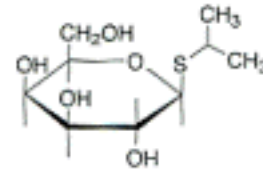


Recombinant Protein Expression and Purification from *E. coli*

Many different biochemistry projects require recombinant protein. This is a simple method for creating crude bacterial lysate of IPTG inducible plasmids. Specific purification of your protein will depend on if it is a fusion protein or the level of expression and solubility. But that is another subject all together...

IPTG Induction

Expression of many proteins in bacteria is controlled by the Lac Operon. An operon is a collection of linked genes under common, coordinate control. Typically bacteria do not use lactose as a source for food, however when enough lactose is added to the cells, lactose binds to repressor proteins and will cause the induction of the production of two different proteins, permease, used to transport carbohydrate and β -galactosidase.



**Isopropyl- beta- D-
thiogalactoside**

MW: 238.30 g/mol

β -galactosidase will hydrolyze the disaccharide lactose to the monosaccharides glucose and galactose, then the bacteria can continue to grow. Many researchers and companies have removed these two genes and placed a gene for a protein that they want. This way, when lactose or its non-metabolized inducer isopropyl beta-thiogalactoside, (IPTG), is added the cell is sort of tricked into making the protein cloned into the lac operon rather than permease and β -galactosidase.

Cell Lysis

There are many methods of breaking the cell membrane and isolating the soluble protein. Until a specific protein has proven difficult to isolate, I prefer to use a combination of sonication and detergent to lyse the cells. Addition of EDTA and PMSF is enough protease inhibitors for first phase of most experiments. BL21 cells are often preferred because they express less proteases. However BL21 cells are not good for maintaining the plasmid due to recombination.

Bacterial Culture - *This is a good general culture guide but if you are working on a class project refer to the class instructions for culturing.* It is good practice to start from a single colony that does not have small satellite colonies around it.

- If starting from glycerol stock, chip off a small piece from the frozen cells. Do not thaw the tube of bacteria. Streak out the cells from the ice chip on a plate with a loop. If necessary, the chip can be directly used to seed the starter culture.
- Pick and add a single colony of *E. coli* to 10 ml of LB media with the appropriate antibiotic. 10 μ l of 1000X or 20 μ l of 500X.
- Incubate at 37°C overnight with shaking in the incubator in Hagen 102.
- Add the whole culture to (use a 250 - 500 ml flask for up to 100 ml cultures - in the 102 incubator and up to 1000 ml cultures should use a 2 liter flask in 102) of LB media with Amp or Kan. **DO THIS FIRST THING IN THE MORNING OR YOU WILL NOT BE DONE UNTIL LATE!**
- Incubate at 37°C in the shaker in Hagen 102 or 104 for 2 to 4 hours or until A550 ~ 0.5-0.7
- Save 1.5 ml of cells as un-induced culture. Centrifuge for 10 min in a microfuge at max speed. Remove supernatant and add 75 μ l of 5X SDS PAGE SDS-PAGE sample buffer and freeze until SDS-PAGE analysis.
- Add stock IPTG and continue to incubate at 37°C for ~ 4-5 hours

- Save 1.0 ml of cells as induced culture. Centrifuge for 10 min in microfuge at max speed. Remove Supernatant and add 75 μ l of 5X SDS PAGE sample buffer and freeze until SDS-PAGE analysis.
- Centrifuge the culture at 5000 x g for 15 min at 4°C. If you have not used the centrifuge before check with Dr Provost before using.
- Resuspend pellet in ~ 25 ml or homogenization buffer (10 mM Tris-Cl pH 7.5) and transfer into a 50 ml falcon tube. Cells can be frozen at this point for several months.

Preparation of Cell Lysate

- Add the following Protease inhibitors and bring volume to 45 ml with PBS or Homogenization buffer. Once in aqueous solution, PMSF has a half-life of about 30 min. Use fresh each time.

| | |
|----------------------------|-------------------|
| 250 μ l of 100 mM EDTA | 0.5 mM Final conc |
| 250 μ l of 200 mM PMSF | 1.0 mM Final conc |

There are two common methods for lysing bacterial preparations to isolate the “lysate”. This preparation is not the cytosol but rather the “guts” of the bacterial cell. It will include the soluble protein expressed by the cell.

- Method 1) Enzymatic digestion of the cell membrane.
 - Make fresh Lysozyme (10mg/ml) add 1/10 vol
 - Shake at 30oC or 37oC in water bath (water is better at temp. transfer) . 30 min
 - Freeze in "liquid N₂" or back in the freezer, then thaw by incubated at 37oC about 15 min
 - It will be very viscous from DNA, thus we need to add DNase 1mg/ml - 1/10 volume and 1M MgSO₄ 1/10 vol. is needed for the DNase to act , shake about 15-30min. Or a short sonication will take care of sheering the DNA.
- Method 2) Mechanical and chemical disruption of the cell membrane.
 - Split into two tubes and freeze in liquid nitrogen and thaw
 - Sonicate 6, 15 sec bursts (60 - 70%) with probe sonicator on ice. Avoid frothing. Cell disruption is evidenced by partial clearing of the suspension. Over sonification can lead to co-purification of host proteins. Do not allow the tip of the sonicator to touch the sides of the sample or leave the sonicator on without the tip being submerged.
 - Add 1.25 ml of 20% Triton X-100 / 25 ml tube and rock for 30 min at 4°C. A typical final concentration of detergent should be 0.5 or 1.0 %. The detergent step can be omitted if there may be a problem with downstream purification steps.
 - Centrifuge at 12000 x g for 15 min at 4°C. Save and combine supernatants.
 - Freeze soluble protein at -20°C for later use.

