CRISPR cell line validation by IP-WB

ENCODE4 - Version 2 - January 2018

Day 1:

NOTE: One tube with 1 x 10^7 cells is good for one antibody reaction. If you would like to run GFP and IgG IP on your gel use two tubes with 1 x 10^7 cells each.

1. Take out two 15 ml Falcon tubes (1 x 10^7 frozen cells each).
2. Add 250 µl 1X RIPA buffer with protease and phosphatase inhibitors (see Reagent Preparation section below) to each cell pellet, pipet up and down, and transfer to 1.5 ml Eppendorf tubes.
3. Incubate for 30 min on ice.
4. Spin nuclear lysate at 14,000 rpm for 15 minutes at 4 ^{0}C. Transfer supernatant to fresh tube, and remove 50 µl aliquot for input lane*. Incubate remaining samples with 2 µg GFP or IgG antibody at 4 ^{0}C with neutator rocking for 12-16 hours (overnight).
   *To the input DNA add 50 µl of 2X Laemmli buffer containing beta-mercaptoethanol, boil and freeze at -20 ^{0}C.

Day 2:

1. <u>For each sample</u> wash 30 µl of 50 % Protein-G agarose beads twice with 1 ml 1X RIPA buffer. Centrifuge at 5,000 rpm for 1 minute at 4 ^{0}C.
   NOTE: We use Protein-G because we use a goat anti-GFP antibody.
2. Add 30 µl Protein-G beads to each tube containing antigen-antibody (Ag-Ab) complex from day 1 step 4, and incubate for 1 hour at 4 ^{0}C while rocking.
3. Centrifuge the beads at 5,000 rpm for 2 minutes at 4 ^{0}C. Discard the remaining supernatant.
4. Wash the Protein-G beads three times with 1 ml of fresh, ice-cold 1X RIPA buffer, and once with ice-cold PBS. Wash for 15 minutes each. Remove all supernatant 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 and freeze at -20 ^{0}C.

Day 3:

1. Load 5 µl molecular weight marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad), 14 µl input, and 7-14 µl sample per lane in a 4-15% gradient precast SDS-PAGE gel.

   Each CRISPR cell line IP-WB should have the following lanes:
   a. Molecular weight marker
   b. Input
   c. IP DNA
   d. Goat IgG IP DNA (for antibody validations)

	MW	Input	IP		IgG		MW	Input	IP		IgG	

2. Attach leads to the gel box. Run the gel at 108 volts for about 1 hour.
3. Discard the stacking gel.
   NOTE: Put the paper tower on top of the stacking gel, then lift it. The gel will come off very easily.
4. Place the pre-wet blot on the gel. Roll blot membrane gently to remove air bubbles.
5. Transfer the separated proteins to nitrocellulose (NC) paper for 1 hour at 250 mA (Bio-Rad power supply).
6. Incubate the NC paper in 5 % milk/PBS with 0.1% Tween 20 (PBST) for more than 1 hour in the cold room on a shaker.
7. Dilute the GFP antibody 1:2000 in 5 % milk in PBST. Incubate each strip of NC paper individually with 1 ml (plastic bag) or 5 ml (small container) of diluted primary GFP antibody. Incubate overnight at 4 ^{0}C on a shaker.

Day 4:

1. Wash blot three times with PBST, 5-10 minutes each wash.
2. Add secondary light chain specific goat antibody (Jackson ImmunoResearch, cat # 205-032-176) 1:10,000 diluted in 5% milk in PBST. Incubate 1 hour at room temperature on orbital shaker.
3. Wash three times with PBST, 5-10 minutes each wash.
4. Place the blot in plastic wrap. Add 750 µl of PICO (Chemiluminescent Substrate, cat # 34078) or FEMTO (Chemiluminescent Substrate, cat # 34096) per strip of NC paper. Incubate at room temperature for 5 minutes.
5. Tap off the solution. Place the blot inside a new sheet of plastic wrap. Tape it securely inside a cassette.
6. 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.

Reagent Preparation:

1. Prepare 1X RIPA buffer from 10X RIPA stock solution
   a. Add protease inhibitor tablets (Sigma Aldrich, cat # 11836145001)
   b. **Right before use** add PMSF (final concentration 0.5 mM)
   c. **Right before use** add DTT (final concentration 1 mM)
      1M DTT: Dissolve 1.54 g DTT in 10 ml PBS, aliquot (~110 µl), and store at -20 ^{0}C.
   d. **Right before use** add 25X phosphatase inhibitor cocktail:
      Mix 50 ml of 100 mM sodium pyrophosphate and 50 ml of 1 M sodium fluoride first and then add 100 ml of sodium orthovanadate (100 mM). Aliquot and freeze.
      - Sodium pyrophosphate (Sigma Aldrich, cat # 221368-100): Make 100 mM stock solution in water
      - Sodium orthovanadate (100mM, Boston BioProducts, BP-440)
      - Sodium fluoride (Sigma Aldrich, cat # S6776-100): Make 1M stock solution in water

2. Prepare 1X transfer buffer by mixing 100 ml 10X transfer buffer + 200 ml methanol. Bring the total volume up to 1000 ml with ddH_2O.