Engineered Polymerases with Altered Substrate Specificity: Expression and Purification

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Polymerase engineering is making it possible to synthesize xeno-nucleic acid polymers (XNAs) with diverse backbone structures and chemical functionality. The ability to copy genetic information back and forth between DNA and XNA has led to a new field of science known as synthetic genetics, which aims to study the genetic concepts of heredity and evolution in artificial genetic polymers. Since many of the polymerases needed to synthesize XNA polymers are not available commercially, researchers must express and purify these enzymes as recombinant proteins from *E. coli*. This unit details the steps needed to express, purify, and evaluate the activity of engineered polymerases with altered substrate recognition properties. The protocol requires 6 days to complete and will produce ~20 mg of pure, nuclease-free polymerase per liter of *E. coli* bacterial culture. © 2017 by John Wiley & Sons, Inc.

Keywords: aptamer • polymerase • protein expression and purification • xeno-nucleic acid (XNA)

How to cite this article:
Nikoomanzar, A., Dunn, M. R., & Chaput, J. C. (2017). Engineered polymerases with altered substrate specificity: expression and purification. *Current Protocols in Nucleic Acid Chemistry*, *69*, 4.75.1–4.75.20. doi: 10.1002/cpnc.33

INTRODUCTION

This unit describes the methodology for purifying *Archaeal* family-B replicative DNA polymerases that have been engineered to synthesize artificial genetic polymers. This protocol is specific to thermophilic polymerases, as it utilizes a heat denaturation step to remove endogenous *E. coli* proteins that are excessively abundant in cellular lysate. After expression and heat denaturation, polyethyleneimine (PEI) precipitation is used to remove contaminating nucleic acids. This step is followed by an ammonium sulfate precipitation to remove excess PEI, which allows the enzyme to be stored indefinitely at −80°C as an ammonium sulfate pellet. The polymerase is then purified by affinity chromatography using either a manual or an automated FPLC protocol to obtain large quantities of highly pure, nuclease-free enzyme for synthetic biology research.

STRATEGIC PLANNING

Choosing an Appropriate Expression Vector and Cell Line for Polymerase Production

There are many factors to consider when designing an appropriate plasmid construct for polymerase expression in *E. coli*. For more information on plasmid design, see Gopal & Kumar (2013). The main points to consider when choosing expression systems for

Current Protocols in Nucleic Acid Chemistry 4.75.1–4.75.20, June 2017
Published online June 2017 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpnc.33

polymerase production are described here. Commercially available plasmids typically have several features that enhance the ease of use and allow tight control of expression of the protein of interest. These plasmids feature an origin of replication ribosome binding-site (Shine-Dalgarno sequence), antibiotic resistance marker (e.g., ampicillin, kanamycin, etc.), and promoter (T7 or T4). Plasmids that control expression using the lac operon also contain the lacI gene for the lac repressor. This molecule binds to the operator and prevents transcription in the absence of allolactose. A non-hydrolyzable analogue of allolactose isopropyl-β-D-thiogalactoside (IPTG) is commonly used to induce expression of a target gene over the entire duration of the growing period. When using vectors that contain a T7 promoter, it is important to use a (DE3) strain of BL21 cells because only these cells carry λDE3 lysogen that encodes T7 RNA polymerase for transcription. Many vectors also encode fusion proteins upstream of the target for ease of downstream purification. It is recommended to use a 6×-polyhistidine tag for polymerase purification due to its small size and high specificity. Because recombinant polymerases are generally not toxic and well tolerated by *E. coli*, using high-copy plasmids (e.g., pET vectors) is recommended to maximize the amount of protein expressed. When using cloning strains such as XL1-Blue or DH5 alpha, vectors must contain a different promoter than T7, e.g., T4, to result in protein expression. After transforming the appropriate cell line with the recombinant plasmid, colonies can be grown overnight and made later into glycerol stocks. This is achieved by mixing the overnight culture and autoclaved 50% (v/v) glycerol in water 1:1 in a cryotube and then storing at −80°C. This unit describes purifying polymerases from a glycerol stock.

BASIC PROTOCOL 1

POLYMERASE EXPRESSION IN *E. COLI*

The following protocol was developed to generate ~20 mg of recombinant, nuclease-free polymerase (~95% purity) from a 1-L culture of BL21 cells grown at 37ºC. The polymerase is purified from *E. coli* lysate following a two-step affinity chromatography strategy, including both nickel and heparin resins. The protocol can be scaled linearly to generate larger quantities of enzyme. Some researchers may use XL1-blue or DH5alpha cells that are engineered to contain reduced levels of endogenous nucleases. In the authors' experience, XL1-blue and DH5alpha cells produce ~6 mg of purified recombinant polymerase from a 1-L culture. While these cells generate less polymerase than BL21 cells, their reduced nuclease abundance avoids the need for a stepwise nickel-heparin purification strategy and instead requires only a single heparin purification step.

Materials

70% ethanol
1.5% LB-ampicillin (100 μg/mL) agar plate (see recipe)
100 mg/mL ampicillin (see recipe)
Polymerase expressing *E. coli* cells (glycerol stock)
Luria-Bertani (LB) medium (see recipe)
1 M IPTG (see recipe)
Lysozyme (0.1 mg/mL), optional
Cell resuspension buffer (see recipe)
10% (v/v) polyethyleneimine (PEI) (see recipe)
Ammonium sulfate powder (Sigma)

Bunsen burner
Inoculating loops
Permanent marker
15º and 37ºC incubators (with and without shaking)
500-mL and 2-L baffled flasks, sterile
Aluminum foil

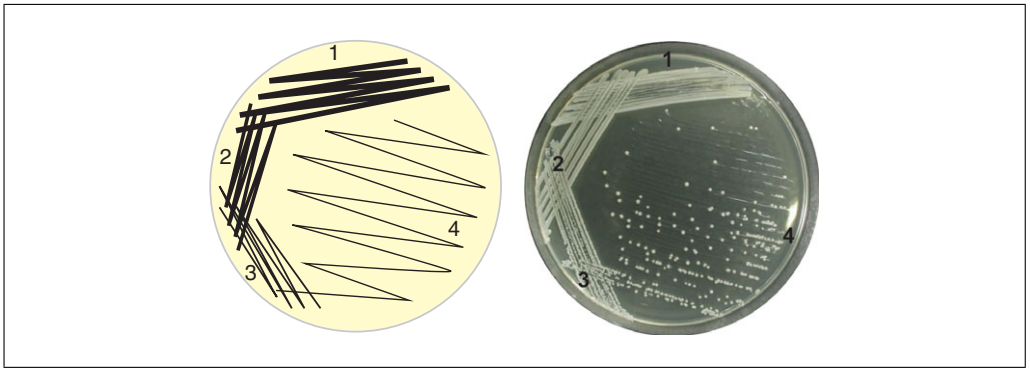

Figure 4.75.1 Growing isolated bacteria colonies on solid agar plates. Obtain an LB agar plate with the appropriate antibiotic. Label the plate with the plasmid name, date, and initials of researcher. Touch a sterile loop to the glycerol stock and then gently spread the bacteria over section 1 of the plate using a back and forth motion (left panel). Sterilize the loop again and spread the bacteria from section 1 to section 2 using the same back and forth motion Repeat the process until all 4 sections of the plate contain bacteria. Incubate plate overnight at 37°C to obtain isolated bacteria colonies (right panel).

