BCH 372
Elementary Biochemistry Laboratory

Laboratory 9
Western Blot Analysis of L-Lactate Dehydrogenase, Part A

The purpose of the next two laboratory periods is to use nondenaturing gel electrophoresis in combination with a Western blot analysis to detect L-lactate dehydrogenase. During the first lab session, the proteins in the samples from the initial stages of the LDH purification and two purified samples of L-lactate dehydrogenase will be separated under nondenaturing conditions. The proteins thus will retain their native secondary, tertiary, and quaternary structures and will be able to interact with antibodies directed against epitopes (immunogenic sites) in their three dimensional structures. Two identical gels will be run in this way. One gel will be stained with colloidal Coomassie Blue to reveal all of the protein bands. The other gel will be used for a Western blot, in which the proteins will be transferred electrophoretically out of the gel onto a PVDF (polyvinylidene difluoride) membrane. The PVDF membrane will be stored until next week's lab. During the second lab session, the Coomassie Blue stained gel will be scanned and analyzed. The PVDF membrane will be treated with a series of reagents including a primary antibody directly specifically against LDH and a secondary antibody conjugated to an enzyme. This will allow the bands that correspond to this particular protein to be revealed.

I. PreLab Preparation

Before the lab, please read Chapters 9 and 10 in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor. You will do a modification of Experiment 10a (Western Blot of LDH). However, rather than casting your own gels, you will use precast polyacrylamide gels that have a 4-15% gradient gel in a plastic cassette. Also, instead of running a gel and cutting it in half, each group will run two gels.

II. Laboratory Procedures

A. Preparation of Polyacrylamide Gels

The purpose of this part of the experiment is to set up the systems for nondenaturing polyacrylamide gel electrophoresis.

1. Each group will be provided with a Bio-Rad Mini-Protean 3 gel system. The system holds two 8 cm x 5.8 cm x 1 mm minigels with 10 sample wells each. To save time, you will use precast Bio-Rad Protean TGX 4-15% polyacrylamide gels in a nondenaturing Tris-Glycine buffer.

2. Wearing gloves, remove a precast Bio-Rad Protean TGX 4-15% polyacrylamide gel from

its sealed pouch. Rinse the gel cassette with distilled water. Remove the green insert from the top of the gel to expose the wells and remove the tape from the bottom of the cassette.

3. Mount the gel in its cassette on one side of the inner chamber assembly, with the shorter plate facing inwards as demonstrated by the instructor and illustrated in the following figures. Note that with these gel cassettes, the shorter plate should fit just under a ridge in the green gasket.

