

Chapter

Growing and Handling of Bacterial Cultures within a Shared Core Facility for Integrated Structural Biology Program

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Abstract

We have established and optimized standard operating procedures for growing and handling bacterial cultures in a shared core laboratory to support Integrative Structural Biology. The Integrative Structural Biology effort within the Biomolecular Research Center allows researchers to generate new knowledge about protein and RNA structure and function. We aim to understand how biomolecules assemble into stable structures and how structural dynamics impacts their function. Here we describe specific procedures for growing and handling bacterial cultures for overexpression and isolation of recombinant proteins, $^{15}\text{N}/^{13}\text{C}$ uniform labeling of recombinant proteins, protein isolation and purification, and analysis of protein solubility that are ideal for implementation in a shared research core laboratory that serves a multitude of diverse customers and research laboratories.

Keywords: protein expression, recombinant protein, isotope enrichment, core facility, protocols

1. Introduction

Shared research core facilities can provide support to campus-wide investigators by providing research infrastructure for the production and purification of recombinant proteins for a variety of research applications. We have designed a research support structure for investigators pursuing research in structural and functional studies that require high yields of pure proteins, particularly suited for structural studies including biomolecular nuclear magnetic resonance (NMR) and small angle X-ray scattering.

The *Escherichia coli* (*E. coli*) expression platform is commonly used for recombinant expression of proteins. The *E. coli* system has several advantages over yeast, insect cells, or mammalian cell expression systems: *E. coli* are relatively easy to handle, the doubling time is short, media are low-cost and there are abundantly established methods for protein expression [1–4]. The *E. coli* expression platform is also well-suited for stable isotope labeling of proteins for biological NMR studies [5–9]. Structural studies of proteins demand large quantities of high purity

protein. Meeting these requirements can be challenging, however, advancements in high-throughput technologies for recombinant expression of proteins have greatly advanced in the last decade or more, in large part due to efforts from large structural genomics and structural proteomics centers [1, 4, 10–12]. The lessons learned and technologies developed from these centers can allow for rapid assessment of different expression strategies, which can be transferred and scaled down to smaller-scale centers and academic labs [3, 4, 13].

In addition to a demand for large quantities of highly pure protein, structural studies also often demand high solubility and stability of the protein in solution. To address this need, a high-throughput fluorescence-based thermal-shift assay, also known as differential scanning fluorimetry (DSF), has been implemented at the large structural genomics and structural proteomics centers [14]. DSF was originally developed as a high-throughput drug discovery assay to screen for small molecules that bind to and stabilize target proteins [15–17]. The DSF screen has been further adapted to optimize buffer conditions by varying the pH, buffer components, detergents, reducing agents and small molecules to screen for conditions that increase the stability and conformational homogeneity of a protein [14, 17–20], which is key in obtaining high-quality structural data.

We have established and optimized standard operating procedures for growing and handling bacterial cultures in a shared core laboratory to support Integrative Structural Biology and have used these in our own research [21–29]. The Integrative Structural Biology effort within the Biomolecular Research Center, a shared core facility, allows researchers at Boise State University and collaborating institutions to generate new knowledge about protein and RNA structure and function. We aim to understand how biomolecules assemble into stable structures and how structural dynamics can impact their function. Here we describe specific procedures for growing and handling bacterial cultures for overexpression and isolation of recombinant proteins, $^{15}\text{N}/^{13}\text{C}$ uniform labeling of recombinant proteins, protein isolation and purification, and analysis of protein solubility that are ideal for implementation in a shared research core laboratory that serves a multitude of diverse customers

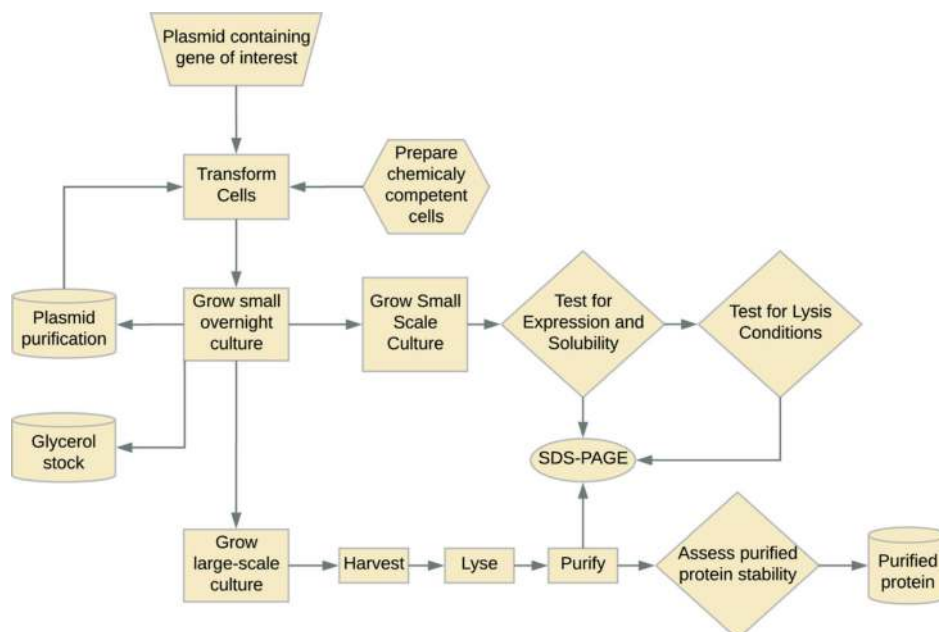


Figure 1.
A protein expression and purification workflow from plasmid to stable purified protein.