UV cuvettes
UV spectrophotometer
Refrigerated centrifuge and rotors JLA 9.1 and JA 25.50 (Beckman)
1-L centrifuge jars
10- and 50-mL disposable pipets
40-mL centrifuge tubes
Sonicator
80ºC water bath or hot plate

Streak plate from glycerol stock (day 1)

1. Clean work surface and tools with 70% ethanol.
2. Ignite a Bunsen burner to create an aseptic environment.

   Be careful to not pass anything above the flame.

3. Place an inoculating loop inside the center of the flame until it glows red hot. Allow loop to come to room temperature, ensuring nothing touches sterile loop region.

   Do not touch the loop, it is very hot and may cause burns.

4. Obtain a 1.5% LB-ampicillin (100 μg/mL) agar plate at 4ºC.
5. Remove a glycerol stock of the desired cell line from −80ºC and place tube on ice.
6. Using the cool, sterile loop, scrape a small amount of the glycerol stock onto the loop.
7. Streak the plate (Fig. 4.75.1) in a zigzag motion to spread the bacteria evenly across the surface of the solid medium.

   It may be necessary to serially dilute the streaks by flame-sterilizing the loop, then pulling a small portion of already streaked cells into a new area of the plate. The goal is to generate single, clonal colonies of bacteria that can be isolated for expression in liquid medium.

8. Place lid on agar plate and label with all necessary information including bacterial strain, plasmid, date, and antibiotic resistance.
9. Return the glycerol stock to −80ºC.
10. Incubate plate upside down overnight in a 37ºC incubator.

Prepare starter culture (day 2)

11. Transfer agar plate from the 37ºC incubator to a designated cell culture area that has been sterilized (as described in steps 1 and 2).

12. Fill an autoclaved 500-mL baffled flask with 100 mL liquid LB-ampicillin (100 μg/mL) medium.

13. Using a sterile inoculating loop, transfer a single colony from the agar plate into the liquid medium.

14. Cover flask with aluminum foil and incubate overnight at 37ºC with vigorous shaking at 225 rpm.

Prepare expression culture (day 3)

15. Transfer 800 μL LB-ampicillin (100 μg/mL) liquid medium to a UV cuvette and use this sample to blank the UV spectrophotometer.

16. Add 200 μL starter culture to the same UV cuvette and mix by inverting the cuvette or pipetting up and down with a filter tip.

17. Measure the OD_{600} value of the starter culture.

18. Calculate the actual OD_{600} of the starter culture by multiplying the measured OD_{600} by 5 to account for the dilution factor.

19. Transfer 1 L LB-ampicillin (100 μg/mL) liquid medium to an autoclaved 2-L baffled flask.

20. Calculate and transfer the appropriate volume of starter culture needed to achieve an OD_{600} value of 0.05 to 0.1 absorbance units, using the following equation:

$$V_{starter} = (V_{expression} \times OD_{600\,expression})(OD_{600\,starter})^{-1}.$$

In general, a 100-fold dilution is required when a starter culture has been grown to confluency.

21. Incubate the expression culture at 37ºC with vigorous shaking at 225 rpm until the LB medium reaches an OD_{600} of 0.6 to 0.8.

The OD_{600} value should double every 30 min. Thus, an initial OD_{600} of 0.05 and final of 0.8 will require four doublings or ~2 to 4 hr.

22. Once the desired OD_{600} is reached, transfer the expression culture from 37ºC incubator to 4ºC and then place into a 15ºC incubator.

23. Induce protein expression when the temperature of the expression culture cools to ~15ºC by adding 500 μL of 1 M IPTG to the culture for a final concentration of 0.5 mM and return the expression culture to 15ºC incubator. Incubate overnight at 15ºC with shaking at 225 rpm.

(Optional) It is often beneficial to collect a 1-mL aliquot of the uninduced and induced overnight expression culture for future SDS-PAGE analysis (Fig. 4.75.3). In such cases, transfer 1 mL of the expression culture into a 1.5-mL microcentrifuge tube. Centrifuge cells 1 min at 1000 × g. Decant the supernatant and resuspend cell pellet in 200 μL of 1× SDS-PAGE loading buffer.

If a 15ºC incubator is not available, once the OD_{600} of the culture reaches 0.6 to 0.8, immediately induce with 1 M IPTG to a final concentration of 0.5 mM and incubate 4 hr at 37ºC with shaking at 225 rpm. Immediately continue to step 24 after the 4-hr incubation.

Lyse cells (day 4)

24. Remove the 1-L expression culture from the 15ºC shaking incubator and transfer to a 1-L centrifuge jar. Fill a second 1-L jar with 1 L of water.

    Ensure that the jars filled with cell culture are appropriately balanced by weighing the masses on a scale with an acceptable tolerance of 0.2 g.

25. Place jars into the centrifuge and centrifuge cells 10 min at 9500 × *g*, 4ºC.

26. Discard supernatant into appropriate disposal container without disturbing cell pellet.

27. Invert jar onto a paper towel and allow the remainder of the medium to drain. Discard the paper towel in an appropriate disposal container.

    Removal of residual medium is crucial for removing metabolic by-products that could impact overall protein purity.

28. Resuspend cell pellet in 35 to 40 mL of cell resuspension buffer. Use a serological pipettor with attached 10-mL pipet to gently scrape excess cell pellet off the wall of the jar and pipet up and down thoroughly until fully dissolved.

    Prevent air bubble formation during resuspension to minimize oxidation of protein, which may result in reduced activity. Do not vortex.

29. Transfer resuspended pellet to a 40-mL centrifuge tube.

    The resuspended culture may be stored up to 1 year at −80ºC. To continue, gently thaw cells in a 24ºC water bath.

30. Place centrifuge tube with resuspended cell pellet into an ice bucket.

31. Lyse cells by sonicating for 5 min using a 15-sec on and 45-sec off protocol.

    Ensure that cell culture is sufficiently cooled before beginning another round of sonication. If necessary, increase the off time. If a sonicator is not available, add lysozyme at a final concentration of 0.1 mg/mL, mix by inversion, and incubate 1 hr at 37ºC.

32. Once sonication is complete, denature the endogenous *E. coli* proteins by incubating the centrifuge tube for 60 min on an 80ºC hot plate or in an 80ºC water bath.

33. Cool sample on ice for at least 30 min to aggregate denatured protein and cellular debris.

34. Precipitate aggregated protein and cellular debris by centrifuging 30 min at 60,500 × *g*, 4ºC.

    If a high-speed centrifuge is not available, centrifuge samples 30 min at 40,000 × g, 4°C.

