Western blotting

1. Make SDS + 10% polyacrylamide gels, running buffer (Maniatis recipes).

2. Load 15 µl sample per well. Run at 80 V until samples pass through stacking gel and enter resolving gel, then change to 100 V.

Check periodically for buffer leakage and add fresh running buffer to top of gel as needed.

3. Set up transfer in transfer buffer (Maniatis recipe).

4. Block for 1 hour in TBS-T + 4% milk powder.

TBS-T = TBS + 0.1% Tween-20

5. Probe overnight at 4°C. For insulin sensitivity, use Cell Signaling antibodies against *Drosophila* PO_4-Akt (#4054) or pan-Akt (#4691) and anti-syntaxin (DSHB 8C-3, as a loading control).

Dilute anti-Akt at 1:500. Dilute anti-syt at 1:10,000. Use TBS-T + milk as the diluent.

Can be reused several times. Save some blocking soln. to use for secondary antibody.

6. Rinse, then wash 3x in TBS-T.

7. Dilute secondaries 1:5,000 in TBS-T + milk. (= 3 uL each antibody + 15000 uL TBS-T-milk)

SECONDARIES are labeled “HRP anti-rabbit” and “HRP anti-mouse”

In GREEN BOX in the refrigerator

anti-Akt are raised in rabbit; anti-syntaxin are raised in mouse.

8. Incubate 1-1.5 hours shaking at room temp.

9. Rinse, then wash 3x in TBS-T.

10. Meanwhile, make up 2 ml per blot chemiluminescent substrate: 1 ml from each bottle

11. After blot is washed and substrate has come to room temperature, add substrate to blot and incubate for 5 minutes.

12. Drain off excess substrate and expose for 15 sec to 15 minutes on Chemi-Doc.