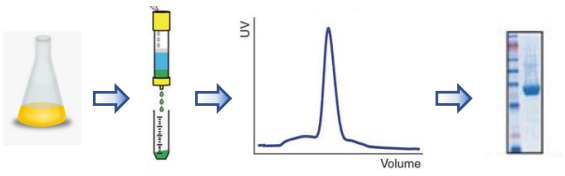
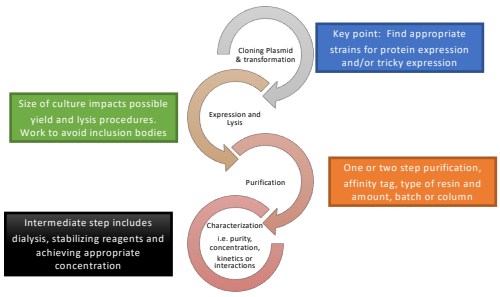
Recombinant Protein Expression and Purification

His Tag Affinity Expression and Purification Tutorial

Part One: Introduction to Expression and Transformation



Experimental flow…



Expression – Getting started

Goal: Produce enough recombinant protein from bacterial culture for planned experiments.

What do you need to get started???

Depends on starting materials…

MDH Protein Expression

Possible yield (mg)

Expression	mg/ml	50 ml culture	100 ml culture	250 ml culture	500 ml culture	1000 ml culture
low	0.25	0.125	0.25	0.63	1.25	2.5
mid	0.4	0.40	0.80	2.0	4.0	8.0
mid-strong	0.7	0.70	1.4	3.5	7.0	14.0
strong	1	1.0	2.0	5.0	10.0	20.0
very strong	5	5.0	10	25	50	100

Very Strong Expression: wgMDH, hMDH1
Mid-Strong Expression: hMDH2
Mid Expression: CS

Mutants vary low to strong – no clear reason!

Expression – Time-Out

How do I pick a bacterial strain for my experiment???

What are you trying to do?

Replicate DNA (plasmid prep)

Reduced expression of endonucleases to improve plasmid yield;
Reduced expression of recombinases to increase plasmid stability
e.g. DH5α, TOP10, XL1Blue, α-select

Great Resource:
Addgene – Plasmid 101
https://tinyurl.com/xhzrby

Express protein

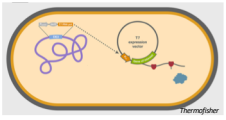
Reduced expression of proteases
- ompT (-) outer membrane proteolysis
- Ion Protease (-)

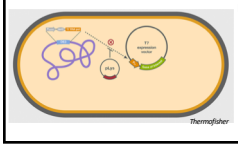
But… not so good for DNA fidelity:
Inactive restriction and methylation system no longer protecting DNA from restriction endonucleases
- Dam and Dcm methylase (-)

Expression – Time-Out Some plasmids need "helpers"

DE3: plasmids with promoters that require the T7 RNA polymerase

- T7 RNA polymerase is a strong, fast enzyme producing high rate of mRNA
- Up to 50% of can be the expressed protein!
- Some plasmids (e.g. **pET vectors**) have the promotor for T7 RNA polymerase
  BUT bacterial cells do not carry the gene – no expression!!!!
- These plasmids need a cell line with a "helper" (DE3)
- "(DE3)" indicates a strain of bacteria infected with λDE3 lysogen adding the gene for the T7 RNA polymerase
- DE3 production of the polymerase is driven by a IPTG Lac promoter





pLys: protect against leaky (basal) expression for toxic proteins

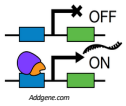
- Background expression means the combination of DE3 and plasmid will still produce some mRNA -> protein without induction
- Cells with pLysS or pLyseE produces a T7 specific protease to reduce background production of the polymerase

rne131: get even MORE expression

- rne gene (Ribonuclease E) increases RNA degradation
- rne131 codes for a C-term truncated protein that does not degrade RNA
- mRNA has longer half-life -> more protein

Expression – Time-Out

What kind of expression strains????