Remove nucleic acids

35. Transfer the clarified cell lysate to a new 40-mL centrifuge tube.

36. Measure the volume of the supernatant using a serological pipette. Then, add enough 10% (v/v) PEI solution to give a final concentration of 0.5% PEI.

37. Mix by inversion until the solution is cloudy and incubate 15 min on ice.

38. Pellet the precipitated nucleic acids by centrifuging 20 min at 60,500 × *g*, 4°C.

39. Transfer the crude lysate to a new 40-mL centrifuge tube and measure the volume.

    Reserve 20 μL of sample for SDS-PAGE gel analysis (Fig. 4.75.3).

Precipitate recombinant polymerase

40. To remove excess PEI, precipitate the protein using ammonium sulfate. Use an online calculator (*http://www.encorbio.com/protocols/AM-SO4.htm*) to calculate the required mass (g) of ammonium sulfate powder needed to achieve the target concentration of 60% ammonium sulfate. When making the calculations, input the PEI-treated supernatant volume measured in step 36, temperature of 4°C, and an initial and final concentration of ammonium sulfate of 0% and 60%, respectively. For example, 24 mL of PEI-treated supernatant will require 9 g of ammonium sulfate.

    Alternatively, 2 vol of 100% saturated ammonium sulfate solution at 4°C may be added. However, this protocol drastically increases the total volume of the sample needed for centrifugation and can make this step in the workflow more time-intensive and laborious than if powdered ammonium sulfate is used.

41. Add the desired amount of ammonium sulfate powder to the crude lysate solution.

42. Mix by inversion until the solid salt crystals are fully dissolved.

43. Precipitate the protein by incubating for a minimum of 30 min on ice. Centrifuge 30 min at 60,500 × *g*, 4°C.

44. Decant the supernatant and invert the tube over a paper towel to remove excess liquid.

45. Store the protein pellet at −80°C until ready for purification.

    The pellet may be stored indefinitely under these conditions.

BASIC PROTOCOL 2

POLYMERASE PURIFICATION AND TESTING

The following protocol details affinity purification of the recombinant polymerase from the ammonium sulfate pellet using a stepwise nickel-heparin purification strategy. The steps are outlined for use with a fast protein liquid chromatography (FPLC) instrument; however, the same steps can also be performed manually if an FPLC instrument is not available. In the manual version, substitute the FPLC with a 30-mL syringe and follow the steps below with one exception: during the elution phase, perform step elutions with increasing imidazole and salt concentrations 5 column volumes (CV) at a time. For the nickel resin, perform 50, 100, 250, and 500 mM imidazole steps. For the heparin resin, perform 100, 250, 500, and 1000 mM NaCl steps. Take care to push the liquid through the column at a flow rate that is slow enough for individual drops to exit the column. Once the protein is purified, the optimal polymerase concentration is determined for XNA activity by performing a primer extension assay with xNTP substrates with titrating and analyzing the greatest amounts of polymerase. For Kod-RI, synthetic tNTP substrates are used in the reaction. Each reaction is then analyzed by denaturing urea-PAGE. The optimal polymerase concentration is defined as the lowest polymerase concentration that produces the most amount of full-length TNA product.

Materials

Nickel buffer A (see recipe)
Nickel buffer B (see recipe)
20% ethanol
Recombinant polymerase protein pellet (see Basic Protocol 1)
4× Nu-PAGE loading buffer
Mini-PROTEAN TGX Stain-Free gel
1× TGS running buffer (see recipe)
Unstained protein ladder (ThermoFisher)
Nickel elution diluton buffer (see recipe)

Heparin buffer A (see recipe)
Heparin buffer B (see recipe)
Nuclease-free water
10× ThermoPol buffer (NEB)
IR800 PBS8 primer (Integrated DNA Technologies):
5′-/5IRD800//iSp18/GTCCCCTTGGGGATACCACC
L11 Library (Integrated DNA Technologies):
5′–GGATCGTCAGTGCATTGAGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGTGGTATCCCCAAGGGGAC – 3′ (N = A:T:C:G = 1:1:0.3:0.3)
1 mM each tNTP mix (tATP, tTTP, tCTP, tGTP)
Polymerase dilution buffer (see recipe)
Polymerase
10 mM $MnCl_2$ (see recipe)
Stop buffer (see recipe)
20% Urea-PAGE solution (see recipe)
10% (w/v) ammonium persulfate (APS, see recipe)
N′,N′,N′,N′,Tetraethylmethylenediamine (TEMED, Pierce)
Acrylamide concentrate (see recipe, also available from National Diagnostics)
Acrylamide diluent (see recipe, also available from National Diagnostics)
10× TBE buffer (see recipe)
Absolute ethanol (Sigma)
70% ethanol, −20°C
Manganese hexahydrate (Sigma)
0.1 M NaOH
5 mL HisTrap HP Resin (GE Healthcare)

40-mL centrifuge tubes
Refrigerated centrifuge
50-mL conical tubes
5-mL Nickel HP columns (GE)
FPLC system (AKTA)
Superloop (size varies)
Fraction collector (AKTA)
1.5-mL microcentrifuge tubes (Eppendorf)
75º and 90ºC heating blocks
Electrophoresis apparatus (BioRad)
Power supply
Geldoc (BioRad)
5-mL Heparin HP resin columns (GE Healthcare)
Spectrophotometer (Nanodrop)
200-μL PCR tubes
Thermal cycler
Gel plates (19.7 × 16– and 19.7 × 18.5–cm)
Spacers (1.5-mm thick)
Comb (20-well)
50-mL beaker
Magnetic stir bar and stir plate
PAGE electrophoresis apparatus
50-mL plastic syringe
Vortex
Refrigerated microcentrifuge
LICOR Odyssey cLX imager

Perform nickel purification (day 5)

1. Remove cell pellet from −80°C storage and resuspend in 40 mL of 1× Nickel buffer A in a 40-mL centrifuge tube.
2. Centrifuge 5 min at 60,500 × *g*, 4°C.
3. Transfer supernatant to a 50-mL conical tube for loading onto the superloop.
4. Install a 5-mL Nickel HP column onto the FPLC system, ensuring the lines are completely sealed.
5. Rinse the FPLC lines and nickel column with 5 CV of water to remove any EtOH from the FPLC system.

   It is important to flush the system with water before introducing the buffer to prevent the precipitation of salt in the lines or columns at the buffer-EtOH interface. When running liquid through the column, ensure the flow rate and pressure does not exceed 10 mL/min and 0.5 MPa, respectively.

6. Place line A1 into nickel buffer A and line B1 into nickel buffer B. Ensure the lines are completely submerged and no air enters the system.
7. Place line A2 into filtered water and line B2 in 20% ethanol.
8. Load recombinant polymerase protein sample into the designated superloop.

