Biology 446-1
Laboratory Exercise 8
cell lysate preparation, immunoprecipitation and Western blot

Objective: To gain experience in preparing cell lysate from cultured cells and in using antibodies to precipitate its antigen from the cell lysate to identify associated proteins by co-immunoprecipitation.

Background

In many field of research in life science, it is often advantageous to lyse cells under nondenaturing conditions. Lysate obtained under these conditions preserves native protein structure and enzyme activity as well as many protein complexes as they exist in the intact cell. If whether a specific gene of interested is expressed in the cell and a good antibody against the gene product is available, the easiest way is to assay the existence of the protein in the cell lysate, providing that the protein is soluble in the lysate conditions. This is often the case when the expression of a gene is dependent on the signal transduction through certain pathways or when the activity of an enzyme is dependent on the cell cycle stage, cell type or an intact upstream signal pathway. Immunoprecipitation and Western blot are often used for this purpose.

Co-immunoprecipitation from the cell extracts is the most rigorous demonstration of a physiological interaction between two proteins. In numerous recent studies, true physiological interactions were subsequently shown by co-immunoprecipitation following identification of the putative interaction by means of other powerful high-throughput approaches, such as two-hybrid screen. In fact, it is desirable to confirm interactions identified by two-hybrid screen by co-immunoprecipitation because the high sensitivity of two-hybrid makes it frequently report false positives.

During this lab exercise, we will use cultured PC-3 cells. (Sterile cell culture procedures must be followed in order not to contaminate the cells. In addition, these are transforming immortalized cells and care must be taken so as to not expose yourself to the cells. Proper disposal procedures must be followed to dispose biohazardous waste). Cells will be grown to near confluence prior to the lab and heat-shocked at 42 °C for 2 hours by your instructor. You are going to prepare cell lysate under nondenaturing conditions, use an antibody to HSP 40 to precipitate HSP 40 along with other proteins that associated with it. Heat shock proteins are chosen because these proteins are involved in important cellular processes like chaperoning of nascent polypeptide chains and protecting cells from the accumulation of improperly folded proteins.

Your will then separate the proteins in the precipitation on an SDS-PAGE and Western blot will be used to detect the Hsp 40 to check whether your immunoprecipitation worked. Your will also use an antibody to Hsp 70 to detect any Hsp 70 that is brought down by anti Hsp 40 through their association.

Protocol:

Lab Day 1

A. Preparing lysate: (keep everything ice cold with inhibitors)

1. Obtain a culture dish from your TA and place the dish on ice, aspirate media, wash (10 cm dish) with 5 ml ice cold PBS.
2. Aspirate PBS, repeat PBS wash once more.
3. Add 0.6 ml lysis buffer (we will use PTY buffer), scrub the plate with a cell lifter.
4. Pipet the lysate from the dish into an eppendorf tube that is placed on ice.
5. Spin the tube at 10,000 rpm at 4 °C for 10 minutes.
6. Transfer the supernatant to a fresh tube with proper label. At this stage, the sample can be stored at –80 °C for future use.

B. Immunoprecipitation:

1. Save 50 μl of your lysate a 1.5 ml tube for late use and divide the rest into two eppendorf tubes. Label one of the tubes as sample (S) and the other as control (C). Add 15 μl of anti-Hsp 40 to the sample tube. Rock both tubes for 1 hour at 4 °C.
2. Add 60 μl of protein A-Sepharose slurry to each of the tubes. **Note: resuspend the Sepharose beads by inverting before use**. Rock both tubes for another 30 minutes at 4 °C.
3. Spin the protein A-Sepharose down with a microcentrifuge at 1000 rpm for 45 seconds. Remove and discard the liquid. BE CAREFUL NOT TO REMOVE THE BEADS. Add 400 μl PBS and resuspend the Sepharose beads by inverting the tubes a few times.
4. Repeat the PBS wash (step 3) four more times.
5. Add 100 μl of Sample Buffer (SDS gel-loading buffer) to each of the tubes and boil them for 5 minutes.

   GIVE YOUR SAMPLES TO YOUR TA TO STORE THEM AT –20 °C.

