Western blots essay

Protein isolation for a western blot uses detergents and mechanical force to separate seeded cells from their container. Eukaryotic cells are attached to the surface of a flask by Catherine. In the past, we've separated the cells from the flask by breaking these bonds with a protease, but In order to keep the proteins Intact, a different method needs to be used to extract the proteins.

In protein extraction for a western blot, we use detergents to else the membrane and free the proteins.

The benefit of using a detergent is that it will dissolve the membranes and loosen routines embedded In It, A cell scraper is also used to mechanically remove proteins and cells. Mechanical lists would break open the membranes but wouldn't free the membrane embedded proteins. One of the membranes broken open is the lissome.

The lissome contain proteases that would destroy our target proteins. To compensate for this, we add a protease Inhibitor. This will inhibit protein cutting and depopulation's of a few phosphorescently proteins, such as cycle.

To give the detergent time to fully dissolve the membrane, the solution was put on ice (figure 1) n order to slow down protein activity while the detergent breaks the membranes.

Standard Curve: To establish the concentration of the proteins Isolated, It Is compared against known concentrations in the form of a standard curve. The unknown concentration is made up of the extracted proteins and RIP buffer,

so known concentrations of ABS can be created in RIP buffer. The known concentration’s absorbency readers can be read In a spectrophotometer, and graphed based concentration vs.. Observance. A standard line can be calculated and the line’s equation be Interpreted (PRNG*b).

Duplicate wells were used to reduce error in the standard line. The line’s equation can be used to estimate the concentration of the unknown. Substitute the absorbency of the unknown Into the Y’ of the equation and calculated the or concentration. This Is an example of a quantitative value, because It Is based on known values and an empirical unit is assigned to the data collected.

The equation loses its accuracy outside the range of the line.

If the unknown solution’s absorbency is found outside the range then it should be diluted and the absorbency should be recalculated. The range of the known samples Is called the linear range because of he graph appearing to be linear in this range. The new solution’s concentration will be multiplied by the dilution factor to obtain the actual concentration. SD-PAGE SD-PAGE stands for Sodium decoded sulfate- Polysaccharide gel electrophoresis. The polysaccharide gel Is made up a matrix of pores that can fit 5-2, 000 KDE size proteins.

Smaller or larger pores depend on the varying size of the molecules being examined. Creamily works best for the proteins being examined. The SD is used to uniform the charges on the protein being examined. The SD weakly binds to the backbone of the protein, and lingering It.

The running buffer added also contains SD. Kaleidoscope and blanks A kaleidoscope is added to one of the wells to serve as standard to measure the uneconomical analysis needs to be done on them. When a band from the extraction is created it can be compared to the multi colored known weight of the band in the kaleidoscope.

This can give the examiner the relative size of the protein. This can be considered a semi quantitative value because the data is based on the relationship to something else.

Another semi quantitative value of protein concentration can be interpreted from the kaleidoscope. If we know the amount of kaleidoscope then we can compare the size of the kaleidoscope band to the protein band. We can estimate if the protein’s concentration relative to being greater or less than the kaleidoscope using relative band size. The kaleidoscope can also serve as a positive control.

It controls for false negatives, because if the kaleidoscope is seen then we can assume that the transfer of the proteins to the membrane was successful, and any lack of protein is due to improper loading or microchemistry analysis. The blank can operate as a negative control to test for false positives.

If the blank Anne has staining then the results cannot be trust because there should have no proteins in that lane. Sample denaturing When the extracted protein is run through the gel, it is important to have the proteins linearity to run accurately through the matrix of the creamily.

In order to denature the proteins, the sample is put in a boiling water bath. With the help of B- merchantable, the cells tertiary and quaternary structure can be broken apart.

B- merchantable will reduce the sulfide bonds in cytosine. The cytosine bonds are important component's in the structure of the protein. The B-merchantable is deed to the protein with the xx protein buffer. The buffer also contains SD.

As stated previously. SD binds to the backbone of the protein, and luminaries it. The heat will open the protein, B-merchantable will but the bonds, and SD will keep the protein open.

Running the gel The gel is run at 200 volts and 100. The higher the volts, the faster and less accurate the bands will be, and the hotter the system will become.

The converse is also true, but too slow and the solution will diffuse throughout the gel. The gel is set up so the positive end is near the wells and the negative end is on the other side of the gel. The proteins will be pushed from the wells by the negative charge and they will be attracted to the positive end. The process of the gel running is monitored because of the bronchiole blue(figure 2).

It will run the fastest through the gel because of its size, so when the blue molecule reached the end of the gel then the electricity can be turned off. The potential problems with the running of the gel include forgetting to remove the tape from the cassette and improperly denaturing the proteins.

