Purification and Characterization of T7 RNA Polymerase

Goal. The gene for a protein believed to be His-tagged T7 RNA polymerase has been overexpressed in *E. coli* cells. The goal of this study is to isolate, purify and characterize the His-tagged protein that has been over-expressed in order to confirm that it is T7 RNA polymerase.

Experimental Design Strategy. Many analytical methods can be employed to confirm the identity of the overexpressed protein. The purification and characterization of T7 RNA Polymerase to be done here has 5 parts:

1. Cell lysis
2. His-tag affinity chromatography
3. Buffer exchange via ammonium sulfate precipitation
4. Protein concentration determination via the Bradford Protein Assay
5. MW determination via SDS-PAGE.

Students will be provided with a frozen pellet of *E. coli* cells corresponding to 100-200 mL of bacterial culture. The cells must first be lysed open and the protein separated from other biological macromolecules, cellular organelles, membrane components and any other cellular components. Cell lysis can be performed using chemicals (such as NaOH), enzymes (such as lysozyme), or some form of physical agitation or disruption (such as homogenization or sonication). Here, students will use ultrasonication to lyse open the bacterial cell pellets after resuspension.

Proteins can be artificially tagged with histidine residues at either the N- or C-terminus to deliberately exploit metal affinity chromatography as a purification strategy. The purification can be so efficient, due to the high specificity of ligand binding to the resin, that only one-step of purification is needed oftentimes. Here, students will use Novagen’s His-Bind® resin charged with Ni²⁺ to purify any His-tagged protein in their bacterial cells using affinity chromatography. Bound protein must be eluted with a molecule that will compete with the His-tag for binding to the Ni²⁺ on the resin. A reasonable competitor molecule would be one that has a similar structure to histidine, but with more degrees of freedom for movement and at substantially higher concentration than the His-tagged protein. The conventional reagent used to elute His-tagged proteins is imidazole at concentrations on the order of 200 mM.

Protein eluted from a metal affinity chromatography column would typically be captured in buffer containing a relatively high concentration of imidazole, which interferes with many subsequent methods such as colorimetric protein assays, gel electrophoresis, and some activity assays. Therefore, a buffer exchange step is required prior to analytical testing or storage of the protein until later analysis. There are many strategies for conducting buffer exchange including dialysis, immunoprecipitation, and filtration. One economical approach is simply ammonium sulfate precipitation followed by resolubilization of the precipitated purified protein in an imidazole-free buffer. Students will end the day’s work by performing ammonium sulfate precipitation on their samples prior to storage at 2-8°C.
MATERIALS AND REAGENTS

**Bacterial Overexpression**

- LB Broth
- LB Agar
- 10 mg/mL Ampicillin
- IPTG
- Inoculating Loop
- Flame
- Bacteria
- Autoclave

**Metal Affinity Chromatography**

- 100 mM Phenylmethanesulfonyl fluoride (PMSF) - a protease inhibitor
- 100% (w/v) Sodium Deoxycholate - a detergent
- Sorvall RCS Super-speed ultracentrifuge
- FiberLite® F13-50cy rotor that accepts 50-mL conical tubes (Piramoon Technologies, Inc.)
- Branson Sonicator Model 250A
- Shimadzu UV1601 UV-visible spectrophotometer
- Novagen His-Bind® Resin
- Bio-Rad Glass Econo-Column (ID: 1.0 cm; Length: 10 cm; Max Vol.: 8 mL)
- Tygon Tubing, Formulation R-3603
- 50 mM Nickel Sulfate
- Ammonium Sulfate

**Binding Buffer**

- 20 mM Tris-HCl, pH 7.9
- 0.5 M NaCl
- 0.05% (v/v) Tween 20 (detergent)
- 5 mM Imidazole

**Wash Buffer**

- 20 mM Tris-HCl, pH 7.9
- 0.5 M NaCl
- 0.05% (v/v) Tween 20 (detergent)
- 60 mM Imidazole

**Elution Buffer**

- 20 mM Tris-HCl, pH 7.9
- 0.5 M NaCl
- 0.05% (v/v) Tween 20 (detergent)
- 200 mM Imidazole

**Strip Buffer**

- 0.2 M Tris-HCl, pH 7.9
- 0.5 M NaCl
- 0.1 M EDTA
EXPERIMENTAL PROCEDURES

Performed by the TA - Growth of E. coli Cells Overexpressing T7 RNA Polymerase

Day 1:
- Prepare the agar solution according to the manufacturer’s instructions.
- Autoclave the agar solution then allow it to cool to ~50 °C.
- To the agar solution, add 1 μL of 100 mg/mL ampicillin for every μL of agar. Pour the agar onto a petri dish and allow the agar to solidify.
- Streak the plate with the IPTG-inducible recombinant expression vector containing His-tagged T7 RNA polymerase using a sterile inoculating loop. Cover the petri dish with its top and wrap parafilm around the circumference.
- Leave the plate inverted at 37 °C for ~16 hours (overnight).

Day 2:
- Check the agar plate for bacterial growth. Store the plate at 2-8 °C until further use.
- Make LB broth according to the manufacturer’s instructions. Autoclave the broth in ~500 mL aliquots in 2L Erlenmeyer flasks. Keep the flasks covered with foil to deter contamination. Also make a flask containing 100 mL of LB broth in 500 mL flask to generate a starter culture. Allow the broth to cool.
- Add enough ampicillin to the starter culture for a 60 μg/mL solution. Allow the solution to mix for 10 minutes.
- Inoculate the starter culture with a single colony from the agar plate.
- Leave the starter culture at 37 ºC for 16 hours with vigorous shaking (i.e. setting #4 on shaking incubator).