and research laboratories. Here we outline a general workflow of essential steps in protein expression and purification that includes plasmid amplification, mini-expression screening, optimized larger-scale protein production, protein isolation and purification, and characterization of optimized experimental solution buffer conditions (**Figure 1**).

2. Materials

All reagents listed in this chapter are commonly available from commercial vendors. A chemical hygiene plan including storage, shelf life, and safety of all chemicals should be in place at the institution.

2.1 Preparation of chemically competent cells

1. Ice.
2. Lysogeny broth (LB) medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl. Sterilize by autoclaving and store at room temperature.
3. Super optimal broth (SOB, a.k.a. Hanahan's Broth) medium for DH5 α cells: 20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.186 g/L KCl. Adjust the pH to 7.0 with NaOH. Sterilize by autoclaving and store at room temperature.
4. Culture tubes and flasks.
5. Incubator/shaker.
6. Centrifuge tubes.
7. Serological pipettes.
8. Repeating pipettor.
9. Dimethyl sulfoxide (DMSO).
10. Competent Cell (CC) buffer: 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 15 mM CaCl₂, 55 mM MnCl₂, 250 mM KCl, pH 6.7. Dissolve all components except MnCl₂ and adjust the pH to 6.7 with KOH. Then add the MnCl₂ and filter sterilize the solution over a 0.22 μ m filter.

2.2 Transformation of cells for expression of desired plasmid

1. Ice.
2. LB or super optimal broth with catabolite repression (SOC).
3. Culture tubes and flasks.
4. Incubator/shaker.
5. Centrifuge tubes.
6. Serological pipettes.

7. Competent cells.
8. LB-agar plates containing the appropriate antibiotic.
9. Plasmid DNA.
10. Heat block set.

2.3 Calculating efficiency of competent cells

1. Transformed colonies on LB-agar plate (see Section 3.3).

2.4 Inoculating overnight cultures

1. LB.
2. 15 mL conical tube.
3. Sterile inoculating loop.
4. Appropriate antibiotics.
5. Shaker/incubator.
6. Sterile aluminum foil or culture tube cap.

2.5 Glycerol stocks

1. 50% glycerol solution (autoclaved).
 - a. Make the 50% glycerol solution by diluting 100% glycerol into water.
2. Screwtop cryogenic vials.
3. Liquid nitrogen.

2.6 DNA plasmid purification

1. Resuspension buffer: 50 mM Tris-HCl, pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA), 20 µg/mL RNase A.
2. Lysis buffer: 200 mM NaOH, 1% w/v sodium dodecyl sulfate (SDS).
3. Precipitation buffer: 3 M potassium acetate, 2 M glacial acetic acid, 4°C.
4. Wash buffer: 70% ethanol.
5. 95% (or 100%) ethanol.
6. TE buffer: 10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA.

2.7 Testing for protein expression and solubility in *E. coli*

1. LB.
2. Appropriate antibiotics.
3. Incubator/shaker.
4. Microcentrifuge tubes.
5. Centrifuge.
6. Isopropyl β -D-1-thiogalactopyranoside (IPTG).

2.8 Lysing cells

1. Induced cells suspended in lysis buffer with a protease inhibitor cocktail, 0.1 mg/mL DNase I, 1 mg/mL lysozyme and 0.1 mg/mL 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF).
2. Sonication buffer.
3. Ice-saltwater bath.
4. Probe sonicator equipped with 1/2 inch tip.

2.9 Gel electrophoresis, protein quantification

1. Electrophoresis system.
2. 4–12% Bis-Tris mini gel.
3. Sample loading buffer: 10% glycerol, 0.14 M Tris Base, 0.1 M Tris-HCl, 2% lithium dodecyl sulfate (LDS), 0.5 mM EDTA, 0.02% Blue G250; 0.006% phenol red, 1.25% 2-mercaptoethanol, pH 8.5.
4. Running buffer: 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.2.
5. Coomassie Blue stain.
6. Protein molecular weight marker.

2.10 Testing lysis conditions for solubility

1. Buffer A: 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mg/mL lysozyme.
2. Buffer B: 50 mM Tris pH 7.5, 2 M NaCl, 5 mM EDTA, 1 mg/mL lysozyme.
3. Buffer C: 50 mM Tris pH 7.5, 100 mM NaCl, 50% detergent, 1 mg/mL lysozyme.