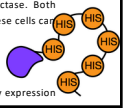


BL21

- Gram-negative E.coli from human intestine
- Most commonly used for protein expression
- Missing galactose and outer membrane and cytoplasm protease expression
- Descendant of the B strain used in early phage studies
- Does not include T7 polymerase so the "(DE)" means additional genes are expressed => BL21 and BL21(DE3) are NOT the same thing!!!!!
- Many biotech companies produce variants of the BL21 supporting protein expression

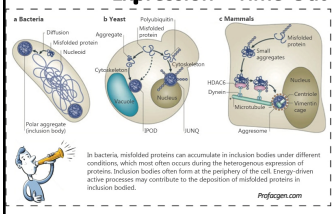
Helpful Bacterial Expression Strains

- **Tuner** - a modified BL21 with a *lacZY* (lac permease) mutants. IPTG in wild-type cells is difficult to control dosing to regulate lac operon-driven protein expression. Mutations of both permeases allow for dose response concentration of IPTG.
- **Origami** - a modified K-12 E. coli strain with mutations in thirodoxin reductase and glutathione reductase. Both result in increased ability to support Cys-Cys disulfide bonds. (better folding – thus "origami"). These cells can only be used with Amp^r strains. OrigamiB is a combination of Tuner and Origami.
- **Rosetta** – These derived Tuner cells with additional tRNAs to address low codons found in bacteria. Rosetta-gami are combinations of the Origami and Rosetta strains for missfolding rare codons
- **ArticExpress** - BL212 derived cells with two chaperone proteins from *Oleispira antartica* bacteria. This allows cells to grow and express/fold proteins at colder temps which helps avoid inclusion body expression



Expression – Time-Out

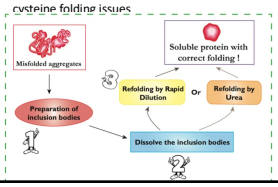
What are inclusion bodies????



An aggregation of insoluble proteins often mis-folded in difficult to isolate

Up to 70-80% of proteins expressed in *E. coli* are found in inclusion bodies

Often due to toxic nature of the protein or cysteine folding issues



Proteins can be isolated from inclusion bodies
Using high speed centrifugation – then dissolved

Proteins must be unfolded and refolded to achieve
Native structure and function

Expression – Transformation

A process to introduce plasmid DNA into a bacteria cell

Competent Cells

- Efficiency: measure of how much DNA enters the cell
- Made competent by chemical or electrical means
- Slight thawing significantly reduces efficiency

Select bacterial strain based on goal:
DNA prep = DH5α or like
Expression = BL21 type

Materials:	Reagents:
Ice/bucket	DNA
Tubes	Competent cells
Shaker/incubator 37°C	SOC/SOB/NYZM/LB media
Incubator 37°C	LB agar plates + antibiotic
Heat/water bath 42°C	

Check plasmid for antibiotic resistance.
Kanamycin requires outgrowth;
Ampicillin does not

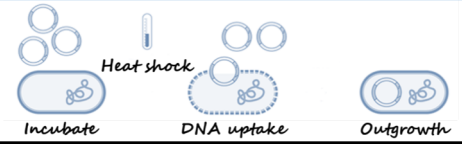
Outgrowth allows expression of the antibiotic resistance proteins encoded in the plasmid backbone so that they will be able to grow once plated on the antibiotic containing agar plate.

- Kanamycin inhibits RNA processes – cells will not replicate in its presence
- Ampicillin inhibits membrane crosslinking – cells will replicate and removal of Amp can "catch up" – no need for outgrowth

Expression – Transformation

Key issues –

- Level of competent efficiency.
  - Low – subcloning used for higher concentrations of plasmid
  - High – ultracompetent used for multiple plasmids, low conc, SDM
- PCR products and ligated samples need higher competent cells
- Spread on two plates (high and low volume) "just to be sure"…
- Sometimes using less DNA (not volume) increases numbers of colonies!



University of San Diego

Biochemistry Lab

Bacterial Transformation Protocol

DO NOT LET CELLS WARM UP! This will allow the holes in the cells to seal and your experiment will not work!

