Lab Manual/ Journal
Addison Bond
Dr. Chen
Honors 497, Fall 2019

9/23/19

- Met with Tiannan for the first time and learned how to subculture HL1C cells. Learned how to create my own cell median but will need a refresher in order to make it on my own.
- The median contains 5% FBS + 1% PS and DMEN as the main stock solution.
    - From my understanding, the FBS is for cell growth (?), the PS is an antibiotic, and the DMEN contains glucose i.e. fuel for the cell.
- In order to use the median with the cells, all solutions must be taken out of the fridge and placed in a water bath of 37 degrees Celsius. You cannot mix the median with the cells if the median is not warmed to this point!
- Created a 1→ 4 plate subcultures.

9/30/19

- Checked on cells from last week. I let them grow too long (they got to 100% cultured which is not ideal, would rather have them at 70-80%).
- From the 5 plates I had in the incubator, 4 of them were frozen and one was used to subculture.
- The freezing process looks like this:
    - Use median of 20% FBS + 5% DMSO + 1% PS
        - More FBS is used here because the cells will need more nutrients and energy if they are to withstand the freezing state.
        - DMSO is toxic, but it prevents the H_2O inside the cells from freezing. This allows for them to be frozen without being destroyed.
    - Remove the old median via suction
    - Add 1mL of trypsin to digest cells, wait 1-2 minutes for completion
    - Remove trypsin via suction
    - Add 1mL of freezing median to plate to wash cells
    - Using pipet, remove the median with the cells into a small test tube
    - Repeat this for the other 3 plates
    - Place the tubes into the 4-degree Celsius freezer for 30 minutes
    - After this, place the tubes into the -20 degrees Celsius freezer for 2 hours
    - After this, place the tubes into the liquid nitrogen freezer for up to a year
- Dr. Chen would like for me to check on the frozen cells next week to see if they have survived. I will need to contact Tiannan to learn how to unfreeze the cells.
- Dr. Chen also has told me I need to print off my biosafety training certificate and put it into the lab binder.
- From the 1 remaining plate, I subcultured a 1→6 plate and will check on the growth of the cells on Wednesday afternoon, October 2nd.
- For the 1-6 plate subculture, I digested and washed the cells as described in the freezing section. When washing the cells, I used 3mL of median (non-freezing median). Divided 6 ways, that leaves 500 microliters in each plate.

10/2/2019

- 2 days have passed since I left the 1-6 subculture grow. After viewing all 6 plates, the cells look healthy but have proliferated to be 100% grown.
    - This needs to be addressed with Dr. Chen because these cells were supposed to grow slower in order to not have to check up on them as frequently.
- Am going to read the 995-pdf article, *Function of Vitamin A in Metabolism* and Matt Goff's Methods and Materials.

10/3/2019

- Met with Dr. Chen this morning briefly

10/4/2019

- Did 2x 1-6 cell subculture in new medium to make sure the cells don't grow too fast.
- Medium used was a 1-10 dilution
    - Used this to stop the cells from growing too fast.
    - This was DMEN, 0.5% FBS and 1% PS
- I came back in the afternoon to try to learn how to treat the cells with the different treatments described in Matt Goff's dissertation.
    - Groups: Control, insulin, RA, insulin + RA
- Treatment of cells involves the creation of a new medium
    - There are 12 plates, 3 plates for each of the divisions described above.
    - The new medium used was not FBS – it is 2% HS. This is used when treating cells in this nature.
    - This was the base solvent used for the new mediums, including the control group. The control group is just the medium + 10 micromolar of absolute ethanol since the ethanol is used with retinoic acid (this is just to ensure everything is consistent across the 4 different groups; it will have no affect).
    - 10 mL of the 2% HS solvent was added to 4 different 50mL test tubes, one for each group.
    - The different solutes (insulin, RA, and insulin + RA) were added to the respected tubes; 10micromolars of each to each tube.
    - The tubes were placed in the -4degree Celsius fridge until they will be used as the new medium for the cells this Sunday, 10/6/2019.
- The stock solution of retinoic acid is 5mM and this procedure calls for 1micromolar. A 1:5 dilution was performed to get the 1 micromolar of RA.
    - 200 micromolar of RA was added to 800 micromolar of absolute ethanol.
        - ALWAYS ADD THE SOLUTE TO THE SOLVENT!
    - RA degrades rapidly in light, so whenever you are handling it you must use brown glass bottles.
    - Do not spill RA and be very careful when handling it. It can cause birth defects in women.
- Insulin was borrowed from Aaron Anderson. He had already prepared a test tube of 10 nanomolar of insulin.
    - Insulin needs to be carefully handled. You cannot freeze, unfreeze, refreeze the insulin due to water crystals forming and freezing on the proteins. This will cause

