peptides. The best solvent to use will depend on the solubility properties of the peptides. Acidic peptides can be dissolved in water or basic buffers, whereas basic peptides can be dissolved in water and acidic solutions. For very hydrophobic peptides, try dissolving the peptides in a small amount of organic solvent such as DMSO, methanol, or isopropanol and then dilute with water. Do not use DMSO for cysteine-containing peptides as it may oxidize the side chain of cysteine [7]. Also, peptides used for co-crystallization should have high purity (>95 %).
3. All buffers used in the ÄKTA FPLC protein purification system, including water (for washing) and 20 % ethanol (for storage), must be filtered through 0.22 μm filter and degassed for 2 h. Store buffers at 4 °C. β-Mercaptoethanol (BME) or DTT should be added to buffers before use.
4. The optimal imidazole concentration in the binding buffer is protein dependent. Typically, 20–40 mM is suitable for many proteins.
5. Small-scale expression test is recommended before proceeding to a large-scale protein production in order to optimize expression conditions. The conditions can be varied including inducer concentration and induction time and temperature.
6. Inducing protein expression at a lower temperature, between 15 and 20 °C, may help increase yields of properly folded protein [8]. Lowering the temperature gradually from 37 to 15 °C can reduce the induction of *E. coli* heat-shock protein 70 (HSP70), which is sensitive to rapid temperature change.
7. The choice of lysis buffer depends on protein properties and purification strategy. In this protocol, the lysis buffer is the HisTrap HP column-binding buffer, which is chosen because the first step of purification is Ni^{2+} affinity chromatography.
8. The pressure cell must be pre-chilled at 4 °C prior to use. If the French Press process takes more than 30 min, cool the pressure cell at 4 °C for 10–15 min and then continue.
9. Alternative method for cell lysis. Use sonication to lyse cells. Keep the cell suspension on ice during sonication. Sonicate the cells using a number of pulses (10 s) followed by pauses (20 s) for cooling. Sonication lyses cells by liquid shear and cavitation. It also shears DNA; thus DNase I is not needed.
10. Avoid introducing air into the FPLC system as it may affect resolution and cause a noisy baseline in the chromatograms.

Keep the pump running while connecting the column to the system. Use a "drop-to-drop" technique.

11. Select the concentrator based on the protein size. For a protein that has a molecular weight of 40 kDa, a 30K MWCO concentrator is appropriate to use.
12. Steps in Subheadings 3.3 (Lyse Cells) and 3.4 (Protein Purification) together can be completed in 5 days. It is recommended that after cell lysis purification should be started immediately to avoid protein degradation. Experiment timeline: Day 1, all steps in Subheading 3.3, **steps 1–4**, in Subheading 3.4; day 2, **steps 5–7** in Subheading 3.4; day 3, **steps 8–12** in Subheading 3.4; day 4, **steps 13–16** in Subheading 3.4; day 5, **steps 17–19** in Subheading 3.4.
13. In the sample buffer, the concentration of pH buffer should be 10–20 times lower than that in the crystallization condition or well solution. If a well solution contains 0.1 M Tris–HCl, pH 8.5, then no more than 10 mM pH buffer should be included in the sample buffer.
14. It is recommended that a crystallization trial should be performed to screen for optimal conditions for crystal growth. Promising conditions can be further optimized to obtain crystals with desired quality [9].
15. To make a greased plate, apply Dow Corning vacuum grease to the upper rim of each well.
16. Because a typical size of crystals is small (under 1 mm), crystal picking needs to be performed under a microscope.

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