Announcements

• Chapter 11 Discussion for October 14:
  • C1: Tuesday 10AM in CAS B36
  • C2: Tuesday 3PM in CAS 326
  • C3 & C4: Tuesday 8 – 9:30 PM in LSE B01 (Basement Auditorium)

• Chapter 11 Computer Lab meets first in CAS 330

  B2: Wed 10/15 8-10am
  B3: Wed 10/15 1-2pm
  B4: Wed 10/15 6-8pm
  B5: Thur 10/16 8-10am
  B6: Thur 10/16 1-2pm
  B7: Thur 10/16 6-8pm
  B8: Fri 10/17 8-10am
  B9: Fri 10/17 1-3pm
  BC: Fri 10/17 6-8pm
  BA: Mon 10/20 12-1pm
  BB: Mon 10/20 4-6pm
  B1: Tue 10/21 4-6pm

*When you are done with the computer lab, or when time runs out, you may return to SCI162 to complete any remaining Chapter 3 work*
Chapter 3: Purification of Lactate Dehydrogenase (LDH)

**Purpose of Week 3:**

F) Run 3P-D sample on an affinity chromatography column to isolate LDH

G) Consolidate purified LDH column fractions & concentrate by ultrafiltration
LDH Purification Process – Weekly Overview

**Week 1**
- Disruption of tissue (Cell lysis)
- Clearing the cell lysate
  - Homogenization achieved by blending minced tissue + buffer
  - Separation via centrifugation
  - Pellet – Cell Debris
  - Supernatant – Cell Extract (lysate)

**Week 2**
- Removal of additional contaminants
  - Protein precipitation via ammonium sulfate fractionation
  - Further separation by affinity chromatography

**Week 3**
- Isolation of LDH
  - Concentrate by ultrafiltration (Week 3 or 4)
**LDH Purification Process – Fractions**

Fractions:
(bold = containing LDH)

1. **Disruption of tissue** (Cell lysis)
   - Homogenization achieved by **blending** minced tissue + buffer

2. **Clearing the cell lysate**
   - Separation via **centrifugation**
     - Pellet – Cell Debris
     - Supernatant – Cell Extract (lysate)

3. **Removal of additional contaminants**
   - Protein precipitation via ammonium sulfate fractionation

4. **Isolation of LDH**
   - Further separation by **affinity chromatography**

**Purified Protein**

- First cut:
  - 1P
  - 1S

- Second cut:
  - 2P, 2S

- 3P-D*

*Concentrate by ultrafiltration (Week 3 or 4)*

Wash fractions + NADH eluate
Affinity Chromatography

- AMP moeity of NADH binds to LDH in the NADH-binding site

- We are using Affi-Gel Blue Gel Resin to bind LDH to the column
  - Crosslinked 4% agarose matrix
  - Agarose beads are attached to Cibacron blue F3GA ligand
  - Cibacron blue F3GA is an analog of AMP (mimics the substrate of LDH)

NADH is later used to elute LDH since NADH binds more tightly than AMP
Affinity chromatography & ultrafiltration

3P-D – dialyze
Activity Assay for total Units & calculate volume to load 5000 U onto column

Run affinity column

Flow-through Fraction – Buffer & unbound proteins
Collect in flask & assay for activity

Wash Fractions – Additional unbound proteins
Collect in test tubes until $A_{280} < 0.1$

NADH eluate Fractions – purified LDH
Assay each fraction for activity & pool fractions with highest activity ($\leq 10 \text{ mL}$)

Ultrafiltration
(Concentrate to ~1 mL)

Purified (concentrated) LDH
Affinity chromatography setup

i) Preparing the column
   • Packing the column with resin
   • Equilibration

ii) Sample application & running the column
   • Binding LDH to the beads
   • Elution
Pipet Affi-gel resin into column

Let resin settle, then drain buffer to just above surface. **DO NOT let column dry out!**

Equilibrate column with buffer

Drain buffer to just above surface. Column is now ready to load.
Apply dialyzate to column with transfer pipet, then drain to just above surface & collect flow-through.

Wash column with buffer & collect fractions until $A_{280} < 0.1$.

Drain buffer to just above surface. Elute LDH with NADH solution. Collect & assay fractions.