them to be less active, leading to the necessity of using more insulin, which will deviate from the method and cause adverse results.

- Moving forward:
    - Come into the lab on Sunday 10/6/2019 to check the 12 plates, switch their mediums, and autoclave materials needed to make my own insulin.
    - Email Aaron Anderson when you are using the autoclave.
        - Use either 1 or 3, sign your name, run liquid cycle, press start, and wait 1.5-2 hours until the cycle is completed.

10/6/2019

- I did my first autoclave in Autoclave 1 and emailed Aaron Anderson to confirm.
- 2 of the 12 cell plates had overgrown, but no contamination has been noted.
    - The rest had all reached around 70-90% confluence.
- Dr. Chen advised to not leave the cells outside of the 37 degree Celsius and 5% CO2 environment for a prolonged amount of time. This can change the pH of the cells and cause them to undergo stress which is obviously not wanted.
- The cells were placed back in the incubator to recover since I had left them out for too long of a time.
    - Dr. Chen advised to spread the plates out singularly and not stacked in order for the medium to reach 37 degrees quicker.
- To replace the suction container, add 10% bleach and allow for everything to be denatured.
- To replace the ethanol used to clean the materials before placing them in the hood: use 70% ethanol and fill the rest up with double distilled water.
    - This used because 70% draws the water out of the environment of the bacteria.
- When treating cells as I just did today with anything
    - Do not use trypsin. Trypsin is used to break up the cells and allows for them to grow. This is why you use trypsin when you are subculturing the cells.
    - Since the mediums were prepared (Friday) before coming to lab today (Sunday), all I did was suction out the old medium and replace it with 3mL of the new respective medium.
        - A different pipet tip was used to give the plates 3 mL of their new medium but the same tip was used to suction out all the old medium for each of the 12 cells.
        - Only 3 plates were out at a time. This was to make sure the cells were not placed under any more stress than needed by being outside of their 37 degree and 5% CO2 incubation settings.
        - The 2 plates that were overgrown were treated with the control medium.
    - Plates were placed back in the incubator not stacked and will be checked in 2 days.
    - Clean-up was the usual. The 4 test tubes were now empty and were thrown away into the biohazard container.
- Autoclave:

10/14/19

- I took some time off after a stressful and sick week and am now back in the lab. Last week, I tried to learn how to test the cells for glycogen and protein lysing, however my cells had not grown enough to be able to extract data.
    - This can be traced all the way back to when I subcultured them and treated the cells. I did not perform subcultures appropriately as I was still learning the methods. When performing a 1→4 split, I used the original plate as part of the new 4. This is not acceptable and lead to certain plates (the ones described) being way overgrown and over 100% confluent.
    - In addition, I treated the cells with insulin, RA, and insulin + RA too early. Subculturing helps the cells to grow and to differentiate. When subculturing is halted, differentiation cannot occur, and the cells stop growing. This means that they need to reach close to 100% (but not all the way) confluence prior to treating them with the new mediums.
- Today, I recovered 3 plates I had forgotten about in the incubator and used them to perform a 3→12 split. Aaron and I decided this was appropriate to move forward even though these cells had reached 100% to >100% confluence given that treating them with the medium later on will negate to some degree the epigenetic differences that can arise when cells reach 100% confluence.
    - I used Aaron's method today for subculturing and liked it much better.
    - Knowing that I was to use 12 plates with 3mL of medium in each, I placed around 40mL of 5% FBS + 1% PS in a 50mL test tube. After treating the plates with trypsin, I placed them in the incubator to digest which provided me with much better results.
    - After enough time had gone by (which was longer than 2 minutes, it was closer to around 10. I did this by eye gage rather than by a stopwatch so I could actually see some of the cells begin to break away from the plate, indicating that it was ready to take out the trypsin and give them fresh medium) I removed the trypsin carefully with the pipet and NOT the suction machine.
    - 3 mL of medium was placed in each of the 3 plates and then this total of 9mL of medium + cells was added to the 50mL test tube.
    - In this regard, the cells will be evenly distributed since this test tube will provide the cells and medium for each of the new 12 plates.
- The 12 plates were left growing in the incubator and will be checked at a later date (perhaps tomorrow or Wednesday prior to leaving for fall break).