   Reserve 20 μL of sample for SDS-PAGE gel analysis (Fig. 4.75.3).

9. Set up the following FPLC method in the method editor.

   a. Method Settings: Ensure the correct column is checked (5 mL Nickel HP) and that the correct channel column is selected if using a multicolumn switch. Selecting the column will bring in the max operating conditions for the column (10 mL/min or 0.5 MPa).
   b. Equilibration 1: Equilibrate with 5 CV of 100% buffer B to ensure the column is completely clean. Use a flow rate of 8 mL/min. Send volume to waste.
   c. Equilibration 2: Equilibrate with 5 CV of 100% buffer A to ensure the column is equilibrated in binding buffer. Use a flow rate of 8 mL/min. Send volume to waste.
   d. Sample application: Set the appropriate volume of sample to pass through the superloop. Pass the sample through the column at a flow rate of 2.5 mL/min to ensure the sample has ample time to bind to the column. Ensure that the flow-through is being properly collected via the outlet valve into a suitable container. Set the outlet valve to the out line and change the fractionation method from fixed volume to fixed outlet.
   e. Wash: Wash the column with buffer A until a stable UV is maintained at a flow rate of 5 mL/min. Use a stable UV time of 2 min and a maximum wash time of 20 min. This will guarantee between 2 and 20 CV of washing. Send volume to waste.
   f. Elute: Using a linear gradient from 0% to 100% buffer B, elute the protein over 10 CV. Set fractionation conditions to fixed volume and collect suitable volume depending on the outlet container dimensions.
   g. Final Wash: Wash the column with 5 CV of buffer B and then 5 CV of buffer A at a flow rate of 8 mL/min. Send volume to waste.
   h. Equilibration: Equilibrate the column with 5 CV of water using line A2, then 5 CV of 20% EtOH using line B2 at a flow rate of 5 mL/min for storage. Send volume to waste.

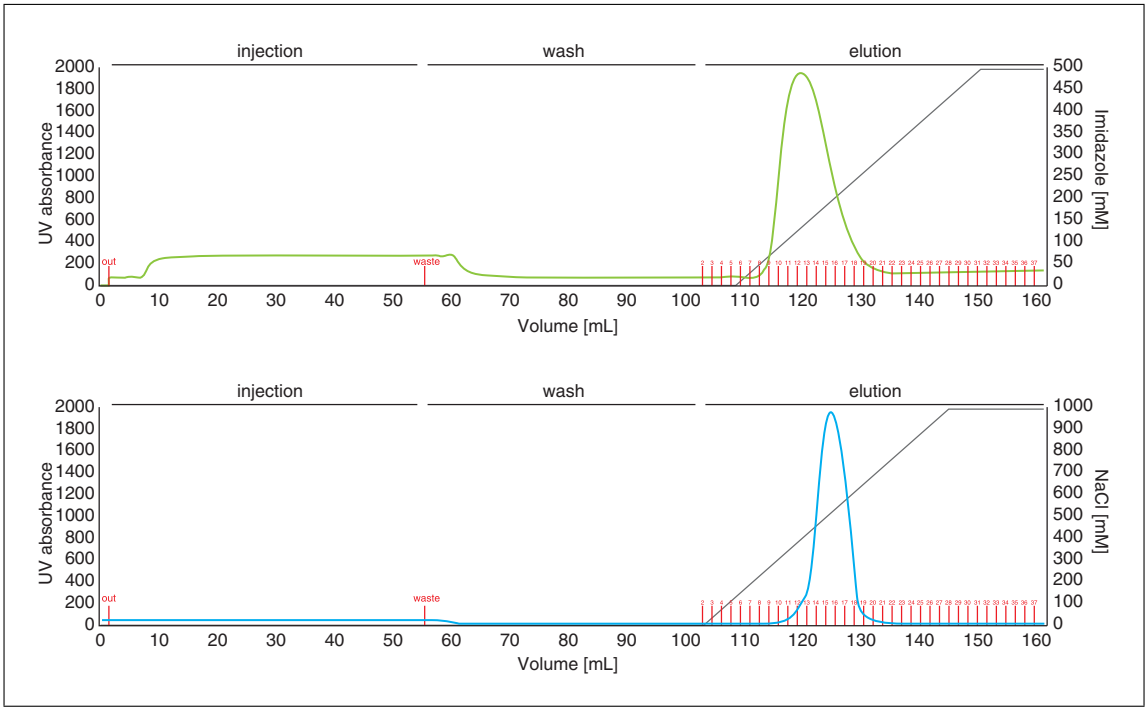

Figure 4.75.2 Stepwise FPLC purification using nickel-NTA followed by heparin affinity chromatography. Elution profile observed for a polymerase from a nickel column (top panel) and linear imidazole gradient (20 to 1000 mM) over 10 column volumes. The peak is symmetric with a slight tail at higher elution volumes. Most polymerases elute with an imidazole concentration between 150 and 250 mM. Elution profile observed for a polymerase from a heparin column (bottom panel) and linear NaCl gradient (50 to 1000 mM) over 10 column volumes. The peak itself is highly symmetric without any visible tails. Protein elutes in the 500-mM NaCl range.

10. Save the method and run it from the system control panel. Designate the correct location for the saved data.
11. Once the program has been initiated, load the appropriate tubes or plates onto the fraction collector, and ensure that the apparatus is properly positioned for collecting the elution fractions.
12. Wait for the program to finish running.
13. Using the trace data from the computer, identify the fractions containing the protein.

    These will be the fractions containing a large peak in the UV data (Fig. 4.75.2).

14. Set aside 20-μL aliquots of flow-through and each elution fraction into separate 1.5-mL microcentrifuge tubes for SDS-PAGE analysis (Fig. 4.75.3).
15. Store protein up to 6 months at 4°C until ready for the next purification.
16. Remove the 5-mL Nickel HP column and store in its designated location.

Analyze purified protein by SDS-PAGE

17. Combine 20 μL of protein sample with 7 μL of 4× Nu-Page gel loading buffer.
18. Denature sample by incubating 10 min at 90°C.
19. Set up the electrophoresis apparatus and SDS-PAGE gel (Mini-PROTEAN TGX Stain-Free gel) following the manufacturer's instructions.
20. Fill with 1× TGS buffer until the buffer level reaches the designated line (~1 in. from bottom of gel).