If the tape was not removed from the cassette then the circuit cannot be completed and the proteins will not leave the well. If the proteins are not properly denatured then the proteins will not be able to properly travel through the creamily.

If this happens then the proteins will not show up, but the kaleidoscope will, as that doesn’t need to be denatured. Sandwiching the membrane The proteins are transferred onto a nitrocellulose membrane. The membrane is made to have a high affinity for proteins, so the proteins will transfer and stick to it by the transfer sandwich. The time the sandwich runs at AAA and for so long (7 the proteins have a risk of penetrating through the membrane.

The transfer is done n an ice bath because the length of time for the current produces a lot of heat. The membrane is set into transfer buffer to help fix the proteins to the membrane.

The buffer unifies the charges of the membrane and proteins. The membrane is soaked in blocking solution (ABS) to occupy the unbound areas of the membrane. A problem that may arise during this step is not orienting the charges correctly. If this is done incorrectly then the proteins can run off the gel, and the membrane will appear clean.

The current could not be correctly established, and the proteins could never leave the gel. If this is done then the proteins will show up on the commies stain. Commies staining A commies stain is done to examine if a problem occurred in the transfer of the proteins to the membrane.

This tests the gel for the presence of proteins.

Commies is a strong dye that will bind to any proteins in the gel. The dye can be rinsed from the gel with a De-staining solution, but it cannot be washed from the proteins. This is a valuable test for determining a mistake in a failed western blot. Microchemistry with western blots Monoclonal and polygonal antibodies are added to the membrane in a similar cushion to previous exercises. In a western blot, the marker is not a florescent marker, but instead a peroxides (horseradish peroxides). The peroxides is added to the secondary antibodies.

When a reduced dye (vector nova red) is added with Hydrogen peroxide, the peroxides breaks the hydrogen peroxide into water and a free oxygen. The oxygen will oxidize the vector red changing it from colorless to red. This is a qualitative measurement because the data recorded is whether the band showed up it not. Methods: Day l: Protein extraction: I obtained a flask of TTT mouse fibroblast cells from the CO incubator.

The flask as rinsed with ice cold PBS to remove secreted proteins and left over media from the flask. Once the flask was rinsed, RIP buffer (Radio nonparticipating assay buffer).

The buffer contains ionic detergents, SD (sodium decoded sulfate) and Sodium decolletage, and a nonionic detergent, Triton x-OHIO. A protease inhibitor was also added to the solution. The cells were also mechanically lased by being scraped from the flask with a cell scraper and pipettes up and down. The solution was transferred to a micro centrifuge tube, and left to incubate in four degrees Celsius fridge for forty-five minutes for the RIP to dissolve the membrane.

After the incubation period the tube was spun to remove cell membrane particles at 14, ramps for fifteen minutes. The supernatant was collected and stored for later use.

Protein concentration assay Five standard solutions (50 ml) were made with ABS (Bovine serum albumin) and RIP buffer at varying concentrations (figure 3). Fifty millimeters of the unknown protein extract was added to a final tube. The standards and protein extract were grouped together and processed for an absorbency reading. To all the tubes, lull of ARC reagent I was added, vortexes, and left to incubate at room temperature for nee minute, and then lollop ARC reagent II was added, vortexes and spun at discarded.

To all the tubes, lull of reagent A+S and lull reagent B were added and vortexes to mix. Lull of each tube was added twice in adjacent wells to a 96 Nell plate. The 96 well plate was read in a spectrophotometer for their absorbency ‘ aloes. The absorbency values were graphed concentration vs.

. absorbency, and a regression line was calculated. The regression line’s equation was obtained (figure 4). Using the equation, the absorbency of the unknown protein extract was substituted onto the equation, and a concentration was calculated in GU/LU. Day II Preparing solutions for gel electrophoresis A standard and two blanks were made as controls for the gel-electrophoresis.

The kaleidoscope was prepared by adding lull of kaleidoscope solution to lull PBS and lull xx protein loading buffer (B-mechanization and SD).

Two blanks were made by adding lull PBS to lull xx protein loading buffer. Three of identical solutions were made with PBS and the protein extraction to bring the total amount of protein in the solution to chug and the volume to lull. lull of xx protein loading buffer was added to the three tubes of the protein extractions. The proteins in these solutions needed to be denatured to optimize the Nesters blot.

The solutions were heated in a boiling water bath (figure 5). Special zaps were needed to keep the solutions from bubbling out of the tube. The tube was then placed in an ice bath for five minutes. Preparing the polysaccharide gel electrophoresis A Bio-Radar SD-page gel pre-cast (4% stacking, 4-15% gradient separating gels containing 10 wells with 1. Mm spacers; can load lull) was obtained and removed from its pouch. The comb was removed, the wells were rinsed with distilled water, and the tape on the bottom edge of the gel cassette was removed.