10/16/19

- Today's lab was jam packed. The agenda: check cells for confluence, treat them, and subculture.
- All 12 plates were of a confluent level where they could be treated. There was an issue though. Since I only had 12 plates and I needed to treat 12, I would have no plates remaining. Dr. Chen had an idea: use 3 for control, 3 for RA, and 2 for insulin + RA. I used the method previously described in the manual. The same 1 micromolar RA and 10 nanomolar of insulin were used to treat the cells and they were left in the incubator once completed.

- An issue arose here that is a possible contamination factor: I forgot that the cells were to be treated in their existing plates. I (incorrectly) placed the medium described in 10 clean plates instead of on the plates with cells.
    - Be prepared to have to redo this due to contamination.
- From 2 plates left over, Dr. Chen advised to do a 2x 1 → 7 split since I won't be able to check on them for 5 days. This was not 0.5% FBS (realized this upon reflection now). However, the large split should allow for the cells to reach confluence and not be an issue by Monday.
- Side note: DO NOT USE 2% HS FOR HL1C CELLS!!! This is only used to differentiate the L6 cells and not needed for the HL1C cells.
- For Monday 10/21: check on the cells that were treated, check on the 14 plates that were subcultured and be prepared to treat 12 cells with 2 remaining.
    - 12 for treatment (3 in each of the 4 groups)
    - 2 for subculture (don't know if this needs to be a 2x 1→7 split as well?)

10/21/19

- Notes for the day can be found in the lab notebook

10/23/19

- Objectives of the day:
    - Subculture 2x 1→7 plates (completed)
    - Treat the 12 plates with control, RA, insulin, and insulin + RA (completed)
- Learned how to aliquot insulin today from Aaron.
    - See lab manual for exact procedure
- Talked to Dr. Chen about my progress. He was very reassuring about my speed and made it known that he wants me to do things on my time and find balance. He mentioned this is the busy part of the lab. Once I have repeated this three times, I should have enough data to be able to process and come in on my own time to do data analysis.

10/25/19

- Today I harvested 8 plates for glycogen assessment and 4 plates for protein analysis (from the 12 treatment plates)
    - Procedures were completed according to the lab manual I have
- The trypsin was left in the subculturing plates too long on Wednesday and hardly any cell growth has occurred in the 2x 1→7 subculture.
    - This is good since I will not be able to check on the plates until Wednesday the 30th
- NEW PROCEDURE FOR CELLS:
    - Let them grow to confluence for 5 days
    - Treat them for 2 days
    - Harvest them after these 2 days
    - Store them in -80 freezer until you can complete the harvesting procedure

10/30/19

- Didn't have Aeron or Tiannan in the lab today, but it wasn't too much to do.
- I subcultured plates same as always (2x) 1→7 split.

- I also treated 12 plates.
    - I THINK THIS BATCH WILL BE GOOD FOR DATA
    - THIS IS THE ONLY BATCH I CAN RECALL USING 0.5% FBS FOR TREATMENT
        - Dr. Chen reminded me today when I met with him that for subculturing cells, I need to be using 0.5% FBS. I do not know if this will have significant effects on the data.
- Went over some math in Dr. Chen's office.
- He sent me some articles to read and mentioned that after reading I could potentially have something to write about.
- Moving forward:
    - On Friday (11/1/19): harvest the 12 plates that were treated.
        - Will most likely keep it consistent and use 8 for glycogen and 4 for protein.
    - On Monday (11/4/19): treat the 12 plates I subcultured today.
        - ASK DR. CHEN ABOUT THE 2 PLATES HERE THAT SHOULD BE SUBCULTURED.
        - If I were to subculture them then I should harvest them on Friday. This does not work because I will be in Dallas. I do not want to waste the cells and need to continue to subculture them.
    - On Wednesday (11/6/19): harvest the 12 plates from Monday.

