



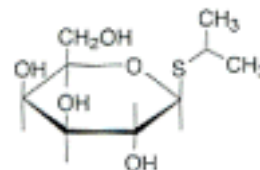
## Biochemistry Lab II Recombinant Protein Expression Protocol



### INTRODUCTION - Protein Expression / Induction

Many different biochemistry projects require recombinant protein. This protocol 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 and/or its level of expression and solubility. But that is another subject all together...

Expression of recombinant protein in bacteria is often controlled by the Lac Operon. The lac 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  $\beta$ -galactosidase.



Isopropyl-beta-D-thiogalactoside  
(IPTG) MW: 238.30 g/mol

Many researchers and companies have removed the genes for  $\beta$ -galactosidase and permease but kept the upstream regulatory DNA of lactose induction. In place of these two genes researchers have inserted a gene for a protein that they want the bacteria to produce instead of  $\beta$ -galactosidase and permease. This way, when lactose is added to a growing culture of bacteria the cells are sort of tricked into making the protein cloned into the  $\beta$ -galactosidase site rather than permease and  $\beta$ -galactosidase. One problem is that lactose is quickly degraded (metabolized) in culture, so in place of lactose a non-metabolized mimic of lactose called isopropyl beta-thiogalactoside, (IPTG) is used. IPTG is used to induce the expression of protein.

Optimal expression of protein depends on a couple of critical factors. Cultures from freshly transformed cells grown induced in 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 bacteria cell. Time and temp of induction as well as IPTG concentration can all contribute the level of expression and the addition 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. 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, 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!

Antibiotic	Stock Concentration	Storage	Working Conc (dilution)
Ampicillin (Sodium Salt)	50 mg/ml in water (500X)	-20°C	100 $\mu$ g/ml (2 $\mu$ l of stock/ml)
Chloramphenicol	34 mg/ml in EtOH (200X)	-20°C	170 $\mu$ g/ml (5 $\mu$ l of stock/ml)
Kanamycin	25 mg/ml in water (500x)	-20°C	50 $\mu$ g/ml (2 $\mu$ l of stock/ml)
Streptomycin	10 mg/ml in water (200X)	-20°C	50 $\mu$ g/ml (5 $\mu$ l of stock/ml)
Tetracycline HCl	5 mg/ml in EtOH (100X)	-20°C	50 $\mu$ g/ml (10 $\mu$ l of stock/ml)

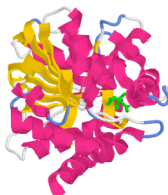
### SHORT PROTOCOL

Day 1 - From an isolated colony (transformed/glycerol stock/streaked plate) grow 10 ml overnight culture

Day 2 - Expand into 50 ml media and culture for 2-3 hrs

-Induce and culture for 4-6 hrs

- Centrifuge, save pellet. Freeze for later or lyse and clarify cells.



## Biochemistry Lab II

### Recombinant Protein Expression Protocol



#### FULL PROTOCOL -

**Culturing Cells** - The flow of the experiment is to first start with a small overnight culture. 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 3-6 hours. The cells must be centrifuged and the pellet stored by freezing or immediately lysed and clarified from cellular debris. Read the *QiaExpressionist* pp 49-5 for background on expression (link on research and lab page).

Starting your culture should always be done from an isolated colony from a freshly streaked plate. Do not go directly from a glycerol stock into your starter culture. It may be tempting but you can lose your plasmid this way. Don't trust the plates that have been around for too long (a month or so). They may look good but are likely dead or contaminated with a mold or fungus or some other nasty critter. If the plates are old, either transform a new set of cells or chip of a bit of frozen glycerol stock from the top of the tube with a pipet tip (do NOT let the frozen cell thaw) and spread on an LB Agar plate with antibiotic. Culture overnight in the 37°C incubator and store the new plate with the edge wrapped in parafilm in the fridge (4°C).

#### Bacterial Culture:

- If starting from 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 fresh 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 (in wood shelf in front of lab) with a plastic or metal loose fitted top.
- Incubate at 37°C overnight with rotating in the incubator. The culture should be cloudy with growth in the morning.
- Add the 5 ml of culture to 50 ml of LB media (in falcon tube in fridge) with Amp or Kan/Chlor (use a 120 - 150 ml flask) DO THIS FIRST THING IN THE MORNING OR YOU WILL NOT BE DONE UNTIL LATE! Use cotton batting to plug the flask (in cabinet in front of class). Do not use parafilm or other closures. Do not use falcon tubes
- Incubate at 37°C in the floor model shaker (SL206) for 2 to 4 hours or until A550 ~ 0.5-0.7. Test OD using LB as a blank.
- Add IPTG for a final conc of 1.0 mM and continue to incubate at 37°C for ~ 4-5 hours
- Transfer the culture to a 50 ml conical (falcon) tubes and centrifuge the culture at 5000 x rpm for 15 min at 4°C in the swinging bucket rotor. The "old school" centrifuge at the front of the room can be used for 20 min. If you have not used the centrifuge before check with Dr Provost before using. USE A BALANCE TUBE! Save and wash your used tubes.
- Resuspend pellet in 5 ml of phosphate buffered saline (PBS). Cells can be frozen at this point for several months.



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### Preparation of Cell Lysate

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.**
  - Resuspend pellet using a glass pipette. NOT the blue Ranin Pipets. Ensure the pellet is homogenous.
  - Use Stock 10X Lysozyme (10mg/ml - in freezer Re-use old enzyme) add 1ml for every 10 ml of re-suspended pellet..
  - Shake at 30°C or 37°C in water bath (water is better for temp. transfer) . 30 min
  - Freeze in "liquid N<sub>2</sub>" or in the freezer -80°C., Then thaw by incubation at 37°C for about 15 min
  - The lysate will be very viscous from uncoiled DNA, thus you will need to perform a short sonication. This will take care of sheering the DNA.
  
- **Method 2: Mechanical and chemical disruption of the cell membrane.**
  - Resuspend pellet using a glass pipette. NOT the blue Ranin Pipets. Ensure the pellet is homogenous.
  - 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 3000-5000 x rpm 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.
- 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 gauge 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  $\mu$ l of both the bacterial samples and the appropriate purification samples.

### **Purification of His Tagged Fusion Proteins –**

Follow the directions for the 10 ml of lysates you used for MGH.