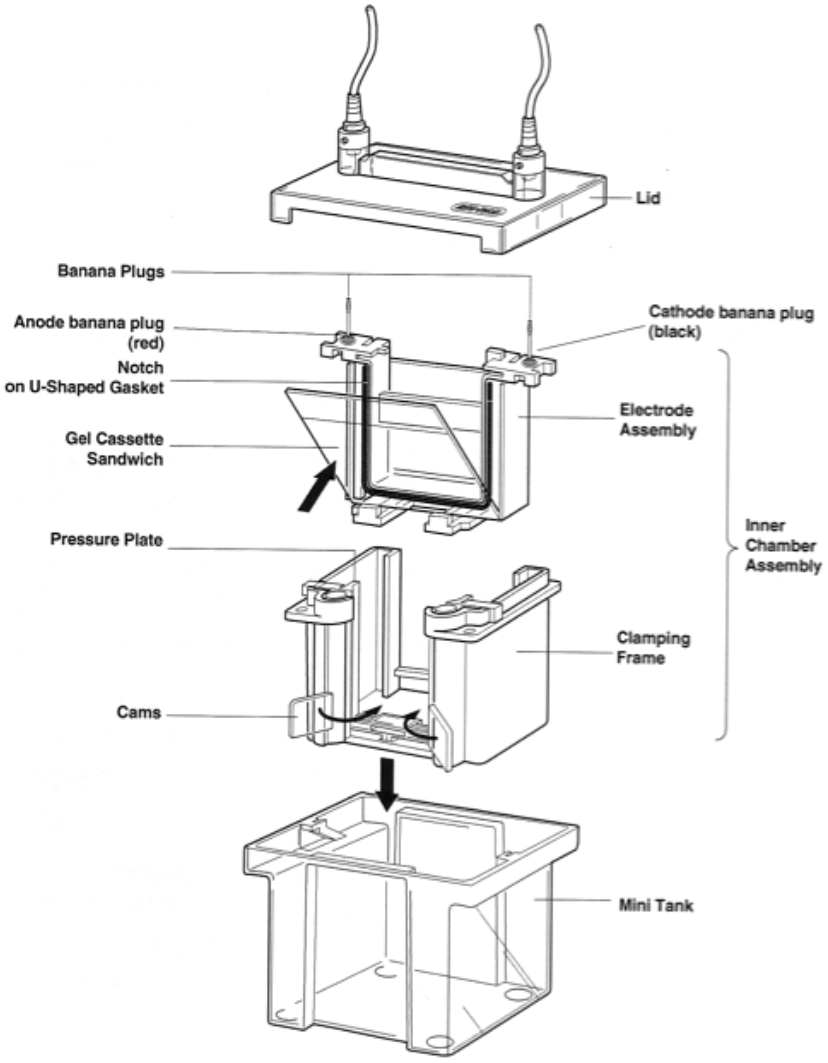


Fig. 2. Assembling the Mini-PROTEAN 3 cell.

4. In the same way, remove a second precast Protean TGX 4-15% polyacrylamide gel from its pouch, rinse it with water, and mount it on the other side of the core assembly. Mark one gel **A** and the other gel **B** with a Sharpie marker.

5. Clamp the gels into the clamping frame and place the core assembly with the gel cassettes in the buffer box.

6. Add Tris-Glycine running buffer to both the central (upper) buffer chamber and the lower buffer chamber. This buffer contains 25 mM Tris and 192 mM glycine but no SDS or other compounds that would denature the proteins. Fill the central buffer compartment until the buffer extends over the short plate and the wells are completely filled with buffer. Fill the lower buffer chamber with buffer until it reaches the bottom of the wells. Using a plastic transfer pipet, rinse out the wells with electrophoresis buffer so that there are no air bubbles in them.

B. Preparation of Samples

The purpose of this part of the experiment is to prepare the samples for the electrophoresis and Western blot.

1. Retrieve the following fractions from the freezer box or from your set of refrigerated centrifuge tubes.

crude extract (**CE**)
40% ammonium sulfate pellet (**40P**)
40% ammonium sulfate supernatant (**40S**)
65% ammonium sulfate pellet (**65P**)
65% ammonium sulfate supernatant (**65S**)

2. Based on the protein assays that were done last week as part of Laboratory 8, start to fill in the following chart with the **protein concentration** of each sample in **mg/ml**.

fraction	protein conc. (mg/ml)	dilution factor to give 2 mg/ml	sample volume	water volume
CE	_____	_____	_____	_____
40P	_____	_____	_____	_____
40S	_____	_____	_____	_____
65P	_____	_____	_____	_____
65S	_____	_____	_____	_____

3. Now calculate how much each fraction must be diluted to give a final protein concentration of 2 mg/ml. **If a fraction has a protein concentration of < 2 mg/ml, it does not need to be diluted.**

For example, if a particular fraction has a protein concentration of 13.4 mg/ml, the dilution factor would be calculated as follows:

$$\frac{\text{initial protein concentration}}{\text{final protein concentration}} = \frac{13.4 \text{ mg/ml}}{2 \text{ mg/ml}} = 6.7$$

dilution factor = 1/6.7

That is, the original solution is 6.7 times more concentrated that you want the final solution to be. A particular volume of the 13.4 mg/ml solution would need to be diluted 1/6.7 to give a final concentration of 2 mg/ml of protein. Put the dilution factors into the chart shown in step # 2.

4. After doing this calculation for each of your five fractions, make an appropriate dilution of each fraction in a 1.5 ml microcentrifuge tube so that you have a total volume of 100 µl at a concentration of 2 mg/ml. Put your calculations into the table in step # 2 and label the tubes carefully.