11/1/19

- Harvested the 12 plates, 8 for glycogen and 4 for protein.
- The lysis buffer used is RIPA. I got it from Aaron. Need to do some more research and understanding on this. He mentioned he would teach me how to make it so that I can know more about it and understand its methods.

11/4/19

- Created new media for cells
- Treated my 12 plates
- Did a 1→ 7 subculture on the advice from Aaron since I won't be in the lab on Friday.

11/6/19

- Harvested my 12 plates
    - Today went really well and efficiently.
    - I made new 30% KOH, PBS, and 190 proof ethanol.

11/11/19

- After coming back from being in Dallas for the weekend, I found that these 7 plates were contaminated and had to be thrown away. However, there is hope from the 1 plate that was left behind from the initial 1-7 split.
- I have a fear that it is the media of my cells that is contaminated, in which case the new batch is now ruined.
- Need to meet with Dr. Chen to discuss the specific protein markers I should be looking for in the data analysis as I prepare to start my western blot.

11/13/19

- The good news is that the plates were not contaminated, and I moved forward in the experiment. All I did was expose myself to the protein measurement method using the BSA assay.

11/14/19

- Met with Dr. Chen in his office to discuss Western blotting. See lab notebook, need to transfer here.
- Did not split the plates today, letting them grow to confluence.

11/15/19

- Emailed Aaron to learn how to do the protein measurement BSA assay.
- I had 4 plates that were grown to confluence. In order to maintain the consistency of the experiment, I did 2x 1-7 split as opposed to the 3x 1-4 split that I emailed Aaron about. The bad thing is I did not realize this until I had already added trypsin to the cells, so I discarded 2 plates.
- I now have 14 plates growing in the incubator with 5% FBS, 1% PS in DMEN. This is consistent with the procedure done previously.
- In 5 days (Wednesday the 20th), I will treat the cells.
    - REMEMBER TO USE 0.5% FBS WHEN TREATING!

11/18/19

- I need to check my cells
    - I think they are contaminated again. This leads me to believe that it is my media that is the source of contamination.
    - I will tell Aaron about this an email Tiannan to see if she has spare plates or cells I can use to begin a new culture.
- I need to make 0.5% FBS media for treatment procedure on Wednesday
    - This is now irrelevant if the cells are contaminated- I won't be treating them.
- I need to complete glycogen harvest
    - Add 37% HCl to equal 3M of final concentration
    - Place them in 100 C water bath for 2 hours
    - Measure with glucose liquicolor kit
- The glycogen harvest was not successful. The readings from the Glomax machine did not provide data that was consistent with the standards. Additionally, several mistakes were made throughout this process:
    - The HCl used was most likely compromised
        - It did not gas off as the lid was removed.
    - There were no positive or negative control groups added to the sample. This is important because a positive and negative control is needed to evaluate and base data off of.
    - Tube caps were not placed on the tubes and the lids were popped off and some tubes were even on their sides.
    - The Glomax machine could have not been working properly.

11/20/19

- A repeat of the glycogen harvest was done today in hopes to attain data from the remaining glycogen tubes from Monday.
- Appropriate steps were taken to assure that no foreseen variables were present (all steps not taken on Monday were taken today).
- In addition to the glycogen harvest, I attained a new cell plate from a fellow student who is also doing research on HL1C cells.
    - My cells were contaminated again, leading me to assume more strongly that this is a result of contaminated media.
    - I have 1 new plate from her at confluence, so I split this in a 1-4 split advised by Dr. Chen and Aaron.
    - On Friday, I will take the 4 plates and do a 1-4 split again.
        - Have 4 plates
            1. 1-4
            2. Use as a safety net
            3. and 4. FREEZE in negative 80.

11/22/19

- I froze 2 plates and subcultured 1 in a 1-4 split.

Submitted to Chancellors Honors College 12/11/19.