SDS- PAGE Analysis

- For each procedure heat samples for 3 to 5 min in boiling waterbath. There is a beaker with a clear/white tube holder in the center isle of the biochem lab by the pH meters. Use this to boil your samples.
- For bacterial samples the sample is likely to be very viscous due to genomic DNA. Sheer the DNA by subjecting the sample to a 27 guage needle two or three times. The needles are found near the hot plate near the pH meters. Please clean out by rinsing several times with water before returning to its holder.
- Load 25 - 30 μ l of both the bacterial samples and the appropriate purification samples.

Down-stream processing- Simple starting points

Size exclusion chromatography - separation by SEC requires that the sample has a small volume. A rule of thumb is that the sample should be 50 to 100 times smaller than the bed volume. There are several ways to concentrate protein but one of the easiest and cheapest is to precipitate with a chaotropic salt, ammonium sulfate. Addition of the salt removes the layers of water from the protein's hydrophilic amino acids to cause protein aggregation and hence precipitation. Addition of aqueous solution to precipitated protein will allow the refolding of proteins. Most proteins will precipitate at 70% ammonium sulfate.

- To obtain a 70% saturated solution, slowly add 47.4 grams of solid ammonium sulfate per 100 g of protein solution.
 - This step should be conducted at 4°C (in the cold room)
 - Use a stir plate
 - Add the salt slowly as to avoid high local concentration of salt as it dissolves.
- Stir at 4°C for 15 to 30 min.
- Centrifuge sample at 10,000 g for 20 min
- Remove supernatant and resuspend pellet in desired volume. The solution should be clear if it is fully soluble.

Remember that the sample now contains a high concentration of salt that will need to be desalted or dialyzed before additional chromatographies can be used. When using SEC consider the final buffer you wish your protein to be in and use that as to elute your protein in.

Glutathione S- Transferase (GST) Fusion Proteins

- Prepare the appropriate sized column
- Equilibrate the column with five column volumes of GST Binding Buffer
- Apply the sample (lysate) save the flow through fraction as one fraction.
- Wash the column with four column volumes with GST Binding Buffer.
- Repeat above step two additional times. Save each wash as separate fractions.
- Elute protein with 10 column volumes of Elution Buffer. Collect purified protein in 6 equal sized fractions.

GST Binding Buffer (250 ml)50 mM NaH₂PO₄ or PBS

25 mM NaCl

0.03g

Adjust pH to 8.0 w/NaOH

GST Elution Buffer (50 ml)50 mM NaH₂PO₄ or PBS

300 mM NaCl

250 mM Glutathione (reduced)

Adjust pH to 8.0 w/NaOH

0.345g

0.877g

His Tagged Fusion Proteins -

- Equilibrate 2 ml of 50% Ni-NTA media slurry by flowing 20 ml of 6His-Wash Buffer through a small column.
- Transfer the beads to the 50 ml falcon tube with lysate and mix by rocking at 4°C for 60 min.

- Load the lysate and bead mixture into a capped column. Let the beads settle before opening and letting the non-binding proteins through.
- Save the flow through as one fraction.
- Wash the column twice with 12 ml of 6His-Wash Buffer and collect 6 ml fractions.
- Elute the column 4 - 6 times with 1.0 ml of 6His Elution Buffer and collect 1 ml fractions.

6His Wash Buffer (250 ml)

| | |
|--|--------|
| 50 mM NaH ₂ PO ₄ | 1.725g |
| 200 mM NaCl | 2.922g |
| 2 mM Imidazole | 0.03g |

Adjust pH to 8.0 w/NaOH

6His Elution Buffer (50 ml)

| | |
|--|--------|
| 50 mM NaH ₂ PO ₄ | 0.345g |
| 300 mM NaCl | 0.877g |
| 250 mM Imidazole | 0.851g |