2.11 Large-scale expression of recombinant proteins

1. LB.
2. IPTG.
3. Culture tubes and flasks.
4. Incubator/shaker.

2.12 Uniform ¹⁵N/¹³C labeling of recombinant proteins

1. LB.
2. IPTG.
3. Culture tubes and flasks.
4. Incubator/shaker.
5. 10X M9 medium: 340 mM Na₂HPO₄, 220 mM KH₂PO₄, 85.5 mM NaCl, pH 7.4.
6. 10X Ammonium chloride: 93.5 mM NH₄Cl.
7. 20% wt/vol glucose stock.
8. 100 mM CaCl₂.
9. 1.0 M MgSO₄.
10. 10 mg/mL thiamine.
11. 10 mg/mL biotin.¹
12. Antibiotic for plasmid selection.

2.13 Protein purification using immobilized metal affinity chromatography (IMAC)

Immobilized metal affinity chromatography (IMAC) is a common method for affinity purification. A genetically encoded 6-histidine repeat affinity tag can be introduced to the carboxy or amino terminal end of the protein during cloning, which has high affinity for metal ions. The protocol given here is for affinity purification by immobilization of nickel ions with a chelator molecule, nitrilotriacetic acid (NTA) that is covalently bound to agarose; commonly known as Ni-NTA agarose. The following buffers are meant to represent a general starting point. Depending on the pI of your recombinant protein and the propensity to nonspecifically interact with the column material or resident *E. coli* proteins, modifications may need to be made. Additional purification may be necessary, especially when purifying proteins that bind to nucleic acids. A lithium wash may be added to the Ni-NTA purification

¹ The stock solution of 10 mg/mL is above the solubility limit of biotin, do not sterile filter this solution. Simply make the solution with previously sterilized water.

to remove nucleic acids. Ion exchange, heparin affinity, size exclusion chromatography are often added in addition to a nickel affinity purification step.

1. Lysis buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH 8.1.
2. Wash buffer: Lysis buffer plus 5–20 mM imidazole.
3. Elution buffer: Lysis buffer plus 100–300 mM imidazole.
4. Probe sonicator.
5. 1 mg/mL lysozyme.
6. Protease inhibitor cocktail.
7. AEBSF.

2.14 Differential scanning fluorimetry to assess protein stability

1. Low ionic strength buffer (e.g., 10 mM Tris-HCl).
2. qPCR machine with filter set that matches fluorescent dye and equipped with a ramp rate of minimum 1°C/min.
3. A fluorescent dye that will bind proteins.
4. 96-well polymerase chain reaction (PCR) microplate.

3. Methods

3.1 Preparation of chemically competent cells

1. Inoculate 5 mL of LB (or SOB if preparing DH5 α cells) with 10 μ L of appropriate *E. coli* cells² and grow overnight at 37°C and 250 rpm in a shaking incubator.
2. Use the overnight culture to inoculate 250 mL of LB (or SOB if preparing DH5 α cells) and incubate at 30°C until the optical density at 600 nm (OD₆₀₀) is between 0.4–0.6.
3. Chill the culture for at least 10 min on ice. For steps 4–10, keep the cell suspension on ice.
4. Spin the cell suspension for 10 min at 6000 \times g.
5. Gently resuspend the pellet in 50 mL ice-cold CC buffer into 50-mL conical tubes. Resuspend with a 10-mL serological pipette and avoid introducing bubbles.

² Some cell lines have a resident plasmid, such as BL21(DE3) pLysS or pLysE cells and require addition of antibiotics for selection of cells containing those plasmids.

6. Incubate the cell suspension on ice for at least 10 min.
7. Spin for 10 min at $6000\times g$ at 4°C .
8. Gently resuspend the pellet in 9.4 mL ice-cold CC buffer and add 0.7 mL DMSO.
9. Incubate the cell suspension on ice for at least 10 min.
10. Distribute the cell suspension in 50–200 μL aliquots in 1.5-mL microcentrifuge tubes.³
11. Flash freeze the cell suspension in liquid nitrogen and store the tubes at -80°C .
12. At -80°C the cells will be competent for at least 6 months.

3.2 Transformation of *E. coli* cells with plasmid DNA

1. Take competent cells out of -80°C and thaw on ice (approximately 20–30 min).
2. For each transformation, remove two LB-agar plates (containing the appropriate antibiotic) from storage at 4°C and warm to room temperature; optionally warm to 37°C in an incubator.
3. Mix 10–100 pg. DNA into 20–50 μL of competent cells in a 1.5 mL microcentrifuge tube.
4. Gently mix by flicking the bottom of the tube with your finger a few times.
5. Incubate the competent cell/DNA mixture on ice for 20–30 min.
6. Heat shock each tube at 42°C for 45–60 s.
7. Put the tubes back on ice for 2 min.
8. Add 1 mL of LB medium (without antibiotic) to the bacteria and grow at 37°C and 250 rpm in a shaking incubator for 45 min.
9. Plate 50 μL of the transformed cells onto one of the 10 cm LB-agar plate containing the appropriate antibiotic and the remaining 950 μL onto the second 10 cm LB-agar plate.
10. Incubate plates at 37°C overnight.