Update: I have successfully completed 2 of the 3 rounds of treatment. I have to get a third set going as soon as the break ends, and I will be on campus. From those 2 sets, I had consistent data for the glycogen harvest. I am currently working on getting Western Blotting techniques down so that I can assess the proteins collected.

Lab Manual/ Journal
Addison Bond
Dr. Chen
WINTER BREAK WORK

Winter Break 12/16/19

- Typical WB day: morning- run gels and transfer, midday- make more gels and do blocking, afternoon/evening- develop those from yesterday
- SDS- insoluble in cold temperatures, needs to be very warm to dissolve (this is found in the proteins about to be used on WB)
- Before you use the gel, investigate its integrity
- SHORT WELL GOES ON INSIDE FOR ELECTROPHORESIS
- If you check the electrophoresis buffer, you want it to be closer to 7 because then it shows that the instrument is more accurate. Farther away, like to 13 is very inaccurate
    - Never go above 6X for this buffer
- Need to draw up fluid from well with plunger to clean out the wells
- Get the samples in the right order for loading them into the gel AND remember this order!
- Once it is arranged correctly, you can use the ladder (this is a fluid)
- Want to use 20microliters per well of your protein
- Use 5 microliters per well of ladder
- Want to place the sample in the middle of the wells, not on the edges
- Once you load the first ladder, the timer starts to get all the protein loaded into the gel
    - As you move down the wells, the previous wells filled with protein have time to dissolve and diffuse, leading to not very good results
    - Applying a gentle electric field, 50V, keeps the proteins locked in place without causing them to diffuse
- Get pipet between the glass panes- BE VERY CAREFUL HERE
- USE THE SAME LOADING BUFFER IF RUNNING MULTIPLE WELLS
- Once all of it is loaded, turn to 100V and let it run until all the protein is through the stacking gel
- Always record the orientation of the samples!
- Write everything and leave a breadcrumb trail
- Draw out a schematic of how the apparatus looks
- Once electrophoresis is complete, remove the gel and get it ready for the transfer
- Let sit in transfer buffer
- *we are using nylon membranes
- Put the membranes on the positive side because the proteins being used are negative from the SDS
- There can be no bubbles between gel and membrane
- Place in ice in transfer and pour transfer buffer back into the transfer apparatus
- Use 80V for 3(?) hours
- More information to come in the following days as more of the WB procedure is observed.

Winter Break 12/17/19

- When using nylon membrane for transfer, do not use methanol at all. Use a lower voltage for longer amount of time to ensure you are not overheating the membrane.
- When using running and transfer buffers, you can use them about 3 times before they need to be replaced.
- Ladder is only applied to the first well in the electrophoresis
- For the proteins you are going to use, need to prep them.
- Use ddH_2O then DTT then the 6X loading buffer
    - Did not note specific amounts, need to get these down from Aaron.
- Procedure for making 2 gels:
    - **Resolving**
        - ddH_2O: 6 mL
        - 30% acrylamide: 5 mL
        - 1.5 tris HCl: 3.75 mL
        - SDS: 0.16 mL (160 microliters)
        - APS 10%: 0.16 mL (160 microliters)
        - TEMED: 0.01 mL (10 microliter)
    - Add 1 mL of ddH_2O to the gel to settle it and make sure it is evenly spread.
    - After about 15-20 minutes (or once the gel is set) remove the water by simply pouring it out.
    - **Stacking**
        - ddH_2O: 3.5/4 mL
        - 30% acrylamide: 1 mL
        - 1.5 tris HCl: 3.75 mL
        - SDS: 0.08 mL (80 microliters)
        - APS 10%: 0.16 mL (160 microliters)
        - TEMED: 0.01 mL (10 microliter)
    - Add the wells
        - I use the 10 well white combs
        - DO NOT ADD WATER AFTER THIS STEP
    - Wait about 15 minutes
    - Remove the comb carefully
    - Cover the gel in a paper towel and saturate it with water until it is soaking wet.
    - Store the gel in a bag in the -20 fridge for a day before use