Day 3:
- Add enough ampicillin to the large cultures for a 60 μg/mL solution per flask. Allow the solution to mix for 10 minutes.
- Inoculate the large cultures with 1 mL of the starter culture per 100 mL of culture.
- Allow the larger cultures to shake vigorously at 37 ºC until the absorbance of the culture reaches ~0.6 at 600 nm. (This normally takes ~2-2.5 hours).
- Add IPTG to a concentration of 0.4 mM to induce bacteria to produce T7 RNA polymerase. Do this by preparing a concentrated solution and adding a very small volume to the culture. The subsequent vigorous shaking will allow for thorough mixing.
- Incubate at 37 ºC with vigorous shaking for 4 more hours.
- Distribute the culture into 200 mL aliquots in Sepcor bottles. Centrifuge the cells at 12,000 rpm for 15 minutes at 4 ºC.
- Discard supernatant and leave the bottles inverted to drain most residual supernatant. Resuspend the pellet in ~5-10 mL of PBS (or Tris buffer). Transfer the cells to polypropylene tubes.
- Centrifuge the cells in the smaller tubes for 8-10 minutes at 10,000 rpm at 4 ºC
- Discard the supernatant and invert the tubes to drain most residual supernatant.
- Store the pellets at -20 ºC until further use.
Performed by the Students
All materials must be kept chilled during this experiment! Keep track of volumes at every step!

PROTEIN ISOLATION: Cell Lysis Via Sonication 30 min – 1 hour

1. Thaw the pellet on ice or at 2-8 °C for about 15 minutes. Resuspend the pellet in 5 mL binding buffer with careful pipetting.

2. Add 12 μL of 100 mM PMSF (protease inhibitor) in ethanol and 100 μL of 10% sodium deoxycholate (detergent) to the cells. Cover the tube and mix with inversion. Incubate the pellet on ice for 10 minutes. Transfer the cell suspension to a 50 mL conical tube.

3. Sonicate the cells to break them open using 10 pulses for each of 4 cycles. Keep the cells on ice during the breaks. Take care to avoid frothing which represents protein degradation. Transfer the sonicated cells back to your Oak Ridge (round-bottom) tube.
   Sonicator settings include:
   Time = Hold
   Duty Cycle = 50%
   Output = 6

AFFINITY CHROMATOGRAPHY 2-2.5 hours

4. Centrifuge the sonicated cells at 10,000 rpm (12,000 xg) for 30 minutes at 4 °C. Collect the supernatant as the lysate. Meanwhile, charge your column with 5 mL of 50 mM nickel sulfate.

5. In your lab notebook, record the total volume of lysate that you have. Aliquot a 75-μL lysate sample for the gel and absorbance studies. Label your tube "Lysate" and store it on ice.

6. Wash the column with deionized water. Meanwhile, prepare the following aliquots:
   - 15-mL of Binding Buffer
   - 50-mL of Binding Buffer
   - 25-mL of Wash Buffer
   - 10-mL of Elution Buffer

7. Equilibrate the column with 25 mL of binding buffer. Discard the eluent down the sink.

8. Load the lysate sample onto the charged column. Once you load the lysate onto the column, be sure to collect every drop of liquid that comes off of the column until the end. While the lysate is flowing through the column, collect the flow through in a 15-mL conical tube labeled "Lysate FT". Store the Lysate FT on ice.

9. Wash the column with 50 mL of binding buffer. While the binding buffer wash is flowing through the column, collect the flow through in two 50-mL conical tubes labeled "Binding FT". Store the Binding FT on ice.

10. Wash the column with 25 mL of wash buffer. While the wash buffer is flowing through the column, collect the flow through in a 50-mL conical tube labeled "Wash FT". Store the Wash FT on ice.

11. Add 10 mL of Elution buffer to the column. While the elution buffer is flowing through the column, collect a 3-mL fraction, a 4-mL fraction and a final 3-mL fraction, each in 15-mL conical tubes. Store these fractions on ice.

12. Either strip the nickel from the column using copious amounts of strip buffer or discard the columns. Be sure to remove the plastic stopcock from each column before disposal!
13. In your lab notebook, write down the total volume of each of the following solutions that you have:
   - Volume of Lysate FT
   - Volume of Binding FT
   - Volume of Wash FT
   - Volume of Elution #1 (~3 mL)
   - Volume of Elution #2 (~4 mL)
   - Volume of Elution #3 (~3 mL)

14. Calculate the amount in grams of ammonium sulfate required to make a 35% (w/v) solution for each sample in the previous step. Record the amounts in your lab notebook.

15. Weigh out the appropriate amounts of ammonium sulfate for each of the 6 samples above. Slowly add the ammonium sulfate to each aliquot and allow it to dissolve, keeping it cold on ice. For efficiency, precipitation of the Lysate FT can be performed while collecting the Binding FT sample, and so on. Transfer the elution samples to 50-mL conical tubes after completing the ammonium sulfate precipitation.

16. Balance the tubes. Centrifuge these 6 samples at 10,000xg for 10 min at 2-8 ºC.

17. Discard the supernatant from the ammonium sulfate precipitation step. Resuspend the pellet in a Tris or Phosphate buffer at pH near 7 or 8; use of Phosphate buffered saline is fine. Use only about 1-mL of buffer to resuspend the pellet. Store the concentrated samples at 2-8 ºC until further use.