Experiment Number: _____

Date: _____

Purpose: Antibody Validation for Mass Spectrometry
(Light Chain Specific Secondary Antibody)

Cells Line: _____ (Date Prepared-_____)

Antibodies:_____

_____

Day 1: _____

Each blue cap tube (1 X 10^8 cells) is good for 1 sample!

1) Take out _____ 15 ml Falcon tubes, each containing 2 X 10 ^8 of frozen cells for ___ antibodies that to be tested. Add 12 ml of cold PBS into each tube. Thaw cells in a cold room for 1 hour on neutator.

2) Spin the cells at 1,500 rpm for 3 minutes. Remove PBS and add 12 ml of hypotonic buffer. Resuspend cells and leave the tubes on ice for 10 minutes to swell the cells.

3) After swelling cells, dounce homogenize on ice, 30 strokes, to break open the cells. Keep the total time to 15 minutes for the entire hypotonic lysis /homogenization steps.

4) Aliquot lysates into 2 ml tubes. Centrifuge at 600g (rcf) at 4 ^{0}C for 8 minutes to pellet nuclei. Discard supernatant and wash nuclear pellet once with hypotonic buffer.

5) Resuspend nuclear pellets in 1ml 1X RIPA buffer. Incubate for 30 min on ice.

6) Spin nuclear lysate at 14,000 rpm for 15 minutes at 4 ^{0}C. Transfer supernatant to 50 ml falcon tube. Add 1 X RIPA buffer to final 30 ml and remove 100 μL aliquot for input. Split the lysate into six 15 ml falcon tubes (5 ml each). Set up IP as indicated bellow. Incubate at 4 ^{0}C with neutator rocking for 12-16 hours (overnight).

*Add 100 μL of Laemmli buffer containing beta-mercaptoethanol to the input DNA, boil and freeze at -20 ^{0}C.

No.	Antibody	Species	Concentration (μg/μL)	Size(kD)	Test(μg)	Ab(μL)
1					12	
2					12	
3					12	
4					12	
5					12	
6					12	

*Each IP tube has ~1 X 10^8 cells.

Day 2: _____

1. Remove 150 μL of 50 % Protein A/G-agarose for each sample (1X10^8 cells) to 1.5 ml microfuge tubes and wash twice with 1 ml of ice cold 1X RIPA buffer. Spin at 5,000 rpm for 1 minute at 4 ^{0}C.

2. Resuspend the Protein A/G-agarose in 150 μL 1 X RIPA. Add the beads (use another 100 μL 1 X RIPA to insure you get all the beads) to tubes containing Ag-Ab complex and incubate for 1 hour at 4 ^{0}C on neutator rocker.

3. Centrifuge the tubes at 1,500 rpm for 3 minutes, wash the protein A/G-agarose beads 3 times with 10 ml of fresh, ice cold 1X RIPA buffer and once with ice-cold PBS. Wash each time for 15 minutes.

*For washing, only use ONE tablet of protease inhibitor per 50 ml 1X RIPA Buffer.

4. Resuspend Protein A/G-agarose in 800 μL ice-cold 1X PBS and transfer the beads from 15 ml tube to a 1.5 ml eppendorf tube. Wash the beads in 15 ml tube with 400 μL ice-cold 1X PBS (to be sure all the beads are collected) and transfer to the eppendorf tube.

5. Then centrifuge the beads at 5000 rpm for 2 minutes. Elute the antibody-DNA complexes from the beads by adding 55 μL of 2X Laemmli buffer containing beta-mercaptoethanol, boil and freeze at -20 ^{0}C.

Day 3: _____

1) Load 5 μL (Bio Rad dual color) of Molecular weight marker, and 7μL of input, and 2 μL sample/lane in a 4-15% gradient precast SDS-PAGE gel.

Each antibody should have the following:
a) Molecular Weight Marker
b) Input

c) IP DNA
d) Mouse (Rabbit, or Goat) IgG IP DNA

MW		Input		IP		IgG		MW		Input		IP		IgG

2) Attach leads to the gel box. Run the gel at 100 volts. It takes about 1 to 1.5 hours.

3) Discard the stacking gel (Trick: Put the paper tower on top of the stacking gel, then lift it. The gel will come off very easily). Transfer the separated proteins to nitrocellulose paper for 1-1.5 hours at 250 mA (Bio-Rad power supply).

Transfer Buffer: 100 mL10X Transfer Buffer + 200 ml Methanol-→Bring up to 1000 mL with dd H_2O.
*Alternative: ZhiHa prepared the transfer buffer (cold room) which is ready to use.

***DO NOT dump the transfer buffer into the sink.**

4) After transfer, take out nitrocellulose membrane and put it in blocking buffer (5% milk).

5) Turn on Blotcycler and listen for click to make sure vent are closed.

6) Add Wash Buffer (PBS/0.1% Tween-20) to main compartment, till max line ~3liter.

7) Add 15ml Blocking Buffer (5% milk/PBS-0.1%Tween 20 (PBST)) to each tray. Place membrane in respective trays. Cover trays with lid.