General Workflow / Protocol for traditional competent cells

- 20 - 100 µl of competent cells (ICE ICE ICE) into prechilled, labeled tubes.
- QUICKLY, mix 1-5 µl of plasmid DNA (1ng to 100 ng) and mix by tapping of "flicking" the tube with your finger.
- Incubate the mixture on ice for 20-30 min.
- Heat shock at 42° C for 45 sec, immediately place back in ice bucket and incubate for 2 min
- Add 250 µl of SOC and incubate at 37° C with shaking for 45 min. This is called the outgrowth step.
- Plate cells onto appropriate antibiotic containing LB Agar plates. Because it is difficult to predict the level of transfection (cell health, plasmid quality and quantity, overall technical handling and size of plasmid) it is best to spread two plates with a low volume (50 µl) and a high volume (200 µl) of outgrown cell culture.
- Spread with hockey stick, beads or inoculating loops. Ensure the entire culture is *evenly spread* over plate. You *are NOT trying to isolate streak in this procedure*. Give plates a few min for liquid to seep into agar.
- Incubate plates at 37° C overnight with the agar upside down.
- After ~12-16 hours, remove plates from incubator, seal with parafilm and store at 4°C upside-down.

Plasmid Preparation

- If the plasmid concentration is known, dilute plasmid with water (mol bio grade) to 0.05 mg/ml (aka 0.05 µg/µl).
- Use 1 µl of diluted DNA for each transformant. Calculate and record the mass (ng) of DNA used for the transformation.
- If the plasmid concentration is unknown, use 1 µl of DNA.
- Record the antibiotic resistance and other information on your plasmid in your notebook.

Tranformation

- Record the type of competent cells in the notebook - what cell strain, level of competent cells, if commercial name of company and information on the preparation of cell strain.
- For this class, you will conduct two transformants - one transformant plated for the appropriate antibody (two volumes - you decide how much for each) and the other will be divided equally onto an LB Agar plate without antibiotic and another LB plate with an incorrect antibiotic.

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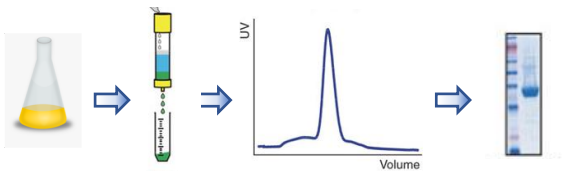
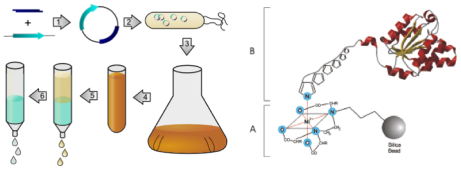
Part Two: Bacterial Culture & Induction, Cell Harvesting, and Cell Lysis



Workflow: Expression and Purification of His Tag MDH

- Expression – use bacteria to make protein
- Lysis – break open bacteria cells
- Purification – use the His Tag
- Characterize – how do you know you have MDH?



Bacterial Cultures: There are typically two reasons for culturing bacteria in our lab. First is to purify DNA and the other to express protein. The volume of culture depends on the plasmid purification method and the final yield you wish to achieve. Please refer to any manufacture's protocols for specifics. Below is a GENERAL guideline that can change depending on the volume of culture you need.

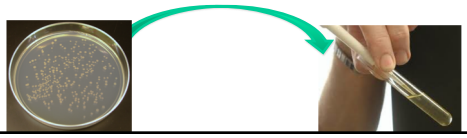
Antibiotic	Stock Concentration	Storage	Working Conc (dilution)
Ampicillin (Sodium Salt)	50 mg/ml in water (500X)	-20°C	100µg/ml (2 µl of stock/ml)
Chloramphenicol	34 mg/ml in EtOH (200X)	-20°C	170 µg/ml (5 µl of stock/ml)
Kanamycin	25 mg/ml in water (500x)	-20°C	50 µg/ml (2 µl of stock/ml)
Streptomycin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

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 60oC 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.

Culturing Cells – Starting your culture should almost always be done from an isolated colony from a freshly streaked plate. In a pinch you can use from glycerol stock 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 wrapped in parafilm in the fridge (4°C).