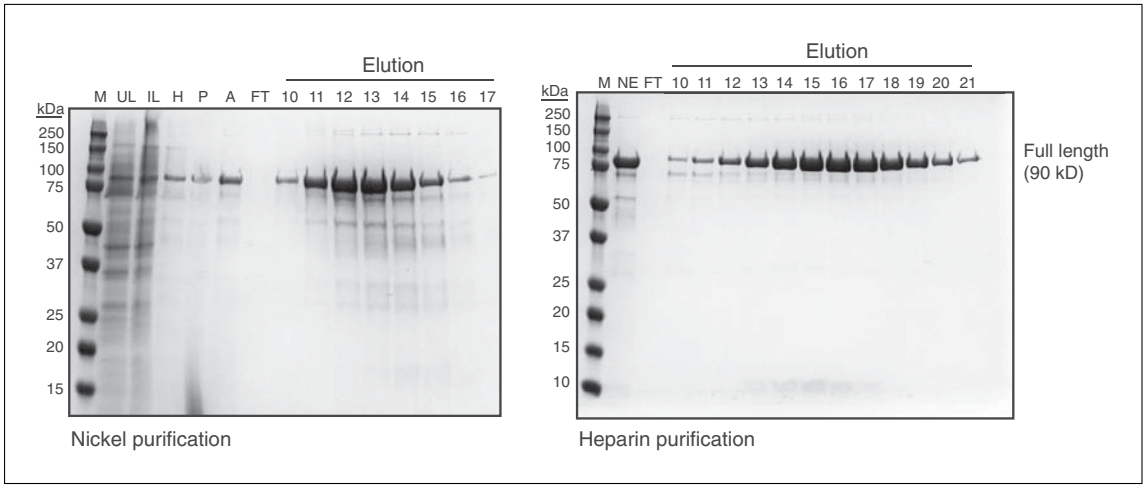

Figure 4.75.3 SDS-PAGE analysis of individual fractions collected by FPLC purification. Eluted fractions collected from the nickel (left panel) and heparin (right panel) affinity purification columns shown in Figure 4.75.2 were analyzed by SDS-PAGE. Elution fractions are shown above the gel. M, marker; UL, uninduced lysate; IL, induced lysate; H, 80°C heat shock; P, 0.5% PEI supernatant; A, 60% ammonium sulfate pellet; FT, flow-through; NE, nickel-elution.

If running more than one gel in the same box, double volume of buffer.

21. Load 15 μL of unstained protein ladder in lane 1 and each sample into lanes 2 through 15. Run gel 40 min at 200 V constant.
22. Remove gel from the cassette following manufacturer's instructions.
23. Image gel (Fig. 4.75.3) using a BioRad Geldoc and the stain-free gel protocol.
24. Combine desired fractions after purity is verified by SDS-PAGE analysis.

Perform heparin purification

25. Dilute the combined nickel elution fractions twelve-fold with 1× nickel elution dilution buffer.

    This will reduce the salt and imidazole concentrations to 50 mM and 15 mM, respectively. Save 20 μL of sample prior to dilution for SDS-PAGE analysis (Fig. 4.75.3)

    It is important for the salt concentration of the sample to be at the same concentration as the loading buffer (~50 mM). Higher salt concentrations (≥100 mM) will prevent the protein from binding to the resin.

26. Install the 5-mL Heparin HP column onto the FPLC system and ensure the lines are completely sealed.
27. Rinse FPLC lines and heparin column with 5 CV of water to remove any residual nickel buffer or EtOH from the FPLC system and column.
28. Place line A1 into heparin buffer A and line A2 into heparin buffer B. Ensure the lines are completely submerged and no air enters the system.
29. Load sample into the designated superloop.

    It may be necessary to load the superloop more than once depending on its capacity. If so, divide the following program in step 30 between multiple runs. For the first load, run steps 30b to 30d. For intermediary loads, only run step 30d. For the final load, run steps 30d through 30h. After each sample application to the column, reload the superloop.

30. Set up the following FPLC method in the method editor.

a. Method Settings: Ensure the correct column is checked (5-mL Heparin HP) and that the correct channel is selected if using a multicolumn switch. Selecting the column will bring in the max operating conditions for the column (10 mL/min or 0.5 MPa).
b. Equilibration 1: Equilibrate for 5 CV with 100% heparin buffer B to ensure the column is completely clean. Use a flow rate of 8 mL/min. Send volume to waste.
c. Equilibration 2: Equilibrate for 5 CV of 100% heparin buffer A to ensure the column is equilibrated in binding buffer. Use a flow rate of 8 mL/min. Send volume to waste.
d. Sample Application: Set appropriate volume of sample to pass through the super-loop. Pass the sample through the column at a flow rate of 2.5 mL/min to ensure the sample has ample time to bind to the column. Ensure that the flow-through is collected via the outlet valve into a suitable container. Set the outlet valve to the out line and change the fractionation method from fixed volume to fixed outlet.
e. Wash: Wash column with heparin buffer A until a stable UV is maintained at a flow rate of 5 mL/min. Use a stable UV time of 2 min and a maximum wash time of 20 min. This will guarantee between 2 and 20 CV of washing. Send volume to waste.
f. Elute: Using a linear gradient from 0% to 100% heparin buffer B, elute the protein over 10 CV. Set fractionation conditions to fixed volume and collect a suitable volume depending on the outlet container dimensions.
g. Final Wash: Wash the column with 5 CV of heparin buffer B and then 5 CV of heparin buffer A at a flow rate of 8 mL/min. Send volume to waste.
h. Equilibration: Equilibrate the column with 5 CV of water using line A2, then 5 CV of 20% EtOH using line B2 at a flow rate of 5 mL/min for storage. Send volume to waste.

31. Save the method and run it from the system control panel. Designate the correct location for the saved data.

32. Once the program has been initiated, load the appropriate tubes or plates onto the fraction collector, and ensure that the apparatus is properly positioned for collecting the elution fractions.

33. Wait for the program to finish running.

34. Using the trace data from the computer, identify the fractions containing the protein.

    These will be the fractions containing a large peak in the UV data (Fig. 4.75.2).

35. Set aside a 20-μL aliquot of flow-through and each elution fraction into separate tubes for SDS-PAGE analysis (Fig. 4.75.3).

36. Analyze the elution fractions by following steps 17 through 23.

37. Combine the desired fractions after the purity is verified by SDS-PAGE analysis (Fig. 4.75.3).

38. Measure the final polymerase concentration using a Nanodrop spectrophotometer.

    Blank with an approximate mix of buffer A/B from the elution profile. Input the MW and extinction coefficient to obtain a more accurate measure of protein concentration. If unavailable, use the A_{280} as an indicator of protein concentration.

39. Store protein at 4ºC until ready for the polymerase activity assay.

40. Remove the 5-mL Heparin HP column and store in its designated location.

Analyze polymerase activity (day 6)

41. Prepare an 80-μL mastermix in a 1.5-mL microcentrifuge tube by adding the following reagents.

    58 μL nuclease-free water
    10 μL 10× Thermopol buffer
    1 μL 100 μM IR800 PBS8 DNA primer
    1 μL 100 μM L11 DNA library or individual template of interest
    10 μL tNTP mix, 1 mM each

    Mix gently by flicking and pulse spin to return liquid to the bottom of the tube if necessary.

42. Anneal the primer and template by first denaturing the sample for 5 min at 75°C then cooling the sample for 5 min on ice.