8) Prepare Primary Antibody. Dilute the testing antibodies in 15ml 5% milk in PBS-T. Add to column labeled P1-P6. ** Make sure that PA and trays are matched. *Save the primary antibody (add 0.02% sodium azide), and store at 4 0 C for reuse, use collection vial. Collection vials should be matched to proper tubing.

9) Prepare Secondary Antibody- Light Chain Specific (Jackson Immuno-research, stored at -80 0 C, once thawed it is good for 6 weeks at 4 0 C). Dilute in 15ml 5% milk in PBS-T. Add to column labeled S1-S6 Mouse (1:10000) Jackson ImmunoResearch (115-035-174); Light chain specific Rabbit (1:10000) Jackson ImmunoResearch (211-032-171); Light chain specific Goat (1:10000) Jackson ImmunoResearch (205-032-176); Light chain specific

10) Start BlotCycler.

11) Place the blot on saran wrap. Add 750 µL of PICO per strip of NC paper. Incubate at room temperature for 5 minutes.

12) Tap off the solution. Place the blot inside a new sheet of saran wrap. Tape it securely inside a cassette.

13) When inside the dark room, place a sheet of film over the blot for the desired length of time. Then feed it into the developing machine. *Redo with PICO (80%) and FEMTO (20%) when any of the bands was faint. Add 750 µL of the substrate per strip of NC paper. Incubate at room temperature for 5 minutes.

Mass-Spec In-Gel Digestion Protocol

Load 5 ul molecular weight marker, 50 ul of IP DNA on NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels. Wash gel with Millipore water and then stain the gel with Coomassie Blue comparable protein gel staining solution. Cut the immunoactive bands and dice them into small pieces (see below).

In-Gel Digest Procedure

1. Wearing gloves and sleeve protectors, wipe down ALL surfaces in the hood with methanol/water moistened lint-free cloth, including the outside of all your tubes (make sure to not wipe off the labeling!), the outside and inside of the Speed Vac and centrifuge, tube racks, bottles etc. Wipe razor blades with methanol-soaked lint-free cloth.
2. Prepare the following solutions:
   25 mM NH_4HCO_3 (100 mg/50 ml)
   25 mM NH_4HCO_3 in 50% ACN
   50% ACN/5% formic acid (may substitute TFA or acetic acid)
   12.5 ng/µL trypsin in 25mM NH_4HCO_3 (freshly diluted)
3. Dice each gel slice into small pieces (1 mm2) and place into 0.65 mL siliconized tubes (PGC Scientific).
4. Add ~100µL (or enough to cover) of 25mM NH_4HCO_3/50% ACN and vortex for 10 min.
5. Using gel loading pipet tip, extract the supernatant and discard.
6. Repeat steps 3 and 4 once or twice.
7. Speed Vac the gel pieces to complete dryness (~ 20 min).
   For low-level proteins (<1 pmol), especially those separated by 1-D SDS-PAGE, reduction and alkylation is recommended. These procedures are performed after step 6.
   a. Prepare fresh solutions:
      10 mM DTT in 25 mM NH_4HCO_3 (1.5 mg/mL)
      55 mMiodoacetamide in 25 mM NH_4HCO_3 (10 mg/mL)
   b. Add 25 µL (or enough to cover) 10 mM DTT in 25 mM NH_4HCO_3 to dried gels. Vortex and spin briefly. Allow reaction to proceed at 56°C for 1 hr.

c. Remove supernatant, add 25 µl 55 mMiodoacetamide to the gel pieces. Vortex and spin briefly. Allow reaction to proceed in the dark for 45 min. at room temperature.
d. Remove supernatant (discard). Wash gels with ~100 µl NH_4HCO_3, vortex 10 min, spin.
e. Remove supernatant (discard). Dehydrate gels with ~100µL (or enough to cover) of 25 mM NH_4HCO_3 in 50% ACN, vortex 5 min, spin. Repeat one time.
f. Speed Vac the gel pieces to complete dryness (~20 min). Proceed with trypsin digest.

8. Add trypsin solution to just barely cover the gel pieces. Estimate the gel volume and add about 3x volume of trypsin solution. This volume will vary from sample to sample, but on average ~5-25 µL is sufficient.
9. Rehydrate the gel pieces on ice or at 4°C for 10 min. Spin. Add 25mM NH_4HCO_3 as needed to cover the gel pieces.
10. Spin briefly and incubate at 37°C for 4 hours - overnight.

Extraction of Peptides

1. Transfer the digest solution (aqueous extraction) into a clean 0.65 mL siliconized tube.
2. To the gel pieces, add 30 µL (enough to cover) of 50% ACN/5% formic acid, vortex 20-30min., spin, sonicate 5 min. Repeat.
3. Vortex the extracted digests, spin and Speed Vac to reduce volume to 10 µL.
4. Either proceed with C18 ZipTip (Millipore) cleanup or analyze with LC-MS. Add 2-5 µL of 5% formic acid. When analyzing low levels of protein, concentrate the petides by eluting from ZipTips using 3µL of elution solution, into a clean 0.65 mL siliconized tube.
5. Use 1µL of the unseparated digests for analysis by MALDI.

Matrices for unseparated digests:
a-cyano-4-hydroxycinammic acid in 50% ACN/1% TFA (10 mg/mL).
2,5-dihydroxybenzoic acid (DHB), saturated solution in water.