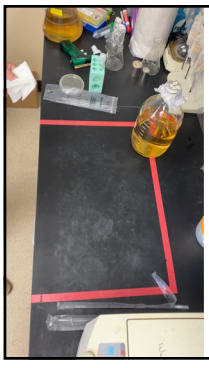
Expression

- Start with clone (resistance?) from plate or frozen glycerol stock
  - Pick a colony and place in starter culture
  - Incubate overnight (o/n) OR limit OD
    - OD – Optical Density – diffusion of light through media (not absorbanc)
    - Some cells stop expressing well if OD is > 0.7 units

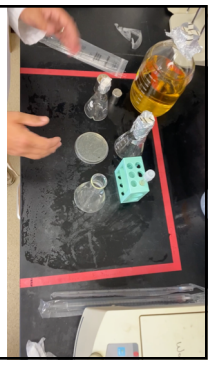


Culture in flasks

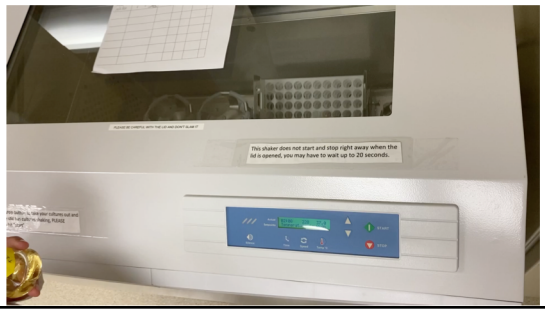
- Transfer portion of starter (inoculate from small initial culture) to larger flask
  - Never use more than ½ of total volume – aeration issues
  - Baffle flask if possible, cap with cotton or open flask cap
  - Orbital shaking while growing to keep media oxygenated and cells from sinking to bottom for healthy growth



Culture
- Inoculation



Culture - Expansion & Induction

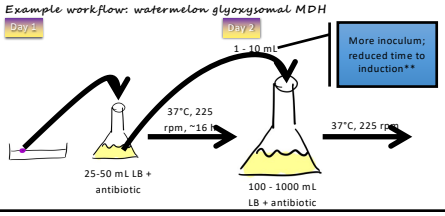


Expression – Let's get synthesizing!

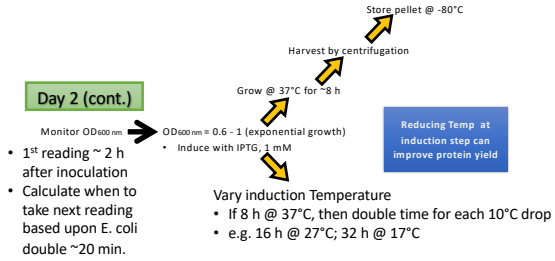
Starting Volumes – LB Media with appropriate antibiotic

- 50 ml culture - use 1 ml of 10 ml starter culture
- 250 ml culture - use 5 ml of 10 ml starter culture
- 1 and 1.5 L cultures use 20-40 ml of starter culture.

Example workflow: watermelon glyoxysomal MDH



Expression – Let's get synthesizing!



- 1st reading ~ 2 h after inoculation
- Calculate when to take next reading based upon E. coli double ~20 min.

Vary induction Temperature

- If 8 h @ 37°C, then double time for each 10°C drop
- e.g. 16 h @ 27°C; 32 h @ 17°C

Expansion of Starter Culture-

- *Expand the culture by adding the starter culture larger volume LB with antibiotic (room temp) and incubate for 1-4 hours until culture density reaches 0.5-0.6 OD600. Top the flask with cotton or a culture flask cap to allow culture to oxygenate without getting environmental contamination. Do NOT seal with parafilm or aluminum foil.*

Protein Expression Induction.

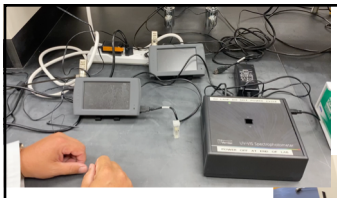
***OPTION 1** ROOM TEMP (20°C) INDUCTION.*
Induce Expression – After culture has reached OD 0.5-0.6 at 600 nm, cool down culture to room temperature by placing in ice.
- induce expression by adding IPTG to a final concentration of 0.1 to 1.0 mM. IPTG is a frozen solution in the -20oC freezer.
- Induce overnight (12-18 hours) at room (20oC) temp. with shaking.

