Biochemistry Lab

Expression and His-Tag Purification

Experimental Plan

Goal: To optimize expression conditions and prepare purified MDH for human mitochondrial MDH and watermelon glyoxysomal MDH.

- Day 1 – Expression and store cell culture in 50 ml falcon tube (well labeled) in laboratory cooler.
- Day 2 – Purification and characterization by SDS PAGE.

General Procedure:

- *Expression*: Express 50 ml culture of **hmMDH (kan)** or **wgMDH (Amp)** using various concentrations of IPTG at 20°C and 37°C. Each culture will also use arabinose to induce endolysin. Use general 50 ml expression protocol, altering protocol as required for the specific expression conditions.
- *Cell Pellets*: Cells will be centrifuged by your instructor and placed in freezer.
- *Lysis and Purification*: Start immediately on Day 2. All buffers must be prepared on Day 1.
- *Characterization*: SDS PAGE, protein assay and enzyme assay as time allows.

GENERAL 50 ml EXPRESSION PROTOCOL: Optimal expression of protein depends on a couple of critical factors. Cultures from freshly transformed cells induced in the early lag phase of growth will typically produce the most protein. Over-expression can lead to the cell expressing and then placing the protein into insoluble bodies called inclusion bodies. Inclusion bodies are kind of the garbage can of the bacterial cell. The time and temperature of induction as well as IPTG concentration can all contribute to the level of expression and the presence of inclusion bodies (something we want to avoid if possible).

To maintain cells that only carry your plasmid, an antibiotic should be included in all phases of growth. Ampicillin acts to damage the membranes of *E. coli* by inhibiting the crosslinking of the bacterial membrane. Another commonly used antibiotic is kanamycin. This drug works by blocking protein synthesis at the mRNA level. It is important to remember that the antibiotic will "break down" (usually a hydrolysis of the compound) above 60°C or if left at room temp for several days. We typically keep concentrated antibiotic in the freezer. While it is not proper to re-freeze thawed solutions, we find little problem re-freezing unused antibiotic.

Remember –***ALWAYS*** check if you have included an antibiotic. ***ALWAYS*** know which antibiotic to use. Unless you are transforming cells all media should contain antibiotic(s). ***ALSO*** – some cell lines have two plasmids each with a different resistance, ensure you add both antibiotics to these cultures or you will end up with only one plasmid maintained!

Antibiotic	**Stock Concentration**	**Storage**	**Working Conc (dilution)**
Ampicillin (Sodium Salt)	50 mg/ml in water (500X)	-20°C	100μg/ml (2 μl of stock/ml)
Chloramphenicol	34 mg/ml in EtOH (200X)	-20°C	170 μg/ml (5 μl of stock/ml)
Kanamycin	25 mg/ml in water (500x)	-20°C	50 μg/ml (2 μl of stock/ml)

Culturing Cells – The flow of the experiment is to first start with a small overnight culture. The culture is provided for you. Then expand part of that culture into a new larger volume of media. Once the culture is in early log phase, induce the culture and incubate for another 3 hours at 37°C and/or overnight at room temp (20°C). You will chose the concentration of IPTG based on Qiagen's book, QiaExpressionist. *For background reading on expression use the links on the lab webpage.*

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Bacterial Culture:

- If starting from a glycerol stock, chip off a small piece from the frozen cells. *Do not even slightly thaw the tube of bacteria*. Streak out the cells from the ice chip on a plate with a loop or pipet tip. If necessary, the chip can be directly used to seed the starter culture. You can also start from freshly transformed cells. It might be necessary to screen for colonies that are high expressors.
- Pick and add a single colony of *E. coli* to 10 ml of LB media with the appropriate antibiotic. (See above chart for your calculations when using antibiotics.) Place into a large test tube with a plastic or metal loose-fitted top.
- Incubate at 37°C overnight with shaking or rotating. The culture should be cloudy with growth in the morning.
- Expand by adding 5 ml of overnight starter culture to 50 ml of LB media (in falcon tube in fridge) with Amp or Kan (use a 120 – 150 ml flask). Use cotton batting to plug the flask. Do not use parafilm or other sealable closures. Do not use falcon tubes
- Incubate at 37°C in the floor model shaker for 2 to 4 hours **or** until A600 ~ 0.4-0.6. Test OD using LB as a blank. Blank will be in a covered/sealed cuvette in the cold box.
- Add IPTG for a final conc of 0.1 to 1.0 mM *AND induce endolysis with arabinose* (500X in freezer) continue to incubate at 37°C for ~ 3-4 hours OR overnight at 20°C
- Transfer the culture to a well labeled, 50 ml conical (falcon) tube. Place tube in cold box in the biochem laboratory. Your instructor will centrifuge the culture at 5000 x rpm for 15 min at 4°C in the swinging bucket rotor. Pellets will be drained and frozen for later lysis and purification.

Preparation of Cell Lysate: XJ Autolysis™ *E. coli* strains are a new alternative for bacterial transformation and lysis. These strains are efficiently lysed following arabinose-induced expression of the bacteriophage λ endolysin protein, coupled to a single freeze-thaw cycle.

- Resuspend cells in 5 ml of His Binding Buffer by pipetting until no clumps remain.
- Add PMSF (1.0 mM final concentration) and DNAse A (50 µg/ml final concentration). Use appropriate volumes based on stock concentrations. ONLY ADD AT TIME OF LYSIS. PMSF has a 30 min half life. DNAse A is a protein and will denature over time.
- Freeze cells in dry ice isopropanol bath. Thaw cells in warm water with rotation. Repeat for a total of two times.
- Centrifuge at 8,000 x g for 15 min at 4°C.
- Save supernatant. Resuspend the pellet in 5 ml of buffer, save 100 ul for analysis, then discard pellet.

***Preparation of Ni-Agarose Beads/Resin (50 – 200 ml culture)*:**

- Prepare 2.5 ml of beads by transferring 5 ml of a 50% slurry of beads into a clean column. Wash and equilibrate the column by running 20 ml of His Elution Buffer followed by 20 ml of His Binding Buffer through the column. This SHOULD be done ahead of time!

Purification:

- Save 100 ul of lysate supernatant.
- Add clarified lysate to the washed beads in the column. Use a spatula and/or a transfer pipette to suspend the beads. Tightly cover with parafilm and incubate with rocking for 30 min at room temp.
- Replace the column on the stand and allow most of the beads to settle, then open column. Add frit back to column. ***Reapply the flow thru***. This is the non-binding protein.
- Wash beads with ***25 ml of His Binding Buffer***.
- Wash the column with ***25 ml of His Wash Buffer***. This will remove some of the weakly binding protein. Save as one fraction.
- Elute the protein with ***10 x 2 ml of His Elution Buffer***. Save ALL fractions in microfuge tubes.
- Check each fraction for total protein and determine purity by SDS-PAGE – coomassie stain.

His-Binding Buffer:	***His-Wash Buffer:***	***His-Elution Buffer:***
• 50 mM Tris-Cl (pH8.0) • 0.5 mM NaCl • 0.1 mM EDTA	• 50 mM Tris-Cl (pH8.0) • 0.5 mM NaCl • 20 mM Imidazole • 0.1 mM EDTA	• 50 mM Tris-Cl (pH8.0) • 0.5 mM NaCl • 300 mM Imidazole • 0.1 mM EDTA