

## **Magnetic Beads Antibody Validation (Light Chain Specific Secondary Antibody)**

### **Day 1:**

Each blue cap tube (5 X 10<sup>7</sup> cells) is good for 6 samples!

- 1) Take out 5 X 10<sup>7</sup> 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 °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<sup>7</sup> cells). Incubate for 30 min on ice.
- 6) Spin nuclear lysate at 14,000 rpm for 15 minutes at 4 °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 °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°C for 10 minutes and freeze at -70 °C.

\*Each IP tube has ~8.3 X 10<sup>6</sup> cells.

### **Day 2:**

- 1) Wash 30µL of Goat Anti-Rabbit(or Anti-Mouse) IgG Magnetic beads (for each sample) 3 times with 1X PBS buffer (no protease inhibitor/PMSF/DTT) before use.
- 2) Transfer Ag-Ab complex from Day1 step6 to each tube containing 30 µL Magnetic beads and incubate for 2 hours at 4 °C with neutator rocking.

3) Place tube in Magnetic particle concentrator to pull beads to the side of the tube. Save the supernatant (30  $\mu$ L) to check lysate for protein clearance. Discard the remaining supernatant. Add 30  $\mu$ L of 2X Laemmli buffer containing beta-mercaptoethanol, boil at 90<sup>0</sup>C for 10 minutes and freeze at -70 <sup>0</sup>C.

Saved supernatant concentration will be  $\sim 1.4 \times 10^4$  cells/mL.

4) Wash the Magnetic 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. 5 minutes each wash.

5) Elute the antibody-DNA complexes from the beads by adding 45  $\mu$ L of 2X Laemmli buffer containing beta-mercaptoethanol, boil at 90<sup>0</sup>C for 10 minutes and freeze at -70 <sup>0</sup>C.

IP eluate concentration will be  $\sim 1.8 \times 10^5$  cells/mL.

### Day 3: Western Blotting

1) Denature protein samples at 90 <sup>0</sup>C for 5 minutes.

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

3) Load 5  $\mu$ L (Bio Rad dual color) of Molecular weight marker, 14 $\mu$ L of input and supernatant and 7  $\mu$ L 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 °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 °C, once thawed it is good for 6 weeks at 4 °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**.