- *Refreeze unused IPTG.*
- *Note – for very difficult expression proteins you may need to express at 16°C. In that case, induce for 24-48 hours.*

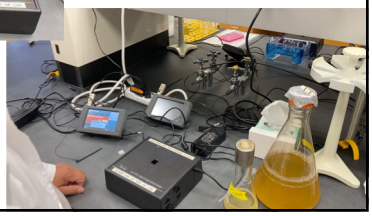
***OPTION 2** 37°C INDUCTION*

- *Induce Expression (see note below) – After culture has reached OD 0.5-0.6 induce expression by adding IPTG to a final concentration of 0.5 mM. IPTG is a frozen solution in the -20oC freezer.*
- *Induce for 3 to 4 hours at 37oC with shaking.*
- *Refreeze unused IPTG.*

Culture
- expansion & early growth

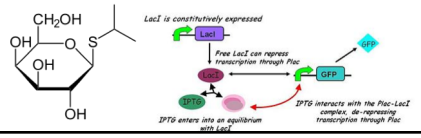


Culture
- Checking for density



Induction

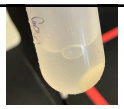
- Allow cells to grow to moderate density before inducing e. coli to express protein
  - 0.4-0.6 OD before starting culture
  - Cells grow exponentially! Growth starts slowly and speeds up fast at the end. DON'T overgrow! Impacts health of cells and expression levels.
  - Some plasmids are leaky – may need to add glucose to decrease expression until IPTG is added

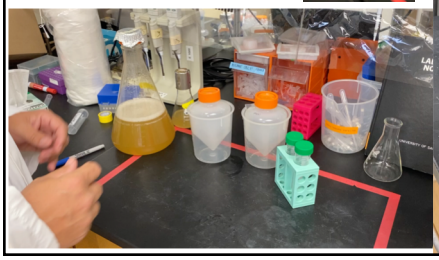


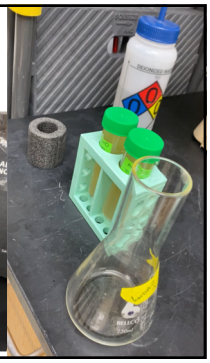
Cell Harvesting

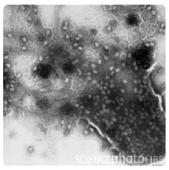
- Carefully balance tubes and centrifuge cells for pellet – can be frozen for later processing

Culture Harvest - centrifugation









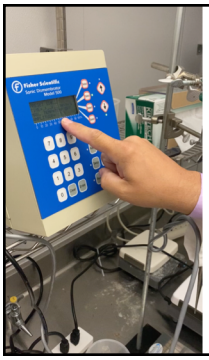
Cell Lysis

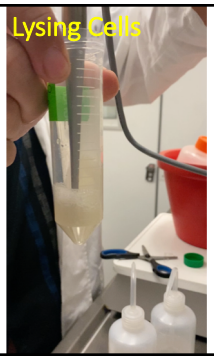
Low equipment/novel approach – autolysis

- XJ autolysis cells (BL21 strain) from Zymo Research - with second arabinose induced plasmid driving expression of bacteriophage gamma endolysin
  - Endolysin – hydrolytic enzyme degrading cell wall
  - Allows cell to be damage after freeze-thaw

Lysozyme/Sonnication (without TX-100)

- Addition of PMSF to inhibit released proteases
- βMercaptoethanol - help maintain redox state of protein
- DNAse A and sonication reduce DNA viscosity large preps
- Centrifuge membrane from soluble portion = lysate





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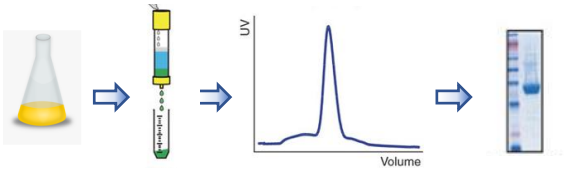
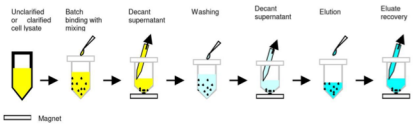
Part Three: His Tag Recombinant Protein Purification and Characterization

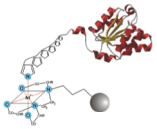


Purification Modes – step elution.