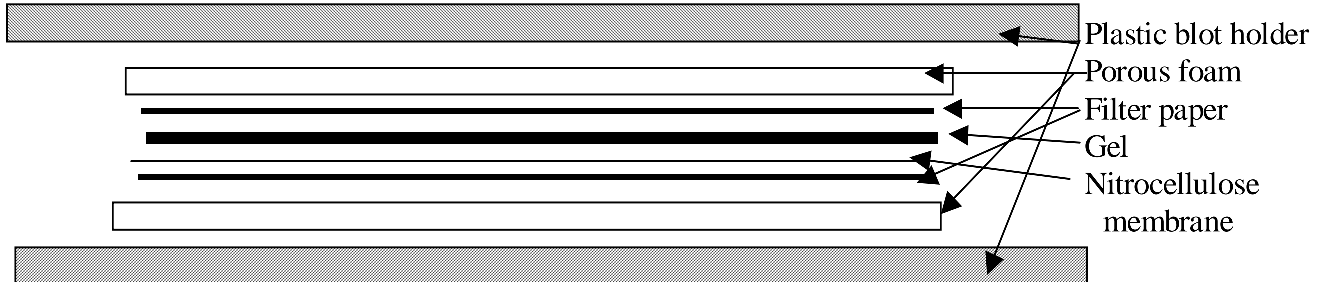
LAB DAY 2

6. Load 15 μl of the samples into the wells of a gel assigned to you. (Protein molecular weight markers will be loaded by volunteer(s)).
   Load the samples in the following order.
   G1-L G1-S G1-C G2-L G2-S G2-C M G1-L G1-S G1-C G2-L G2-S G2-C
   where G1, G2, G3 denote group one, two and three, respectively. L, S, and C denote lysate, sample and control, respectively. Samples from other groups should arrange in a similar manner but with an empty well at different places to differentiate the gels from one another.
7. Run the gel at 100 volts until the dye front reaches the bottom of the gel.

C. Western blot:

1. Crack the shell using a paint scrapper or spatula and remove the top casing wall. Use the paint scraper or razor blade to cutoff the bulging “foot” of the gel that was used to make contact with the lower running buffer chamber.

2. Wearing gloves, assemble a blotting sandwich as shown in the diagram bellow. Do not allow air bubbles to be trapped between the various layers, especially between the filter paper / membrane/ gel interfaces. It is helpful to wet the upper surface of each layer with transfer buffer before applying the next component.



3. Place in the blotting chamber. Note that the blotting buffer pH is high (~10) so that most proteins will be negatively charged; in addition all the proteins will still have a large amount of SDS bound to further add to this negative charge. As such, they will move away from the negative electrode and toward the positive electrode. Be sure to place the sandwich in that orientation. Otherwise, the proteins will move away from, and not toward the membrane.
4. Transfer at 40 V and then cross over to constant current, or manually set the transfer to constant current at the current reading at 40 V. Run the transfer overnight. The TAs will remove the nitrocellulose membrane, which will now contain the proteins, and perform the following step:
5. Remove the nitrocellulose membrane, and transfer it to blocking buffer. This blocking buffer contains a high concentration of non-target protein (we will use TBS or PBS containing 10% fat free milk) that effectively saturate all the binding sites on the membrane. The blot will be stored for you until the next lab session in this solution.

LAB DAY 3

6. Incubate the first half of the membrane with 10 ml blot incubation buffer (TBS or PBS containing 1% fat free milk) containing 2 μl α-Hsp 40 and the second half of the membrane with 10 ml blot incubation buffer containing 2 μl α-Hsp70. Incubate with shaking for 40 minutes at room temperature.
7. Remove and discard the antibody solution (this solution can be used again if stored at 4 °C for a couple of weeks).
8. Wash 3 times, with shaking, for 5 minutes with TBS or PBS buffer containing 0.1% tween-20; discard each wash.
9. Incubate the membrane with 10 ml blot incubation buffer containing 1 μl of Horse Radish Peroxidase conjugated goat anti rabbit (if the primary antibody is produced in rabbit) antibody for 40 minutes at room temperature.
10. Wash the blot as in step 8, but do not discard the last washing solution, yet.
11. After step 10 is finished, prepare the detection reagent by adding 5 ml detection reagent A and 5ml of detection reagent B. Discard the washing solution and pour the detection reagent on to the membrane. Incubation for 1 minute at room temperature.
12. Wrap the membrane in a piece of plastic film. Make sure the membrane is flat and there is no bubble between the membrane and the wrap.

13. Tape the membrane to an x-ray cassate and expose a piece of x-ray film to the membrane in the dark room for 1 minute. Develop the film using the developer and fixer in the dark room. Depending on the intensity of the bands on the film this step may need to be repeated with a longer or shorter exposure time.
14. Once the film is dry, the TA will scan it and send the image to you for your documentation and report.

Report: in the discussion of your report. Pay attention to the following question.
Does the lysate contain Hsp 40 and/or Hsp 70? Are the antibodies good for Western bolt? Are the antibodies good for immunoprecipitation. Does Hsp 40 interact with Hsp 70? If your result cannot give definite answers to any of these questions, so state it and explain why, if possible.

Buffers:

NETN:

20 mM Tris-HCl (pH 8.0)
1 mM EDTA
100 mM NaCl
0.5% (v/v) Nonidet P-40 (Igepal CA 630)

2X SDS gel-loading buffer:

100 mM Tris-Cl (pH 6.8)
4% (w/v) SDS (electrophoresis grade)
0.2% (w/v) bromophenol blue
20% (v/v) glycerol
200 mM dithiothreitol or β-mercaptoethanol
* can be stored at room temperature if the without adding thiol reagent reducing agent.

PBS:

137 mM Nacl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4
pH 7.4

TBS:

137 mM Nacl
2.7 mM KCl
25 mM Tris
pH 7.4

PTY lysis buffer:

50 mM HEPES, pH 7.5
50 mM NaCl
5 mM EDTA
1% (v/v) Triton X-100
50 mM NaF
10 mM $Na_4P_2O_7$

Inhibitors:

1 mM Na_3VO_4
1 mM PMSF
0.01 mg/ml aprotinin
0.01 mg/ml leupeptin

Transfer buffer:

48 mM Tris (use tris base)
39 mM glysine,
20% methanol,
0.004% SDS, pH 9.2