43. While the sample is annealing, prepare a two-fold polymerase dilution series as follows. Obtain eight 200-μL PCR tubes, add 10 μL of polymerase dilution buffer to tubes 2 through 8, add 20 μL of pure polymerase to tube 1, and then transfer 10 μL from tube 1 to the subsequent tubes while mixing thoroughly.

44. Add 10 μL of 10 mM $MnCl_2$ solution to each of the eight polymerase dilutions and mix well.

45. Pipet 8 μL of the reaction mastermix to nine, new 200-μL PCR tubes and cap tubes.

    The first sample will have no enzyme added to provide a size marker for gel analysis.

46. Place tubes into a thermal cycler set at 55ºC.

47. Add 2 μL of the polymerase-Mn^{2+} mixture to tubes 2 through 8 and cap tubes. Incubate 3 hr at 55ºC.

48. After the 3-hr incubation, quench reaction by adding 100 μL of stop buffer to each tube.

49. Denature the extended primers from the template by heating 10 min at 70ºC.

Prepare urea-PAGE gel

50. Prepare gel plates, spacers, and comb following the manufacturer's instructions.

    Analytical gels are 0.8-mm thick. Using a comb with 14 to 20 wells is recommended.

51. In a 50-mL beaker, add 25 mL of 20% Urea-PAGE solution. Stir the mixture using a magnetic stir bar and plate.

52. Add 200 μL of 10% APS and 10 μL TEMED to the solution and stir for 30 sec.

53. Carefully pour acrylamide solution into the prepared gel plates, ensuring no leaks or air bubbles are present between the plates.

54. Insert comb at the top of the gel and dislodge any air bubbles by tapping on the glass.

55. Allow the solution to polymerize between the glass plates (typically 30 min).

Pre-run urea-PAGE gel

56. Carefully remove comb and bottom spacer from gel plates making sure not to damage gel.

57. Rinse gel with tap water to remove excess acrylamide or urea that may form on the outside of the glass plates, which may prevent a good seal when attached to the electrophoresis apparatus.

58. Place gel plates into the gel electrophoresis apparatus and secure the plates following the manufacturer's instructions.

59. Fill the bottom reservoir of the gel apparatus with 1× TBE buffer. Displace air bubbles that form in the bottom spacer region using a syringe containing 1× TBE buffer.

60. Fill the top reservoir of the electrophoresis apparatus with 1× TBE buffer until the solution is ~1 cm above the gel.

61. Rinse the top wells with 1× TBE buffer to remove any residual polyacrylamide.

62. Connect the apparatus to the power supply and pre-run gel 30 min at a constant power of 8 W.

    The ideal wattage for the gel will heat the glass plates so they are warm to the touch. Temperatures greater than ~70ºC can break the glass. Pre-warming is essential for minimizing the amount of "smiling" (i.e., curving of gel lane bands) that occurs when running PAGE.

Load and run urea-PAGE gel

63. Once the gel has finished pre-running, disconnect the gel apparatus from the power supply and rinse the wells with 1× TBE to remove any residual urea.

    Rinsing the wells prior to sample loading helps visualize the sample as it is being loaded into the wells. The sample can also be mixed with dye to aid in visualization.

64. Load 20 μL of each sample into its corresponding well using a micropipette equipped with a gel loading tip.

65. Reconnect the gel apparatus to the power supply and run gel 1 hr at a constant power of 8 W.

Image gel

66. Carefully separate glass plates without damaging gel.

    The gel should stay attached to one of the glass plates.

67. Lay gel down on the LICOR Odyssey cLX imager, glass plate up.

68. Image gel using appropriate settings.

69. Analyze the gel and determine the polymerase concentration that generates the highest amount of full-length product (Fig. 4.75.4).

Store long term

70. Dilute the protein with polymerase dilution buffer to achieve a final concentration of 10×. For example, if the polymerase obtained highest activity at a 50-fold dilution, dilute original protein stock five-fold.

71. Divide the 10× polymerase stock into 1-mL aliquots and store up to 6 months at 4ºC until needed.

    The addition of glycerol up to 50% final concentration will enhance the long-term stability of the protein by allowing storage at −20ºC. Glycerol addition does change buffering conditions with a slight effect on polymerase activity, so performing the activity assay with the glycerol present is recommended.

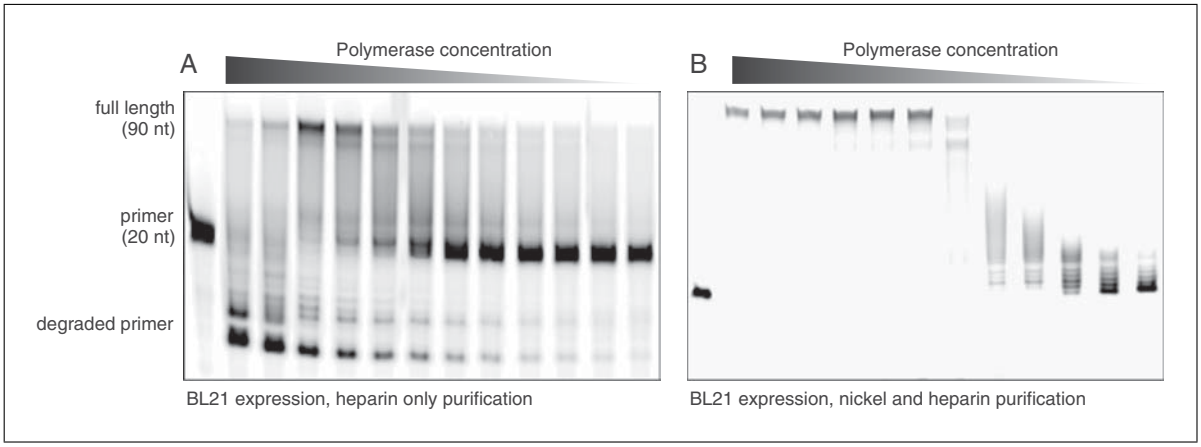

Figure 4.75.4 Polymerase activity assay. Engineered polymerase expressed in BL21 cells and purified using (**A**) a single-step purification with heparin resin or (**B**) a two-step purification using both nickel and heparin affinity purification strategies are assayed for function in a TNA synthesis reaction using a serial (two-fold) dilution strategy to identify the optimal polymerase concentration. The single-step heparin purification strategy from BL21 cells is insufficient to remove contaminating nucleases, as evidenced by significant amounts of degraded primer. By contrast, the two step strategy generates high quality nuclease-free polymerase.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A.

Ampicillin, 1000×

To 1 g ampicillin, bring up to 10 mL with water. Filter sterilize using a 0.2-μm filter. Divide into 1-mL aliquots and store up to 6 months at −20ºC.