Winter Break 12/18/19

- Made new PBS with Aaron. Formula is in the lab.
- Aaron is going to look into a stripping buffer to see if this is effective. If so, then it will cut out a large amount of time and allow for him (and me too) to test multiple proteins by reusing the same membrane.
- My understanding of the WB procedure thus far:
    - Make gels
    - Load gels w protein
    - Run electrophoresis
    - Run transfer

- Block membrane using casein from milk (do this for an hour but can do over night)
- Wash with primary antibodies
  - This is done overnight on the rotator in the -4-degree walk-in freezer
- Wash with secondary antibody
- Develop the film using ECL kit in dark room

12/19/19

- I am working on my own Western's today
- Make sure to warm up the protein from the freezer you will be running in a hot water bath to ensure the SDS is dissolved completely.
- For running the ELECTROPHORESIS, use the 1X running buffer
- Since I am using the 10-well comb, the wells are deeper. With them being deeper, I do not have to worry about the protein diffusing out. The proteins diffuse up and over, with mine being deeper this will not happen very quickly.
- As soon as the electric field is applied, it will grab the protein and pull them back down.
- Use 100V until the proteins are through the stacking gel
- Move voltage up to 135V once the protein gets to about the middle of the gel
- When completed, there should be a straight blue line at the bottom of the gel and the ladder should have different colors and be spaced out throughout the gel.
- Turn off and pour the buffer back into the running buffer container.
- For TRANSFER, cut out your membrane and do not let anything touch it (if your fingers touch it you should be fine)
- Arrange the transfer holder in a tray and pour transfer buffer into the tray (the entire bottle)
- Always put the membrane on the positive side of the holder (meaning the WHITE part) and facing the RED portion of the transfer apparatus when doing the transfer.
- Make sure everything is squared away before closing the transfer holder
- Place into apparatus. This should be in a bin filled with ice. Fill the transfer apparatus with the transfer buffer from the tray
- Once the apparatus is filled with this transfer buffer, fill the bucket with water from the sink
  - This gives the ice more surface area to act on

Lab Manual/ Journal
Addison Bond
Dr. Chen
Honors 498, Spring 2020

1/6/20

- Arrived at lab after taking 2 weeks for Winter Break. I reviewed the film processed from the Western blots from my last day in lab. The B-actin looks good and useable; however, Dr. Chen would like to have the IRS-1, PIRS-1, and Akt run again.

1/7/20

- Attempted to make gels for Westerns. Not enough resources to move forward.

1/8/20

- Looked at new HL1C cells revived from Tiannan's. We prepared the new cell media (5% FBS, 1% PS) for the cells. Aaron and I are going to do a 1-10 split in order to maximize the plates and hopefully get many good samples.
- The cells had no grown to adequate confluence to split. Will look and try to split them again on Friday if they have grown enough.
- Going to meet with Dr. Chen sometime this week or next week. Need to ask if Tiannan would want to incorporate data from JUST glycogen content of cells under variety of treatments. If this is so, I would not have to complete any more westerns and could work on writing and literature review to fill in the gaps in my data.

1/10/20

- The HL1C cell plate from Tiannan was confluent enough to subculture.
- A 1-10 split was done and incubated in the left incubator.

1/13/20

- Each of the 10 plates looks to be about 70-75% confluent after the weekend. No signs of contamination.
- Need to figure out a way to time the 3rd round appropriately.
- I would like to harvest on a Wednesday, meaning they need to be treated on a Monday. Need to do a split on a Wednesday to let them grow to confluence for 5 days.
- Plan:
    - Wednesday the 15th: do 2x 1-7 split and freeze the remaining 6 plates.
    - Monday the 20th: treat the cells with C, RA, Ins, and Ins + RA
    - Wednesday the 22nd: harvest the 12 plates (8 for glycogen and 4 for protein)
    - Thursday the 23rd: Do glycogen assay and BSA
    - Pause and analyze what you have and devise new plan moving forward

1/15/20

- Completed above plan for this date.

3-10-20

- Get each sample into an order where you can see the number and the treatment
    - Separating them out by group
    - See excel for how to do this
- Normalize the OD value for groups from glomax
- Get every cell mass number for 1-3 groups
- Mg/dL will be one graph
- Apply this to the total glucose and that will be the second graph
-