Two gels were placed into a clamping frame (figure 5).

The green clamping arms hold the two gels in place. The chamber, between the two gels, was filled with SD- running buffer (Tries-HCI, SD, Glycerol, BAM, EDT, Bronchiole Blue). The gel wells Nerve then filled with a blank, kaleidoscope, sample 1, sample 2, sample 3, and a blank. The solutions were added to the wells using a fine tip pipette to more accurately load the solutions. The first experiment we used a guide and the second time around we didn’t.

Running the Gel Electrophoresis Four gels were run at the same time, so two clamping frames were used in the electrophoresis run.

We attached the leads to their color coded ends, and ran the gel at 200 V and 100 A for h hour. Transferring the SD-page gel to the nitrocellulose membrane The gels were removed from the glass plates, and placed in cold transfer buffer for 15-20 minutes. The nitrocellulose membrane, sponges and filter paper were soaked in transfer buffer as well (figure 8). A transfer sandwich was made with positive electrode, sponge, filter paper, gel, nitrocellulose, filter paper, sponge and negative electrode (figure 9).

The sandwich was placed into a take of ice, and a current was run for seven hours at AAA.

When it is finished, the membrane was stored in the fridge at four degrees Celsius in membrane blocking solution. Commies staining We placed the gel in container, and added enough commies to submerge the overnight (figure 10). In ours the gel was rinsed twice with double denizen Neater to remove the commies. Gel descanting solution was added and the gel was rocked for 30 minutes.

The gel had all of its commies removed except for the proteins stained with the commies (figure#). On the first attempt, I tried to stain the membrane after the uneconomical analysis failed. Day 3

Uneconomical analysis of the nitrocellulose membrane The membrane was removed from the fridge and rinsed with ODL water. The membrane was incubated in blocking solution for 30 minutes on the rocker. The blocking solution was replaced with primary antibody solution (monoclonal anti- cyclic and monoclonal anti-beta acting made in a rabbit), and incubated for 1. 5 hours.

Ere solution was then rinsed with blocking solution three times for five minutes each. A solution of secondary antibodies (goat anti-rabbit) with horseradish peroxides attached was added and incubated for 50 minutes. The membrane was then rinsed

Ninth washing solution (Tryst-HCI, 0. 05% Teen, nontoxic detergent) three times. The membrane was then ready for the vector red to be added.

The vector red was added to submerge the membrane. Color developed in 15-30 minutes, and bands (or lack thereof) were recorded. Results The concentration of the protein extractions were 1. Eng/LU and 1. Eng/LU for attempt 1 (03/04) and attempt 2 (03/14). Day 2 The commies stain for the first attempt was negative for proteins.

The second attempt the commies showed some proteins still in the gel. Day 3 No bands appeared on either attempt. Conclusion

On both attempts the graphs made from the standards were poor. The data fluctuated strongly and this gave me a lower confidence in my sample. The reason I believed it fluctuated so much was because of poor mixing with the ARC reagent I and II. These steps involved incubation, forgetting, and centrifuging.

Since my data was dispersed and this step required the use of different machines and technique, there Nas a higher risk of something going wrong in this step. I could have also overexposed the wells. If my technique of covering the wells was not uniform then some wells could have been exposed more than others.

This uniformity could account for the skewing in one some data. This unreliability made me question the oddly high concentration of our unknown sample.

In both situations the sample goes out of the linear range of the graph, so my confidence drops further for both attempts. The commies stain for attempt one was clean; however, in this attempt, proteins failed to leave the wells during the current flow. I was sure I removed the tape from the bottom of the cassette, but that mistake would account for these results. If the mistake was not properly denaturing the proteins then our blanks should have ran, UT they didn’t.

The other group on the outside also failed. If one person had their tape on the cassette perhaps it could disrupt the current and as a result, affect another gel.

The second attempt succeeded in the running of the gel, but it failed to proteins were discovered in the commies stain. The kaleidoscope could have a higher affinity for the nitrocellulose, or better repulsion during the sandwich. Since the stain was found in the commies stain, I can say confidently the mistake was in the transfer. Since our gel run failed in our first attempt, we received no results from the uneconomical assay.

The second attempt failed at the commies so no uneconomical assay was going to work there either. On the first attempt I wanted to know if the transfer worked.

I wanted to see if the proteins that didn’t make it far from the well, but still made it a little progress, were transferred to the membrane. If I could confirm this I could be confident in my transfer technique. I tried to stain the membrane with commies to look for transferred protein, but not results could be observed. The commies wouldn’t wash off the membrane.

The mistakes were noted and actions will be taken to not repeat them.