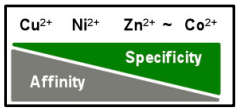
- Batch (centrifugation or magnetic)
- Spin
- gravity column chromatography
- LC/FPLC



Key issues



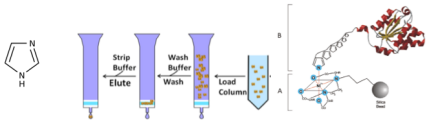
- Clarification
- Loading, washing and eluting (column chromatography)
- Overloading (bind top 10-20% for resolution)
- Inclusion of salt and imidazole in binding and initial wash
- My need other metals for specificity
- Rule of thumb- 10 CV wash…
- Storage



Other Rule of thumb
1liter good expression needs ~20 ml column
100-250 ml liter needs 2-5 ml column

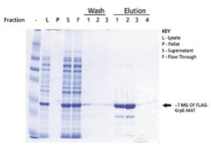
Purification

- Nickle-Agarose (or some other solid support) beads
  - Mix lysate with beads to allow maximum binding
  - Load onto column, allow beads to settle and re-load lysate through
  - Inclusion of low concentration of Imidazole to reduce weak Ni+2 binding.
  - Wash poorly bound proteins (~10 column volumes)
  - Elute (remove from beads) with imidazole



Characterization

- ➢Protein Concentration
- ➢Enzyme Activity (specific activity)
  - ✓Demonstrates purity and active enzyme
- ➢SDS PAGE Analysis
  - ✓Demonstrates purity
  - ✓Protein should migrate to correct size/location on gel



His-Tag Nickle Protein Purification

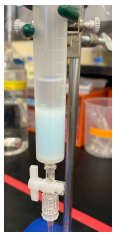
Purification Instructions (per 1000 ml culture adjust to the appropriate volumes):

- ***Preparation of Ni-Agarose Beads/Resin:***
  - Prepare 25 ml of beads by transferring 50 ml of a 50% slurry of beads equilibrate into a clean column. Wash and equilibrate the column by running 200 ml of His Elution Buffer followed by 500 ml of His Binding Buffer through the column. This SHOULD be done ahead of time! Store prepared beads with a few ml of His Binding buffer at RT.
- **Purification:**
  - Save 100 ul of lysate. Add clarified lysate (if frozen, check for ppt material. If there is any clumpy or ppt material or if the lysate is cloudy, centrifuge and keep the supernatant) to the washed beads.

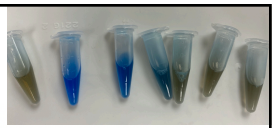
Batch Binding - if protein expression is low or the His-tagged protein binds with low affinity then use a batch purification method.

- Combine the washed beads and lysate onto a drained and capped column or a 50 ml falcon tube for smaller volumes. Use a spatula and/or a transfer pipette to suspend the beads. Tightly cover with parafilm and incubate rocker for 30 min at room temp.
- Replace the column on the stand and allow most of the beads settle then open column.
- ***Optional: Reapply the flow thru.*** This is the non-binding protein. Continue with purification

Column Binding - Flow the clarified lysate through the column. Save the eluate as flow-through in one fraction. If there is a concern with binding efficiency the flow-through can be reapplied. Continue with the purification.



Column Purification Cont…



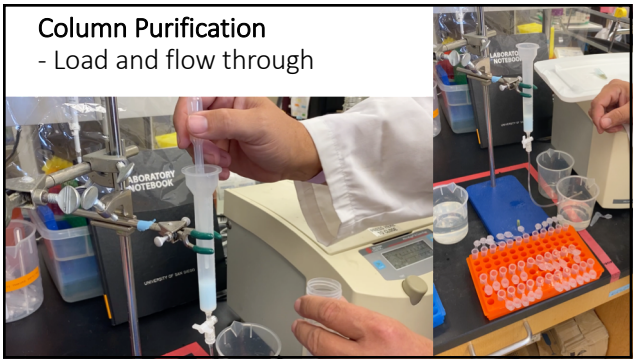
After binding protein to beads

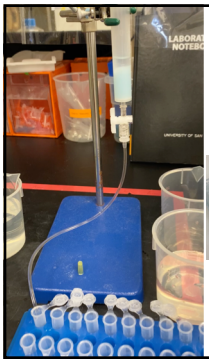
- Wash beads ~ ***250 ml of Ni-Column His Binding Buffer***. Save as flow through wash in one fraction.
- Wash the column with ***~500 ml of His Wash Buffer***. *This will remove some of the weakly binding protein.
  - (check flow through for protein with quick Bradford protein assay).
  - Continue with the His wash buffer until no detectible protein is found in eluate.
- Elute the protein with ***20 x 15 ml of His Elution Buffer***. Save ALL fractions. Check for eluted protein with quick Bradford.
- Check each fraction for total protein and determine purity by SDS-PAGE – Coomassie stain