Loading & running the affinity column.
Chapter 3F-G: Procedures overview

Part F:
I. Column preparation
II. Affinity chromatography
III. Collect & pool purified LDH from column

Part G:
I. Concentration of purified LDH by ultra-filtration

*NOTE: Some groups might not make it to this step. If you have to wait until the following week, turn in your POOLED LDH to TFs
Chapter 3F Procedure

**Column Preparation**

*NOTE: This must be started AS SOON AS YOU GET TO LAB. TFs will give pre-lab talk after column equilibration.*

1. Take 5 mL of 50% affinity resin slurry and load column
   - *Your partner should be testing activity for 3P-dialyzed*

2. Allow slurry to settle for a few minutes to correctly pack column
   - *Do NOT allow column to dry out at any point of the lab!!!*

3. Equilibrate with 10-15 mL of 0.02 M potassium phosphate buffer
   - *If flow rate for column is very slow, contact TF immediately!*
Chapter 3F Procedure

Affinity chromatography: loading the column

1. Measure and record the volume of the 3P-Dialyzed fraction

2. From calculated activity, determine the volume of 3P-dialyzed fraction needed for 5000 U
   • Don’t forget to take an aliquot for dye-binding
   • Heart samples: you may need your entire 3P-D fraction

3. Test the initial “flow-through” for activity
   • Why is this necessary?
   • What does low activity tell you?
   • What does high activity tell you?
Chapter 3F Procedure

Affinity chromatography: washing the column

1. Wash column with 5 mL portions of buffer
   • Collect washes from column in 1-2 mL fractions

2. Measure absorbance @ 280 nm of “wash” fractions on the UV-Vis
   • Which macromolecules are absorbing @ 280 nm?
   • What is the significance of $A_{280} < 0.1$?
Chapter 3F Procedure

Eluting & pooling LDH from the column

1. Begin eluting with 0.2 mM NADH in 0.02 M potassium phosphate buffer
   - What is in the buffer that allows LDH to elute?
   - Why does LDH elute from the column?

2. Collect eluate in 1-2 mL fractions

3. Test eluate fractions for LDH activity

4. Pool the fractions with the highest activity in a 15-mL conical tube
   - Keep total volume below 10 mL
   - Measure and record this volume
Tips: Affinity chromatography

• When adding liquid to the column, be gentle and do not disturb the resin bed

• Never allow any part of the resin to dry out!
  • *When column is flowing, one person must be monitoring it at all times*

• Set up calculation for 5000 U in **pre-lab** procedure!
  • *Have TF check before loading sample*

• If flow rate is very slow, immediately call TFs

• Keep flow-through and all eluate fractions on ice
  • *Why is this important?*

• Pay attention to [NADH] on labels for wash & elution buffer stocks
  • *Why is this important?*

• Properly label all fraction tubes
Chapter 3G Procedure

Concentration of purified LDH by ultra-filtration

• Add sample to ultra-filtration device
• Place in ice tray on top of stir plate
• Spin internal stir bar slowly
• Slowly begin to apply pressure from nitrogen tank
• Collect the flow-through as you concentrate your sample
• Collect sample in eppendorf tube when you have ~1.5 – ~1.0 mL of sample left

• *Place on ice and do not throw your away sample!*
**Tips: Ultra-filtration concentration**

- Keep ultra-filtration device packed in ice in a small plastic box
  - Assemble unit similar to last week’s Erlenmeyer flask setup

- Keep nitrogen pressure low and be patient
  - No more than one drop of flow-through every 5 seconds
Week 3: Activity Assays

- Perform LDH Activity Assays for:
  - 3P dialyzed
  - “Flow-through”
  - Eluate fractions
    - Ultra-filtered, purified LDH

- Use aliquots for assays
- Dilute where necessary
- For Weeks 2 & 3 samples, only need 1 dilution for each fraction in $\Delta A_{340}/\text{min} = -0.05$-0.25 range

What do you use to blank your spectrophotometer?
Week 3: Bradford Assays

- Protein Concentration – Bradford (dye-binding) Assay
  - Make new standard curve if doing this week
  - Find protein concentration for:
    - 3P dialyzed
    - “Flow-through”
    - Eluate fractions
    - **Ultra-filtered, purified LDH**
- Use aliquots for assays
- $A_{595}$ should be within linear region of your standard curve
  - Dilute protein when necessary

What do you use to blank your spectrophotometer?
Chapter 3, Week 3 Checklist:

At the end of lab, you should have:

✔ Prepared four new “sets” of fractions (& recorded volumes of):
  o 3P-dialyzed
  o “Flow-through”
  o Eluate fractions
  o Ultra-filtered, purified LDH

✔ Performed activity assays on all fractions

✔ Made a new standard curve & performed Bradford assays (only if had time)

✔ Turned in all aliquots and purified LDH to TFs to freeze
Public Service Announcement:
What will happen if you throw your LDH away...

(you)

... DON'T TALK EVER AGAIN!

(your lab partner)

WHAT I HAZ SEEN CANNOT BE UNSEEN

(your TFs)
Questions?