3.3 Calculating transformation efficiency of competent cells

1. Count the number of colony forming units (CFUs) on the LB-agar plate after transformation (see Section 3.2).

³ A repeating pipettor or a multichannel pipettor speeds up the aliquoting process greatly. This will minimize the time that the competent cells are manipulated, thus increasing their competency. Expect competency of ca. 10^7 – 10^8 CFU/ μg of plasmid DNA.

2. Calculate the transformation efficiency (TrEff) in CFUs/ μ g of DNA using Eq. (1).

$$TrEff = \frac{\# \text{ of CFUs} \times \text{Total vol. of Transformation } (\mu\text{L})}{\mu\text{L of transformation on plate} \times \mu\text{g of DNA}} \quad (1)$$

3.4 Inoculating cultures

1. Add 5–10 mL of liquid LB to a culture tube and add the appropriate antibiotic to at correct concentration. A good negative control is LB media plus antibiotic without any bacteria inoculated. You should see no growth in this culture after overnight incubation.
2. Using a sterile inoculating loop, select a single colony from your LB-agar plate for plasmid purifications and a swipe from 10 to 20 colonies for protein expression (Section 3.2).
3. Add the inoculating loop to the liquid LB with antibiotics and swirl.
4. Loosely cover the culture with sterile aluminum foil or a culture tube cap.
5. After incubation, check for growth, which is characterized by a cloudy haze in the media.
6. For overnight cultures, incubate bacterial culture at 30°C for 12–16 h in a shaking incubator.⁴
7. For long-term storage of the bacteria, you can proceed with Section 3.5.

3.5 Preparation of a glycerol stock

1. Follow Section 3.2 for transforming and plating *E. coli* cells.
2. Follow Section 3.4 for inoculating an overnight culture.
3. Add 500 μ L of the overnight culture to 500 μ L of 50% glycerol in a 2 mL screw top cryogenic vial⁵ and gently mix.
4. Submerge the glycerol stock tube into liquid nitrogen and store at –80°C. The stock is now stable for years, as long as it is kept at –80°C. Subsequent freeze and thaw cycles reduce shelf life.
5. To recover bacteria from your glycerol stock: open the tube and use a sterile loop, toothpick, or pipette tip to scrape some of the frozen bacteria off of the top. Do not let the glycerol stock thaw. Streak the bacteria onto an LB-agar plate.

3.6 Plasmid DNA purification

1. Preheat the TE Buffer in the incubator at 37°C.

⁴ For some applications (especially culturing cells in minimal defined media) cultures should never be overgrown; growing overnight cultures at a reduced temperature, 25–30°C, is suggested.

⁵ Snap top tubes are not recommended.

2. Spin 5 mL of the overnight LB culture at $6000\times g$ for 10 min at 4°C . Discard supernatant.
3. Add 250 μL Resuspension Buffer containing RNase A to the cell pellet and resuspend the pellet by pipetting until homogeneous.
4. Add 250 μL Lysis Buffer. Mix gently by inverting the capped tube until homogeneous. Do not vortex. Incubate the tube at room temperature for 5 min.
5. Add 350 μL Precipitation Buffer. Mix immediately by inverting the tube until homogeneous. Do not vortex. Centrifuge the lysate at $16,000\times g$ for 10 min at 4°C .
6. Add 2–2.5 volumes 95% or 100% ethanol and 1/10 volume of 3 M Na-acetate (pH 4.8) to the supernatant. Invert the microcentrifuge tube to mix. Let stand for 2 min at room temperature.
7. Centrifuge solution at high speed (at least $16,000\times g$) for 15–30 min at 4°C . Discard supernatant.
8. Open and invert the tube on a paper towel to drain it out.
9. Wash pellet by adding 500 μL cold 70% ethanol.
10. Centrifuge solution at high speed (at least $16,000\times g$) for 5 min at room temp. Discard supernatant by pipetting it out of the tube.
11. Dry the pellet by inverting over paper towel for 5–20 min.
12. Resuspend dry DNA with TE.
13. Store plasmid DNA at 4°C (short term) or store the DNA in aliquots at -20°C (long term.)

3.7 Testing for soluble protein expression in *E. coli*

The following protocol is written for proteins expressed under the control of the lac, tac, or T7 promoters. The method as described is a generic protocol that can be expanded to test expression in different strains of *E. coli*, induction temperatures, concentrations of IPTG, or even in the presence of ligands or cofactors.

3.7.1 Protein expression

1. Transform plasmid into an *E. coli* expression strain following Section 3.2.
2. Inoculate a liquid LB culture following Section 3.4.
3. Grow cells for a few hours at 37°C , shaking at 250 rpm. Make sure the tubes are tilted.
4. Monitor the turbidity. Once the culture reaches an OD_{600} of 0.4–0.6 (takes ~2–4 h, depending on the sample), take out 2 mL of the culture. Measure the actual OD_{600} .