Quick Bradford. ~1 ml of 1X Bradford plus ~20 ul or one drop of eluate or sample. If the dye turns blue-ish there is protein present

His-Binding and lysis Buffer:	***His-Wash Buffer:***	***His-Elution Buffer:***
• 50 mM Tris-Cl (pH8.0) • 1 mM Imidazole • 100 mM NaCl • 0.1 mM EDTA • * include 1 mM PMSF made fresh	• 50 mM Tris-Cl (pH8.0) • 300 mM NaCl • 10 mM Imidazole • 0.1 mM EDTA • include 1 mM PMSF made fresh	• 50 mM Tris-Cl (pH8.0) • 50 mM NaCl • 300 mM Imidazole • 0.1 mM EDTA • include 1 mM PMSF made fresh

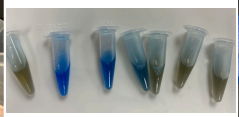
Column Purification
- Load and flow through



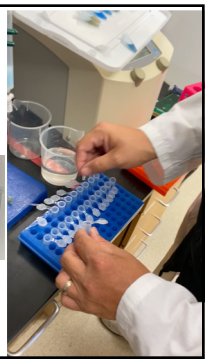


Time to elute bound protein

- Fraction size will depend on column size.

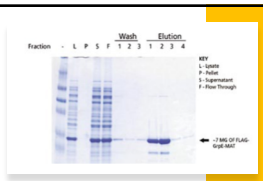


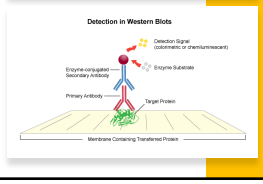
Which fractions should you save?
Concentration vs yield



Characterization

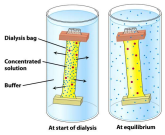
- Protein Assay for Concentration
- Enzyme Activity (specific activity)
  - ✓Demonstrates purity and active enzyme
- SDS PAGE Analysis
  - ✓Demonstrates purity
  - ✓Protein should migrate to correct size/location on gel
- Immunoanalysis /Western Blot Analysis
  - ✓Demonstrates identity of "SDS Band"





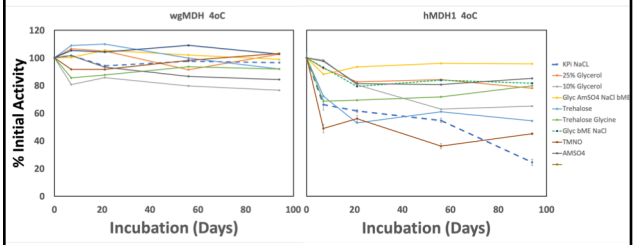
Post Purification Storage & Preparation

- ➢Prevent Aggregation –
  - MDH @ >1mg/ml will PPT
- ➢Dialysis for imidazole & salt removal and buffer exchange
- ➢Glycerol and other additives for stable storage
- ➢DO NOT FREEZE MDH without additions!!!!



Think of C1V1 = C2V2

Keep Your Protein Stable / Active



"Recombinant MDH and CS"

- In some cases, the affinity His Tag may need to be removed for various experiments.
- There is a TEV protease cleavage site between Tag and MDH/CS
- TEV protease is a highly sequence-specific cysteine protease from Tobacco Etch Virus.
- rTEV protease has a $(histidine)_6$-tag and recognizes the amino acid sequence:

  Glu-Asn-Leu-Tyr-Phe-Gln↓Gly.
- The advantage of this enzymatic cleavage is separation of TEV and the his tag from MDH
- The $(histidine)_6$-tag and the $(histidine)_6$-tag rTEV protease will both bind to the column, and the protein of interest can be collected in the flowthrough.