APS, 10%

1 g ammonium persulfate
10 mL deionized water
Store up to 1 month at 24ºC

Cell resuspension buffer, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
500 mM NaCl
10% glycerol
Filter sterilize using a 0.2-μm filter
Store up to 6 months at 4ºC

Heparin buffer A, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
50 mM NaCl
10% glycerol
Filter sterilize using a 0.2-μm filter
Store up to 6 months at 4ºC

Heparin buffer B, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 M NaCl
10% glycerol
Filter sterilize using a 0.2-μm filter
Store up to 6 months at 4ºC

IPTG, 1000×

To 2.38 g IPTG, bring up to 10 mL with deionized water. Filter sterilize using a 0.2-μm filter. Divide into 1-mL aliquots and store up to 6 months at −20ºC.

LB-ampicillin (100 μg/mL) agar plate, 1.5%

7.5 g agar
10 g sodium chloride
10 g tryptone
5 g yeast extract
Bring to 500 mL with water
Autoclave
When cooled to ~37ºC, add 500 μL of 1000× ampicillin solution (see recipe)
Pour into petri dish until a thin, even layer forms on the plate
Store up to 3 months at 4ºC

Luria-Bertani (LB) medium

10 g sodium chloride
10 g tryptone
5 g yeast extract
Bring to 1 L with water
Autoclave
Store up to 1 month at 4ºC

$MnCl_2$, 10 mM

10 mg $MnCl_2$ tetrahydrate
5 mL nuclease-free water
Store up to 2 hr at 4ºC

Nickel buffer A, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
600 mM NaCl
10% glycerol
20 mM imidazole
Filter sterilize using a 0.2-μm filter
Store up to 6 months at 4ºC

Nickel buffer B, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
600 mM NaCl
10% glycerol
500 mM imidazole
Filter sterilize using a 0.2-μm filter
Store up to 6 months at 4ºC

Nickel elution dilution buffer, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
10% glycerol
Filter sterilize using a 0.2-μm filter
Store up to 6 months at 4ºC

Polyethyleneimine, 10% (v/v)

700 mL deionized water
100 mL PEI

continued

Add concentrated HCl drop-wise to the solution to pH <2.0. Stir until the PEI is dissolved (~2 to 3 hr). Maintain pH <2.0 throughout. Add concentrated NaOH drop-wise to the solution to pH 7.9. Bring volume to 1 L with water. Filter sterilize using a 0.2-μm filter.
Store up to 1 year at 24ºC

Polymerase dilution buffer, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
250 mM NaCl
10% glycerol
Filter sterilize using a 0.2-μm filter
Store up to 6 months at 4ºC

Stop buffer

1× TBE
20 mM EDTA
8 M urea
Filter sterilize at 0.2 μm
Store up to 6 months at 24°C

TBE buffer, 10×

1 M Tris base
1 M boric acid
10 mM EDTA, pH 8.0
Store up to 6 months at 24ºC

TGS buffer, 10×

250 mM Tris base
192 mM glycine
0.1% (w/v) SDS
pH 8.3
Store up to 1 year at 24ºC

Urea PAGE solution, 20%

193.3 g acrylamide
6.7 g methylene bisacrylamide
7.5 M urea
Deionized water to 1 L
Filter sterilize using a 0.2-μm filter
Degas using a vacuum chamber
Store up to 1 year at 24ºC in the dark

CAUTION: *Acrylamide and bisacrylamide are hazardous especially in powder form. Prepare in a fume hood using appropriate safety precautions and laboratory apparel.*

COMMENTARY

Background Information

Engineered polymerases

Recent advances in polymerase engineering have made it possible to synthesize nucleic acid polymers with a wide range of chemical modifications, including xeno-nucleic acid polymers (XNA) with backbone structures not found in nature (Chen & Romesberg, 2014). XNA systems have the potential to benefit society by functioning as next-generation affinity reagents and catalysts with future application in molecular medicine, nanotechnology, and materials science. As a result, a major goal of synthetic genetics is to determine the extent to which XNA can replace DNA and RNA as an information coding system. Several distinct types of XNAs have been developed to study

fundamental questions about the structural and functional properties of DNA as well as to increase the chemical toolbox available for nucleic acids research (Anosova et al., 2016). In certain cases, XNAs have been found to exhibit remarkable stability against nucleases that degrade DNA and RNA, suggesting that XNA could find widespread use in future diagnostic and therapeutic applications (Culbertson et al., 2016).

Given the importance of XNA as an alternative genetic system capable of heredity and evolution, many new technologies have been developed that enable XNA polymerases to be generated from natural DNA polymerases. Technologies for evolving XNA polymerases include, but are not limited to, compartmentalized self-replication (CSR), compartmentalized self-tagging (CST), droplet-based optical polymerase sorting (DrOPS), and phage display (Ellefson et al., 2016; Ghadessy, Ong, & Holliger, 2001; Larsen et al., 2016; Pinheiro, Arangundy-Franklin, & Holliger, 2014; Tingjian et al., 2016). This effort has become increasingly more challenging as the structural diversity of the phosphodiester backbone increases relative to natural DNA and RNA, which is why most XNA polymerases function with catalytic efficiencies that are vastly inferior to their natural counterparts.

Nevertheless, researchers have succeeded in developing several examples of functional XNAs by in vitro selection (SELEX). In 2012, the first aptamers were identified with XNA backbones for α-L-threofuranosyl-(3′→2′) nucleic acid (TNA) and hexitol nucleic acid (HNA) (Pinheiro et al., 2012; Yu, Zhang, & Chaput, 2012). These reagents were shown to bind to human α-thrombin, hen egg lysozyme, and the HIV-1 TAR RNA element with affinities in the low-to-mid nanomolar range. In 2015, a 2′-fluoroarabino nucleic acid (FANA) aptamer to HIV-1 reverse transcriptase was developed that could inhibit HIV-1 reverse transcriptase activity in vitro (Alves, Cozens, Holliger, & Destefano, 2015). In 2016, a second TNA aptamer to human α-thrombin was developed using a second-generation TNA replication system (Dunn & Chaput, 2016). Additionally, aptamers have been developed using an expanded repertoire of nucleobase chemistries that have improved the affinity and specificity of these aptamers to low-picomolar affinity (Kimoto, Yamashige, Matsunaga, Yokoyama, & Hirao, 2013). These systems do not require engineered polymerases, thereby increasing their utility in the chemical biology community.

XNA polymers have also shown promise in nanotechnology and materials engineering. GNA, which has a phosphodiester backbone with a single stereocenter was used to construct two identical nanostructures with mirror-image symmetry (Zhang, McCullum, & Chaput, 2008). Following suit, nanostructures composed of FANA, 2′-fluoro RNA (FRNA), HNA, and cyclohexene nucleic acid (CENA) have been demonstrated (Taylor et al., 2016). Because XNAs have diverse helical geometries, one could easily speculate that future work in nucleic acid nanotechnology will leverage combinations of natural and unnatural genetic polymers to expand conformational fold space to regions that are not accessible to natural DNA and RNA. Likewise, XNAs have the potential to increase the range of mechanical and physical properties available for molecular engineering as demonstrated by an expanded bending and flexing space of several DNA analogues (Anosova et al., 2016) This work may rely on polymerase engineering to synthesize the building blocks required for assembly.