5. Transfer the equivalent of 1 mL of cells at $OD_{600} = 0.8$ in a 1.5-mL microcentrifuge tube.⁶
6. Collect cells by centrifugation at $16,000\times g$ on a tabletop centrifuge for at least 1 min. Carefully remove all of the supernatant. This is the uninduced sample. Store the cells at -20°C .
7. Add IPTG to a final concentration of 1.0 mM to the remaining culture. Continue shaking at 250 rpm for 12–16 h at 18°C .
8. Measure the OD_{600} . Collect cells by centrifugation in two tubes containing the equivalent of 1 mL of cells at $OD_{600} = 0.8$ and remove the supernatant. These are the induced samples; one tube will be used to test for expression and the second for solubility. Store the cells at -20°C until ready to test for expression.

3.7.2 Testing for expression

1. Take the tube of uninduced and one tube of induced cells and resuspend each in 100 μL of 1X SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.
2. Boil the samples for 10 min, then cool down to room temperature.
3. Centrifuge for 5 min at $16,000\times g$ on a tabletop centrifuge at room temperature.
4. Take 10 μL of each sample from the top of tube taking care not to disturb the pellet.
5. Analyze the results using SDS-PAGE following Section 3.9 (**Figure 2**), with western blotting if necessary.

3.7.3 Testing for solubility

1. Resuspend the remaining induced cell pellet in 50 μL of lysis buffer containing protease inhibitors and 1 mg/mL of lysozyme.
2. Follow Section 3.8.1 for freeze-thawing to lyse the cells.
3. Spin down in a microcentrifuge at maximum speed for 10 min at 4°C .
4. Carefully transfer all of the supernatant into a new microcentrifuge tube. Add 50 μL of 2X SDS-PAGE buffer. This is the soluble fraction.
5. Resuspend the pellet in 100 μL of 1X SDS-PAGE buffer. This is the insoluble fraction.
6. Boil the samples for 10 min, then cool down to room temperature.

⁶ In order to have equal loading on an SDS-PAGE gel, the same amount of cells need to be harvested for gel analysis. To harvest the same number of cells each time, calculate the volume in mL needed of your culture that would be the equivalent of 1 mL of $OD_{600} = 0.8$. E.g. $X \text{ mL} = 0.8/OD_{600}$ of your culture.

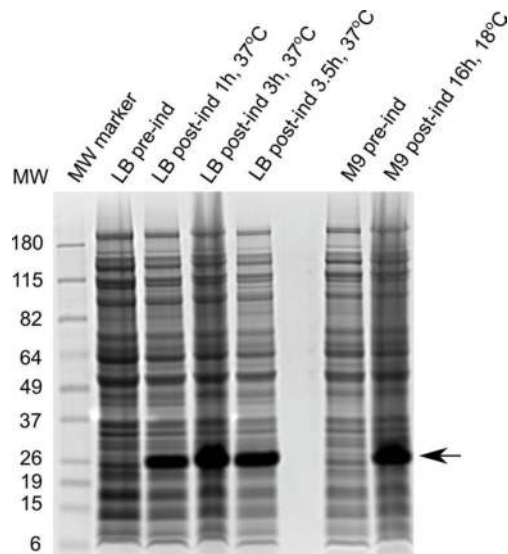


Figure 2. SDS-PAGE gel of pre- and post-induction of an RNA binding protein (RBP) in both rich (LB) and minimal (M9) media. The arrow indicates the recombinant RBP.

7. Centrifuge for 5 min at 16,000× g at room temperature.
8. Analyze 15 µL of each sample using SDS-PAGE following Section 3.9.

3.8 Lysing cells

Traditionally cell lysis can be done with physical disruption or reagent-based methods. Freeze-thaw protocol works best for small volumes (less than 1 mL) in 1.5 mL microcentrifuge tubes. Sonication can be done with smaller volumes using a microtip.

3.8.1 Freeze-thaw

1. Freeze the samples to be lysed (typically 0.1–1.0 mL in a 1.5 mL microcentrifuge tube) in a – 80°C freezer, leave for 15 min.
2. Thaw immediately in a 42°C water bath. Vortex vigorously to mix well.
3. Repeat the two previous steps three more times (four freeze-thaw-vortex cycles in all).
4. Spin the tubes for 5 min at maximum speed in a microcentrifuge.
5. Separate the supernatant (contains soluble protein) from the pellet (contains insoluble protein) by pipetting out the supernatant to a clean tube.

3.8.2 Sonication

1. Prepare ice-saltwater bath by sprinkling salt over packed ice in a container.
2. Place a 50-mL conical tube containing the cell pellet suspended in lysis buffer securely in the ice-saltwater bath.