Adjust pH to 8.0 w/NaOH

Stock Solutions, Media, Reagents and Materials

| Item | Concentration / Volume | Location |
|----------------|--|--|
| Ampicillin | Stock concentration is either 500X (50 mg/ml) or 1000X (100 mg/ml) in H ₂ O. Final concentration = 100 µg/ml Do not add until the media is cool to the touch! | In Plastic bags in the freezer in Hagen 102 |
| Kanamycin | Stock concentration- 500X (25 mg/ml) in H ₂ O. Final concentration = 25 µg/ml. Do not add until the media is cool to the touch! | In Plastic bags in the freezer in Hagen 102 |
| LB Medium | 10 g/L tryptone, 5 g/L yeast extract, 10 g/l NaCl. OR use the pre-mixed powder as directed. Autoclave, but do not fill more than 80% of capacity. For 1 liter cultures, use the 2 liter flasks in Hagen 104. | Individual items are in the cold room in Hagen 102. Pre-mixed powder is in Hagen 104. |
| LB Agar | LB medium containing 15 g of Agar OR use pre-mixed powder as described on the bottle. | See above |
| Culture Plates | The large plates (100 mm) need 10 - 15 ml per dish | In bottom drawer in the southwest corner of Hagen 102 |
| IPTG | Stock concentration is 1M (238 mg/ml in H ₂ O). Final conc. Should be 1 mM. | Stored in plastic bag in freezer in Hagen 102 |
| PBS | 10x Stock Solution <ul style="list-style-type: none"> ○ 80 g NaCl ○ 2 g KCl ○ 11.5 g Na₂HPO₄ H₂O ○ 2 g KH₂PO₄ | Shelve on Hagen 102. Use MilliQ Water to dilute. Only make what you need. |
| Tris-HCl | Trizma and Tris-HCl are one and the same. Tris base is the base form and will need to be pH adjusted differently | Hagen 102 Shelves |

| | | |
|---------------------------|--|--|
| EDTA | Stock solution is 0.5 M Tetrasodium EDTA Salt. Free acid is difficult to dissolve without adjusting pH. | Hagen 102 shelf / King 303 |
| PMSF | Stock concentration = 200 mM in Ethanol. | Hagen 102 shelf / King 303 |
| 20% Triton X-100 | Dissolve 10 ml Triton X-100 into 50 ml total H ₂ O. It may take 1 or more hours rocking to dissolve. The concentrate is very viscous so be certain of used volumes | Stock and concentrated detergent is at Hagen 104 shelf / King 303 |
| 5X SDS Page Sample buffer | See SDS-PAGE gel instructions. Stored in 15 ml falcon tubes. | Hagen 102 shelf / King 303 |
| Sonicator | Be careful to follow directions and clean and dry when done. Use the ear-protection when using. NEVER leave the sonicator on without being in the solution or touch the tip to the side. This includes turning the power down. It must be off when not in use. | King 303 or on the center bench in Hagen 102. |
| Needles syringes | Place needles in sharps container when finished. Wash and replace the barrels back container next to pH meter. | In a holder next to the pH meter in Hagen 102. |
| Plastic Cuvettes | Blank with media only. It is best to use a transfer pipet to save some for the duration of the experiment. | In a drawer in the small room between Hagen 102 and 104. |
| Falcon Tubes | Wash and replace when done. If stress fractures appear, discard. Re-used tubes are fine for many purposes. | In drawer in Hagen 102 |
| Solns SDS-PAGE | Recipe is found on web | Shelf in Hagen 102 |
| Coomasie Stain | Recipe is found on web | Shelf in Hagen 102 |
| Destain | Recipe is found on web | Shelf in Hagen 102 |
| Methanol | | Under north hood-Hagen 102 |
| Glacial Acetic Acid | | Under south hood-Hagen 102 |
| Biorad Gel Apparatus | Make gels day before using. It will take about 2 hours. Typically do this on a Thursday in lab. | Hagen 102. |
| Ethidium Bromide | 10 ng/ml stock. Transfer all solutions and agarose containing EtBr to large container in hood in Hagen 102. | Stored in falcon tube in cold room of Hagen 102. Tube is wrapped in aluminum foil. Take care handling. |
| DNA Loading Soln | | Shelf on Hagen 102 |
| Agarose | 0.8 or 1% is typically enough. Dissolve in TEA or TBE, not water. Add 40 µl Ethidium Bromide (10 ng/ml) to running buffer before preparing gel and using in electrophoresis. | Powder is on shelf in Hagen 102. |

| | | |
|--------------------------|---|--|
| TAE | 50X solution <ul style="list-style-type: none"> ○ 242 g Tris base ○ 57.1 ml glacial acetic acid ○ 37.2 g Na₂EDTA2H₂O ○ QS to 1 liter with H₂O | Shelf on Hagen 102 |
| Centrifuge Tubes | 500 ml tubes need to be washed and returned ASAP. Do NOT use centrifuge without prior clearance from Dr Provost | Tubes are in Hagen 102, smaller tubes are in a drawer near the sink in 102 |
| Imidazole | Use your solutions within a few weeks. Store the solutions you make in the cold room. Be certain to label with your name and the contents of the bottle. Not just elution buffer. | Powder is on the shelf in Hagen 102. |
| Ni-Columns. | Pre-made columns will be prepared and placed in the cold room for your use. When you are done, transfer it into the bottle labeled used Ni-Agarose. It is expensive and can be re-used after regeneration. | Cold Room Hagen 102. |
| Bradford Protein Reagent | The 1X solution is stored in a labeled bottle. A 5X solution is purchased from BioRad. | Shelf Hagen 102 |
| MDH assay | OAA, NADH (DNPH), and other materials are solids in the freezer. OAA can be prepared and saved as a frozed solution. NADH must be made up fresh each time. For other materials ask JP | Freezer Hagen 102 |