For example, suppose a certain fraction has a protein concentration of 5.6 mg/ml. You can calculate how much to use to make 100 µl of solution in two ways:

A. The first is to use the dilution factor you calculated in step # 4

$$\frac{\text{initial concentration}}{\text{final concentration}} = \frac{5.6 \text{ mg/ml}}{2.0 \text{ mg/ml}} = 2.8$$

The dilution factor is 1/2.8, so:

$$\frac{1}{2.8} \times 100 \text{ µl} = 35.7 \text{ µl}$$

For 100 µl, you need to combine 35.7 µl of the stock solution and (100 – 35.7) or 64.3 µl of water.

B. The second is to calculate how much protein you need to add:

$$100 \text{ µl} \times \frac{2.0 \text{ mg}}{\text{ml}} \times \frac{1 \text{ ml}}{1000 \text{ µl}} = 0.2 \text{ mg}$$

$$X \text{ µl} \times \frac{5.6 \text{ mg}}{\text{ml}} \times \frac{1 \text{ ml}}{1000 \text{ µl}} = 0.2 \text{ mg}$$

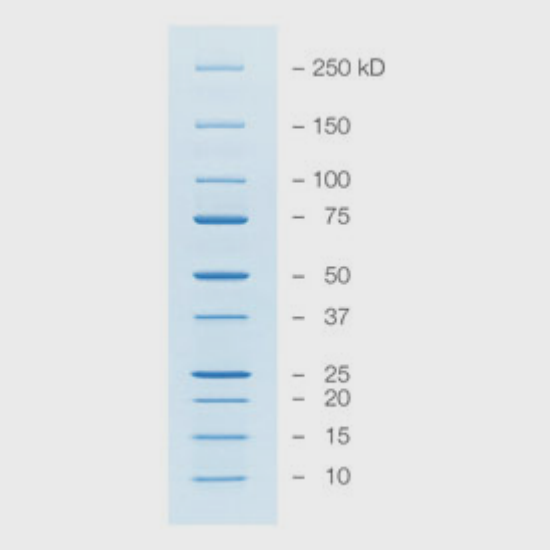
$0.0056X = 0.2$, so $X = 35.7$ µl

If you combine 35.7 µl with 64.3 µl of water, you will have 100 µl at the correct concentration.

5. After you have made up all of the samples, add 100 µl of 2X nondenaturing protein sample buffer to each of the tubes. This buffer contains 20% glycerol, 130 mM Tris base, and 30 mg/100 ml bromphenol blue. This will give a final protein concentration of 1 mg/ml and a total sample volume of 200 µl.

6. You will be provided with tubes containing 50 µg of bovine heart LDH (**HLDH**) or 50 µg of bovine muscle LDH (**MLDH**) in a volume of 25 µl. Add 25 µl of 2X nondenaturing protein sample buffer to each of these tubes to give a total sample volume of 50 µl.

7. Each group also will be provided with microcentrifuge tubes containing 25 µl of a mixture of unstained proteins standards of known molecular weights (**MWM**).



The mixture contains 10 recombinant proteins that vary in size from 10,000 to 250,000 daltons in a sample buffer. You do **not** need to add anything to this tube.

C. Loading and Electrophoresis of Samples

The purpose of this part of the experiment is to load the samples into the wells of the polyacrylamide gels and to subject the proteins to electrophoresis. You will be able to see the samples and to follow the electrophoresis by looking at the bromphenol blue tracking dye that is in the sample buffer.

1. Using a P-100 micropipetter and **long plastic gel-loading tips**, load the volumes of each sample into the wells of one of the gels as indicated below. Note that you will load the samples through the buffer and between the glass plates. You can place a yellow plastic block over the central compartment as a guide. It will take a little practice to see the wells and to load the samples, but once the first sample is loaded, the rest will be quite easy. Note that since your CE and 65P fraction had the most activity, you will load them twice.