3. Insert clean tip of a sonicator in the sample without contacting sides or bottom of the tube.
4. Set the output power, cycle, and timer to the optimal settings (e.g., five short bursts of 15 s followed by intervals of 30 s for cooling).
5. Keep the suspension at all times on ice.

3.9 Gel electrophoresis

1. Add ~100 μg of protein to SDS sample buffer.
2. Heat the sample at 70°C for 10 min.
3. Load the entire volume of sample onto a 4–12% Bis-Tris mini gel.
4. Run the gel at 200 V for 35 min.
5. At the end of the electrophoresis, wash the gel in deionized water three times.
6. Stain the gel with Coomassie Blue stain for 1 h.
7. Wash the gel with deionized water extensively until the water is clear.

3.10 Testing lysis conditions for solubility

The solubility of a protein depends strongly on the composition of the lysis buffer. Using the procedure described below, the solubility of a specific protein can be tested under neutral (Buffer A), high salt (Buffer B), and with detergent included (Buffer C).

1. Follow Section 3.7.1 for the best expressing condition and collect four induced samples.
2. Spin down the cells for 5 min at 12,000 \times g in a microcentrifuge.
3. To each cell pellet, add 100 μL of the appropriate buffer (see Section 2.10).
4. Vortex to resuspend the cells.
5. Lyse cells using the freeze-thaw method (Section 3.8.1).
6. To the supernatant, add 25 μL of 4X SDS-PAGE loading buffer.
7. To the cell pellet, add 125 μL of 1X SDS-PAGE loading buffer.
8. Heat all samples to 95°C for 5 min.
9. Vortex briefly and then centrifuge for 5 min at maximum speed.
10. Load 20 μL on an SDS-PAGE gel; avoid disturbing the pellet.

3.11 Large-scale expression of recombinant proteins

1. Transform plasmid into an *E. coli* expression strain following Section 3.2.
2. Inoculate a liquid LB culture for an overnight growth following Section 3.4.
3. The next day, use the overnight culture to inoculate 1 L of LB with the appropriate antibiotic.
4. Grow cultures at 37°C and 250 rpm shaking until the OD₆₀₀ is ~0.6–0.8.
5. Induce expression of protein by adding IPTG to a final concentration of 0.1 mM.
6. Lower the temperature to 18°C and continue 250 rpm shaking for 12–16 h.
7. Follow Sections 3.7.1 and 3.7.2 to test for protein expression.
8. Harvest the cells by centrifugation at 6000 × g.
9. Suspend cells in lysis buffer and store at –20°C.

3.12 Uniform ¹⁵N/¹³C labeling of recombinant proteins

This protocol is for proteins expressed under the control of the lac, tac, or T7 promoters.

3.12.1 Day 1

1. Transform 10 μL of competent BL21(DE3) cells (or derivatives) with 10 ng of plasmid DNA and plate cells on LB-agar containing the appropriate antibiotics (See Section 3.2).

3.12.2 Day 2

1. Prepare 50 mL of unlabeled defined medium for overnight culture as follows, in a 200 mL culture flask:
 - 5 mL 10X M9 medium.
 - 5 mL 10X ammonium chloride.
 - 0.75 mL 20% glucose.
 - 50 μL of each CaCl₂, MgSO₄, thiamine and biotin.
 - antibiotic at working concentration.
 - autoclaved water to 50 mL.
2. Inoculate a 5 mL culture (LB with appropriate antibiotic) with several freshly grown colonies (ca. 10–20).

3. Incubate cells in tilted tubes for a few hours at 37°C and 250 rpm in a shaking incubator, until the culture is visibly turbid.
4. Prewarm 50 mL of unlabeled defined medium to 30°C. While warming, centrifuge the LB culture (5 min, 4000× g, 30°C) and discard supernatant.
5. Resuspend cell pellet in 50 mL unlabeled defined media, for a starting OD₆₀₀ of ~0.03–0.08. Grow the culture overnight at 30°C in a shaking incubator.

3.12.3 Day 3

1. Prepare 500 mL of ¹³C, ¹⁵N labeled defined medium as follows, in a 2 L baffled flask:
 - 50 mL 10X M9 medium.
 - 0.5 g ¹⁵NH₄Cl dissolved in 5 mL water and sterile filtered.
 - 1.5 g ¹³C glucose dissolved in 10 mL water and sterile filtered.
 - 500 µL of each CaCl₂, MgSO₄, thiamine and biotin.
 - antibiotic at working concentration.
 - autoclaved water to 500 mL.
2. Prewarm the 500 mL of ¹³C, ¹⁵N labeled defined medium.
3. Centrifuge the overnight 50-mL unlabeled defined medium culture (5 min, 4000× g, 30°C) and discard supernatant.
4. Resuspend the cell pellet in 500 mL of ¹³C, ¹⁵N labeled defined medium, for a starting OD₆₀₀ of 0.03–0.08.
5. Grow culture at 37°C and 250 rpm in a shaking incubator until cells reach mid-log growth (OD₆₀₀ ~ 0.5–0.8).
6. Once cells reach mid-log growth (OD₆₀₀ ~ 0.5–0.8), measure the OD₆₀₀. Calculate the corrected volume (in mL) to take for the sample aliquot equivalent of 1 mL of cells at OD₆₀₀ = 0.8 (See Section 3.7.1 for details).
7. Transfer aliquot to a microcentrifuge tube, and spin it down at maximum speed for at least 1 min at room temperature. Remove the supernatant. This is an uninduced sample. Store the uninduced cells at –20°C.
8. Induce protein expression by adding IPTG based on the optimal values of IPTG concentration, incubation time and incubation temperature (See Section 3.7).
9. After the induced cells have grown for the proper length of time, dilute 200 µL of the culture 10-fold with 1X PBS and measure the OD₆₀₀. To prepare an induced sample, take an aliquot containing the equivalent of 1 mL of cells at OD₆₀₀ = 0.8 and immediately process it as described in Section 3.7.2.

