Antibody Validation (Light Chain Specific Secondary Antibody)

Day 1:

Each blue cap tube (5 X 10^7 cells) is good for 6 samples!

1) Take out 5 X 10 ^7 of frozen cells. Add 3 ml of cold PBS (with protease inhibitor, PMSF, DTT, phosphatase inhibitors) 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 3 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 (7ml homogenizer) 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 microfuge 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 1.5 ml of 1X RIPA buffer (every tube with 5 X10^7 cells). Incubate for 30 min on ice.

6) Spin nuclear lysate at 14,000 rpm for 15 minutes at 4 ^{0}C. Transfer supernatant to fresh tube, and remove 100 µL aliquot (input). Split the lysate into six 1.5 ml eppendorf tubes (~233 µL in each). Add 2µg antibody or normal IgG to each lysate. 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 at 90^{0}C for 10 minutes and freeze at -70 ^{0}C.

*Each IP tube has ~8.3 X 10^6 cells.

Day 2:

1) Wash (5,000 rpm, 1 minute, 4^{0}C) 30µL of 50 % Protein A/**G** –Agarose beads (for each sample) twice with 1X RIPA buffer (no protease inhibitor/PMSF/DTT) before use.

Note: When working with antibody before #198, use Protein A or G depends on the species.

2) Transfer Ag-Ab complex from Day1 step6 to each tube containing 30 µL Protein A/G beads and incubate for 1 hour at 4 ^{0}C with neutator rocking.

3) Centrifuge the beads at 5,000 rpm for 2 minutes at 4 ^{0}C. Save the supernatant (100 µL) to check lysate for protein clearance. Discard the remaining supernatant. Add 100 µL of 2X Laemmli buffer containing beta-mercaptoethanol, boil at 90^{0}C for 10 minutes and freeze at -70 ^{0}C.

Saved supernatant concentration will be ~1.4*10^4 cells/mL.

4) Wash the Protein A/G beads 3 times with 1 ml of fresh, ice-cold 1X RIPA buffer (with protease inhibitor, add PMSF, DTT, phosphatase inhibitors just before use), and once with ice-cold PBS. 15 minutes each wash. Remove all wash as close to the bead layer as possible.

5) Elute the antibody-DNA complexes from the beads by adding 45 µL of 2X Laemmli buffer containing beta-mercaptoethanol, boil at 90^{0}C for 10 minutes and freeze at -70 ^{0}C.

IP eluate concentration will be ~1.8*10^5 cells/mL.

Day 3: Western Blotting

1) Denature protein samples at 90 ^{o}C for 5 minutes.

2) Fill gel box with 700 ml 1x Running buffer.

3) Load 5 µL (Bio Rad dual color) of Molecular weight marker, 14µL of input and supernatant and 7 µL IP and IgG sample/lane on a 4-15% gradient precast SDS-PAGE gel.

Each antibody should have the following:
a) Molecular Weight Marker
b) Input
c) Check Supernatant (Soup)
d) IP DNA
e) Mouse (Rabbit, or Goat) IgG IP DNA

	MW	Input	Soup	IP		IgG		MW	Input	Soup	IP		IgG	

4) Attach leads to the gel box. Run the gel at 80 volts. It takes about 1 to 1.5 hours.

5) 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). (Keep cold in ice bucket).

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

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

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

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

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

10) 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.

11) 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

12) Start BlotCycler.

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

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

15) 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**.