Sample Loading Protocol

well:	1	2	3	4	5	6	7	8	9	10
sample:	CE	CE	40P	40S	HLDH	MWM	MLDH	65P	65P	65S
volume:	10	20	20	20	20	10	20	10	20	20

2. When the gel on one side of the system has been fully loaded, carefully turn the system around and load the wells of the second gel in exactly the same way.

3. Remove the yellow block if you used it and carefully place the lid on the system so that the electrodes are correctly aligned. Insert the leads from the lid into the power supply. Check to see that the power supply is set to run at 200 Volts and 400 mA.

4. Run the gels at 200 Volts for about 45-60 minutes. The large volume of buffer in the lower chamber will prevent the samples for over-heating during the run. Note the position of the bromphenol blue tracking dye throughout the run.

5. When the tracking dye gets to within **1 mm** of the bottom (**below** the lower edge of the green gasket), disconnect the leads from the power supply, turn off the power, and carefully remove the lid.

6. Pour off the buffer from the upper and lower buffer chambers into the designated waste container and remove the cassettes with the gels from the system

D. <u>Staining of One Gel for Total Protein Composition</u>

1. Using the edge of the green plastic tool that comes with your gel system, crack open **one (1)** of the cassettes and carefully pry the two plastic plates apart. The gel will usually stick to one of the plates. Place the plate with the gel side up in a plastic staining dish. With a piece of label tape, label the dish with your initials.

2. Add about 30 ml of filtered deionized water to the staining dish. Shake the staining dish gently for about 5 min on the Belly Dancer platform shaker to wash the gel. The gel will float off of the plastic plate during this process if it has not done so already. Remove the plate from the staining tray. Decant the water and add 30 ml of fresh water. Shake the gel again for about 5 min.

3. Add 20 ml of Pierce Gel-Code Blue Stain Reagent to the staining dish.

4. The instructor will allow the gel to stain overnight to allow maximal development of blue-stained bands.

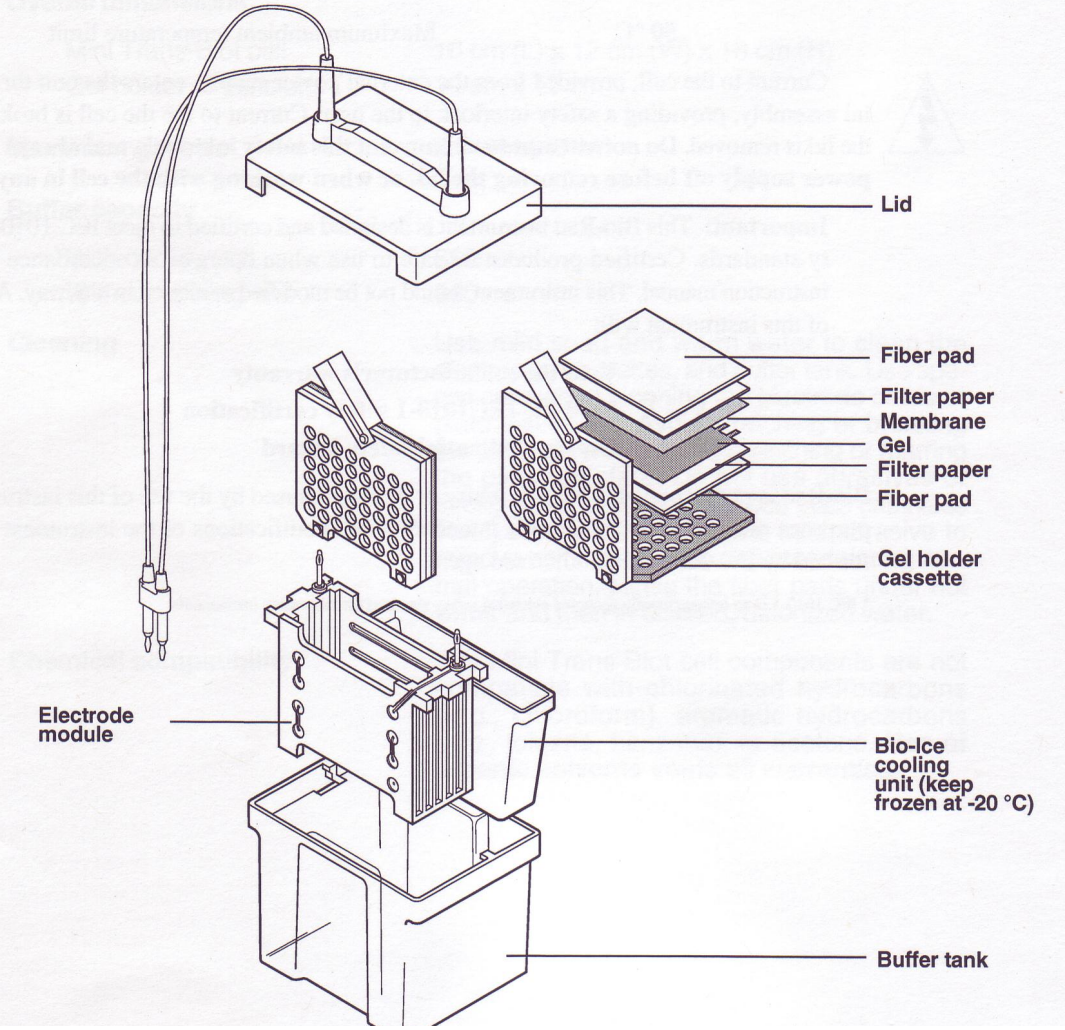
5. The next day, the instructor will decant the stain, wash the gels with two portions of filtered deionized water, and allow them to destain in distilled water until the next lab session.