10. Harvest the cells by centrifugation at 6000× g for 20–30 min at 4°C. Discard supernatant. Store the pellet at –20°C until ready for cell lysis.

3.13 Protein purification using IMAC

1. Resuspend cell pellet in ~35 mL of lysis buffer containing AEBSF, a protease inhibitor cocktail, and 1 mg/mL lysozyme.
2. Lyse cells (See Section 3.8).
3. Remove 75 µL of lysate and pipette into a 1.5 mL microcentrifuge tube. Centrifuge the 75 µL aliquot for 10 min at 12,000× g at room temperature.
4. Separate the supernatant into a new vial and treat with 25 µL of 4X SDS PAGE sample buffer. To the remaining pellet, add 100 µL of 1X SDS PAGE.
5. Boil separated and SDS buffer-treated samples for 10 min and store at room temperature for further SDS-PAGE analysis.
6. Centrifuge the remaining lysate (ca. 35 mL) at 16,000× g at 4°C for 20–30 min.
7. Filter the supernatant over a 0.4-micron syringe filter.
8. Pre-equilibrate the appropriate amount of Ni-NTA agarose for the amount of protein expressed in desired equilibration buffer; typically, 1–2 mL of settled agarose washed with two column volumes (CVs) of sterile, deionized water followed by two CVs of the buffer.
9. Bind the filtered lysate to the Ni-NTA agarose either batch or column loading. For batch loading, combine the filtered lysate and Ni-NTA agarose and gently rock for 30–60 min prior to pouring into the column. For column loading, pack Ni-NTA agarose into the column and pass the filtered lysate through the column. Collect the flow through eluent for SDS-PAGE analysis.
10. Wash the column with 15 CVs of cold lysis buffer. Collect wash eluent for SDS-PAGE analysis.
11. A step gradient consisting of 15 CVs each of 5, 10, and 20 mM imidazole may be used to determine best washing conditions. Collect wash eluents for SDS-PAGE analysis.
12. Wash the column with 20 CVs elution buffer, collecting 1 mL fractions.
13. Evaluate all collected samples by SDS-PAGE (see Section 3.9)⁷ (**Figure 3**).

⁷ Store lysate cell pellet at –20°C until SDS-PAGE has confirmed that full extraction of desired protein from the pellet is accomplished. Keep all buffers and protein samples at 4°C during purification. Batch vs. column choice will depend on binding properties of the individual protein. The SDS-PAGE of the step gradient imidazole washes will illustrate what is the highest imidazole concentration that can be used as an initial wash to clean off non-binding contaminants. If large amounts of protein remain in the cell pellet, alternative growing methods, such as IPTG concentration adjustment, or alternative purification methods including purification under denaturing conditions, should be considered. Additional purification may be necessary, such as ion exchange, or heparin binding column chromatography.

14. Pool fractions containing pure recombinant protein and dialyze into an appropriate buffer.
15. Clean Ni-NTA agarose by washing with 0.5 M NaOH for 30 min. Wash with five CVs sterile deionized water and store in 30% ethanol for long-term storage. The Ni-NTA agarose may be re-used for the same protein multiple times.

3.14 Differential scanning fluorimetry to assess protein stability

1. Prepare 1500 μL of 18 μM protein in dilution buffer.
2. Add 1.5 μL of 5000X dye.
3. Pipette up and down gently to mix.
4. Divide the protein plus dye solution among 10 vials: 140 μL per vial (some stock solution will remain).
5. Add 80 μL additive to be screened to one of nine vials.
6. Add 80 μL of dilution buffer in the tenth vial as a control.
7. Pipette up and down gently to mix.
8. Transfer 50 μL of protein plus dye plus additive solution (or control solution) to the 96-well PCR plate in triplicate.⁸
9. Cover PCR plate with a sheet of optically clear adhesive and seal each well.
10. Spin 96-well PCR plate in a centrifuge equipped with a plate holder at $800\times g$ for 2 min at room temperature.
11. Place 96-well PCR plate into qPCR machine and run the following program:
 - select total volume per well 50 μL
 - select experiment type melting curve
 - set the following temperatures: an initial 2 min hold at 25°C, increase in increments of 0.5–1.0°C and hold each for 30 s,⁹ to a final temperature of 95°C (with a 2 min hold).
12. Export data for further analysis.
13. Data can be plotted as the fluorescence vs. temperature (**Figure 4**).
14. After buffer optimization, structural data can be collected such as a ^1H , ^{15}N 2D NMR spectrum (see **Figure 5** for example of spectrum).