Critical Parameters and Troubleshooting

Protein expression

Critical parameters for protein expression include the *E. coli* cell strain and growth conditions. The expression may be performed in standard plasmid prep strains like DH5alpha or XL1-blue, standard expression strains like BL21(DE3), or a hybrid cell strain called Acella available from EdgeBio. Choosing a strain requires balancing the benefits and constraints between endogenous nuclease expression and recombinant protein production yields.

DH5alpha and XL1-blue strains are intrinsically nuclease deficient. However, they are poor cell lines for protein expression and their yield is about three-fold lower than that obtained with BL21 cells. Advantageously, however, only a single resin (nickel or heparin) is required for effective purification. By contrast, when expressing in BL21 cells, a two-step purification protocol (nickel and heparin) is recommended to ensure complete removal of endogenous nucleases. The hybrid cell line Acella is an attractive alternative to either because it is a nuclease-deficient strain of BL21. It produces about two-fold less protein than BL21 cells but comes with the added benefit of removing the nickel purification step.

Because engineered polymerases require long incubation times to ensure complete incorporation of modified substrates, even small amounts of nuclease contamination can be problematic. If nuclease contamination is discovered, it is often useful to ensure that all heat/cold shock steps (i.e., 80°C heat shock, PEI precipitation, ammonium sulfate precipitation) are performed at the appropriate temperature and for the indicated duration times. Additionally, combining only the main peak from each column purification can reduce nuclease abundance. If necessary, perform a nuclease test on each fraction before combining them to ensure that nuclease-containing fractions are removed.

Ensuring that proper cell density has been reached prior to inducing protein expression with IPTG is an important constraint for minimizing nuclease contamination. Low protein yields can result when cells are induced at suboptimal induction ranges (i.e., outside the optimal OD_{600} ~0.6 to 0.8 range). In the event that a culture has become overgrown, dilute a portion of the culture with fresh medium and carefully monitor it until it is in the appropriate induction range of OD_{600} ~0.6 to 0.8.

Protein work up

Critical parameters for the protein work up include the temperature and time required to perform each step in the protocol, as unnecessary temperatures and times can lead to protein oxidation.

The hot and cold incubation steps are one of the most critical purification steps in the protocol, responsible for removing nearly 90% of the endogenous *E. coli* proteins present in the crude cellular lysate. This is due to the low thermostability of most *E. coli* proteins. Therefore, it is important to ensure that the full incubation of 60 min at 80ºC and 30 min on ice are performed. If crude protein concentration is not significantly and drastically reduced after the hot and cold temperature treatments, ensure that the hot block or water bath is set to the correct temperature.

Polyethyleneimine (PEI) is used to precipitate nucleic acids from crude lysates. At neutral pH, PEI is a positively charged polymer that can interact strongly with nucleic acid polymers. Depending on the salt concentration, this step can be used to either purify nucleic acid binding proteins along with the pellet or to remove DNA and RNA from the supernatant. At high ionic strength (>1 M NaCl), nucleic acids are removed and almost no proteins remain bound to the pellet. Typically, this salt concentration is not used because it causes undesired *E. coli* proteins to be retained in the supernatant. At medium ionic strength (0.2 to 1 M NaCl), nucleic acids and some proteins will be precipitated and the desired protein will remain in the supernatant. At low ionic strength (<0.2 M NaCl), many nucleic acid binding proteins remain bound to the nucleic acid and co-precipitate. If the polymerase is being lost during the PEI step, titrate the NaCl concentration to determine the highest salt concentration that leads to maximum retention of the recombinant polymerase in the supernatant.

Ammonium sulfate precipitation is performed to remove excess PEI. Protein loss during this step is often due to the solution not being fully saturated with ammonium sulfate. The saturation is affected both by the amount of salt added to the solution and the temperature of the solution during the incubation. Ensure that all steps including centrifugation are performed at 4ºC or a portion of the protein will re-solubilize into solution and not precipitate.

FPLC purification

Commercial FPLC columns have a finely packed matrix with a high-loading capacity. Clogging of the matrix or unpacking of the column can lead to drastic reductions in column efficiency. To avoid this problem, samples should be centrifuged at 20,000 rpm or filtered with a 0.2-μm filter prior to loading onto the column. Care should be taken to avoid introducing air bubbles onto the column during injection. Also, avoid flowing salt buffers in the presence of EtOH, which can cause salt crystals to form in the column.

The highly charged nature of the nickel column leads to a high amount of nonspecific binding in the presence of low salt. Therefore, the nickel purification must be performed in buffers containing at least 600 mM salt and 20 mM of imidazole as a nonspecific competitor. If the column is still eluting large amounts of endogenous *E. coli* protein, try either including a wash with 1 M salt after the binding step to further reduce nonspecific interactions with the column or increasing the starting amount of imidazole up to 50 mM.

Low protein loading onto the heparin column is likely due to a high salt concentration (>100 mM NaCl) in the protein sample. The concentration of salt in the sample should be equivalent to the salt concentration of the loading buffer (~50 mM NaCl). If necessary,

collect the sample, dilute with additional low-salt buffer, and reload onto the column.

Additionally, it is beneficial to periodically clean the affinity column with ~10 to 20 CVs of 0.1 M NaOH. NaOH is highly effective at degrading a wide variety of contaminants including protein, nucleic acid, bacteria, yeasts, endotoxins, and inactivating viruses. A cleaning protocol that involves 10 CVs of water, 10 to 20 CVs of 0.1 M NaOH, followed by a second 10 CVs of water, and finally 5 CVs of 20% EtOH is recommended. At this point the column can be safely stored for future use.

Anticipated Results

One liter of cell expression will yield ~20 mg of purified protein in ~6 mL of buffer. Protein purity will range from 95% to 99%. The optimal working concentration of polymerase is ~1 μM, depending on the fraction of active polymerase following purification. With a molecular weight of nearly 90 kD, the 1 L of culture will produce ~20 mL of protein at 10× working stock.

Time Considerations

The entire protocol takes 6 days to complete. On day 1, the cells are streaked from a glycerol stock onto an agar plate. On day 2, the agar plate is used to inoculate a starter culture. On day 3, the expression culture is inoculated and grown, and then inducted with IPTG and allowed to express overnight. On day 4, the cells are harvested and lysed from which the protein is brought to an ammonium sulfate pellet. On day 5, the nickel and heparin purifications are performed. On day 6, the protein is characterized for activity and purity and then stored at 4ºC or in 50% glycerol at −20ºC.

Acknowledgments

The authors would like to thank members of the Chaput laboratory for testing the protocol and for providing helpful suggestions and comments. This work was supported by the DARPA Folded Non-Natural Polymers with Biological Function Fold F(x) Program under award number N66001-16-2-4061 and a grant from the National Science Foundation (1607111).

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