6. During the next lab period, you will scan and analyze this stained gel and compare it with

the Western blot.

E. Setting up the Western Blot with the Other Gel

1. To transfer the proteins from the other gel to the PVDF membrane, you will use a Bio-Rad Trans-Blot Cell, which is shown in the following figure. It fits into the same buffer tank you used for the gel electrophoresis. Because each group will have only one gel to blot, two groups will share one apparatus. Wear gloves throughout the following steps. Note that you will need to **start equilibrating** the membrane, filter pads, and filter paper in buffer while the nondenaturing gel electrophoresis is being done (steps 2, 3, and 4).



2. You will be provided with a precut PVDF membrane. The membrane is a thin white sheet of material that has been placed between two filter paper sheets and two blue

glassine separation sheets.

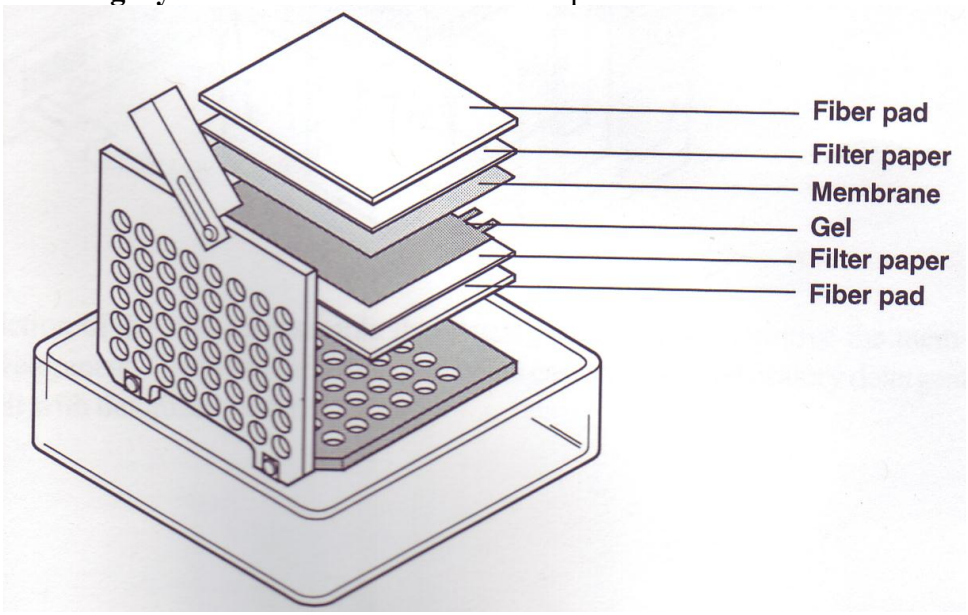
3. Using a pair of forceps, place the PVDF membrane in a glass dish containing 100% methanol for a few seconds. The membrane is normally quite hydrophobic but it will become translucent in this solvent. When it has become clear, carefully remove the membrane from the methanol and place it in a staining dish containing about 30 ml of the running buffer used for the electrophoresis (25 mM Tris, 192 mM glycine).