⁸ Excess solutions are suggested to account for loss due to transfers and sticking to the sidewall of the tubes.

⁹ Slower ramp rates will provide better melting temperature resolution, however not all instruments are equipped with fine temperature resolution.

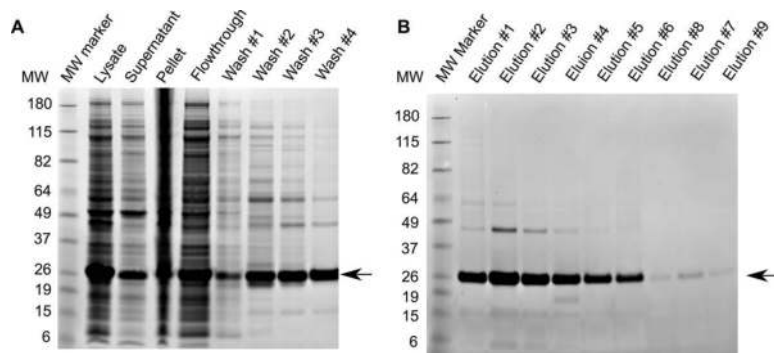


Figure 3. SDS-PAGE gel of a typical Ni-NTA purification of an RBP (arrow indicates the recombinant RBP). (A) Samples appear in the following order: MW markers, Lysate, Supernatant, Pellet, Flowthrough, Wash #1: 50 mM Tris-Cl, 100 mM NaCl, pH 7.7; washes #2-4: 10 mM Imidazole, 50 mM Tris-Cl, 100 mM NaCl, pH 7.7. (B) MW markers, Elutions #1-9: 200 mM Imidazole, 50 mM Tris-Cl, 100 mM NaCl, pH 7.7. Some protein elutes from the column in the wash steps. All fractions are kept and can be pooled after SDS-PAGE analysis.

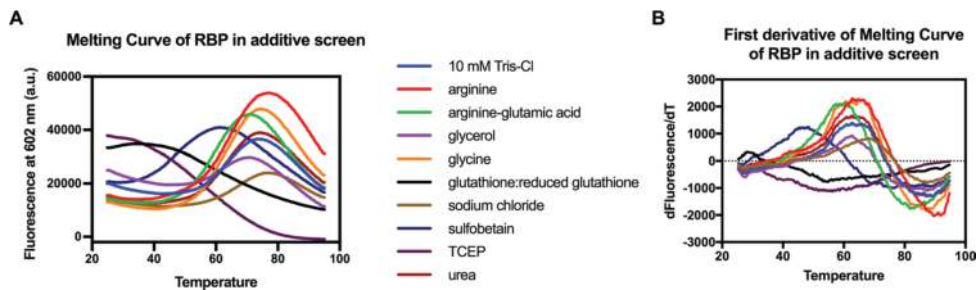


Figure 4. DSF analysis of an RBP in buffer (10 mM Tris-Cl) with different additives. (A) A graph of the fluorescence at 602 nm at increasing temperatures for the surveyed additive screen. The inflection point preceding the peak is the melting temperature. (B) A first derivative plot with a four-point smoothing applied helps to visualize the melting temperature, where the peak is the melting temperature. The legend provides a key for both A and B.

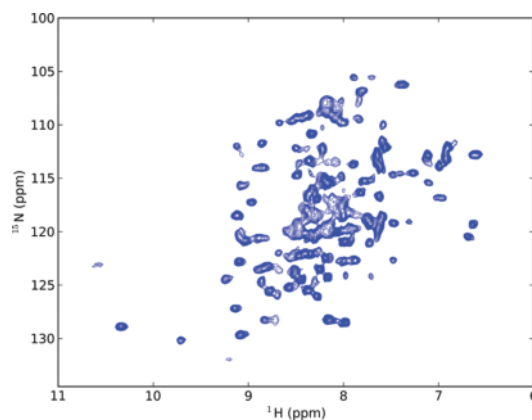


Figure 5. ^1H , ^{15}N 2D NMR spectrum of an RBP prepared using the methods described here.

4. Conclusion

We have described the workflow for protein expression and purification used in our shared core laboratory. These methods for growing and handling bacterial cultures work well for plasmid amplification, mini-expression screening, optimized larger-scale protein production, protein isolation and purification, and

characterization of optimized experimental solution buffer conditions. Future methods can be added as needed by the users of the core and the university research community.

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Conflict of interest

Authors have no conflict of interest.

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