4. Place the two filter paper sheets and the two fiber pads provided with the apparatus in the same staining dish as well. Allow everything to equilibrate in the buffer for about 15 minutes.

5. When the gel electrophoresis has finished, use the edge of the green plastic tool to crack open the other gel cassette. Carefully pry the two plastic plates apart, leaving the gel on one of the plastic plates. BE CAREFUL TO PRESERVE THE CORRECT ORIENTATION OF THE GEL. Use a scalpel to cut off the lower left hand corner of the gel by lane 1.

6. Place the plate in another staining dish containing about 30 ml of the running buffer used for the electrophoresis (25 mM Tris, 192 mM glycine). Allow the gel to equilibrate for about 10 minutes. The gel should float off the plastic plate at this time. Remove the plastic plate.

7. To assemble the system, open the Trans-Blot cassette and place it on some paper towels with the **gray side down**. Then add the components as shown below:



a. Place a pre-wetted fiber pad on the gray side of the cassette.

b. Place a sheet of equilibrated filter paper on top of the fiber pad.

c. Place the equilibrated gel on top of the filter paper. This is a little tricky. You may want to use a spatula. Handle the gel carefully to avoid tearing it, but place it squarely on the paper. Add a little running buffer if necessary to keep everything moist.

d. Use a 16 x 150 glass tube to roll the gel flat on the filter paper and to eliminate any air bubbles.

e. Place the pre-wetted PVDF membrane on top of the gel. Again, handle the membrane carefully and place it squarely on the gel.

f. Use the 16 x 150 glass tube to roll the membrane flat and eliminate any air bubbles.

g. Place another sheet of equilibrated filter paper on top of the PVDF membrane.

h. Use the 16 x 150 glass tube to roll the paper flat and eliminate any air bubbles.

i. Place another pre-wetted fiber pad on top of the filter paper.

8. Close the cassette firmly, being careful not to move the gel and the filter paper sandwich. Lock the cassette with the white latch.

F. <u>Running the Western Blot</u>

1. Slide the cassette into the Trans-Blot module with the gray side of the cassette facing towards the electrodes (the black side of the module) and the white side of the cassette facing toward the red side of the module. In this way, the proteins will move out of the gel towards the PVDF membrane.

2. Another group can slide their cassette into the other slot. If only one group has a cassette to add to the module, put an empty cassette with two moistened filter pads into the other slot.

3. Place a frozen plastic Bio-Ice cooling unit into the lower buffer chamber along with the Trans-Blot module.

4. Fill the buffer chamber with 650 ml of ice-cold running buffer (25 mM Tris, 192 mM glycine).

5. Place a stir bar into the buffer chamber and set the whole system on a stir plate. BE SURE THE HEAT IS OFF. Stir the buffer as fast as possible to keep the ionic

distribution even.

6. Place the lid on the buffer chamber so the electrodes are correctly positioned. Connect the leads to the power supply.

7. Set the limits on the supply as follows:

   VOLTAGE = 30 V
   CURRENT = 90 mA
   TIME = 999 MIN

8. Turn on the system. The instructor will let the systems run overnight to allow complete electrophoretic transfer. The next day, the instructor will disassemble the Trans-Blot cassettes and transfer the membranes to a blocking solution containing 10% dry milk, 10 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 8. The membranes will be refrigerated in this solution until the next lab session.

9. The instructor will also transfer the gel itself to another staining dish and add 20 ml of Pierce Gel-Code Blue stain reagent. The next day, the instructor will decant the stain, wash the gels with two portions of filtered deionized water, and allow them to destain in distilled water until the next lab session. This will allow you to determine the efficiency of protein transfer out of the gel and onto the membrane.