Western Blots

Westernblotting: p-perilipin A, pPKA, HSL, p-HSL, UCP1, beta-tubulin

Part I –Homogenization

(Extraction of protein from adipose tissues)

- Adipose tissues were immediately excised from sacrificed animals, minced, weighed and snap frozen.
- Later, frozen tissues were homogenized using Bullet blender
    - Homogenization buffer (for 2ml)
      100ul of 1M HEPES
      100 ul of 2M NaCl,
      1000ul of 20% SDS
      8ul of 0.5M EDTA
      1ul of 1M DTT
      721ul sterile H2O
      20ul of 100mM Benzamidine (make fresh: 6mg in 0.5 ml)

      ph to 7.4-7.5
      Add just before samples:
      83ul of Protease Inhibitor Cocktail (Halt, Freezer)
      83ul of Phosphatase Inhibitor Cocktail (Fridge) (not necessary for UCP1)

      Tissue to homogenization buffer ratio: 1:2 (e.g. 300mg:600ul).
    - Bullet blender
        - Add 1 scoop of zirconium oxide beads (0.5mm) to each tube BEFORE adding fat
        - Minced tissue should be placed in Eppendorf Safe-Lock tubes
        - To ~150-300 mg tissue, add 600 ul of homogenization buffer. (for RWAT 400 ul is enough)
        - SPEED =6, TIME = 1 minute. Press start

- centrifuge at 12,000 g for 5 min at 4C
    - SDS will precipitate if cfg for >5 min
    - The infranatant was carefully removed and re-centrifuged if the lipid from the fat cake supranatant got mixed into the infranatant fraction.
    - Use 1ml pipette to poke whole in fat cake and remove to the side or out, then use new 1ml pipette to remove infranatant.
- Repeat cfg step and remove infranatant
- Subsequently, the protein extracts were aliquoted (usually 3 tubes) and stored at -80 degrees. One tube (4 tubes total) was maintained with approximately 10ul for BCA protein content.

- BCA kit : Dilute samples 1: 10.

- Prepare reagent mix A: B, 50: 1, e.g. for 10ml: 10ml/51= 0.196 ml (of B)
- Standards come in kit: 2, 1, .5, .25, .125, .0625, 0 ug/ml
- Add 25ul of samples & std
- Add 200ul of reagent mix
- Incubate in 37 degrees for 30min before reading at 562 wavelength absorbance

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Preparation of Samples:

BCA	BCA * 10	10ug load per lane (e.g. for 3 lanes)	Loading buffer 6x (e.g. for 3 lanes)	H2O	1ug load per lane
.8547	8.55	A=10/8.55 *3	B=3.33*3	30- A-B	1/8.55

8.55 ug/ul is divided by 10 to get 10ug/ul; then multiply by # of lanes if pipetting 10 ul per lane

Final loading buffer conc should be 2X

Work on ice, and mix with pipettes to avoid bubbles

Part II (Westernblot)

Gel-cast:

- Prepare poly-acrylamide gels. The ratio for acryl:bis-acryl should be 10%:0.07% for resolving gel and 4%:0.028% for stacking. (This recipe appears to help resolve the proteins at the 55kD-70kD better, thus helping separate the unphosphorylated and phosphorylated perilipin A)

Denature samples (protein, loading buffer, H2O) for 8 min at 95 degrees, vortex, then denature for 8 min at 95 degrees then vortex again:

(Turn on machine 5 min before)
Achieves linearity of proteins; Take time, no ice necessary, protein already denatured

Loading samples:

- Load samples and ladder (lane 1: 10ul, lane 15: 1ul)
- 10ul samples for each lane, but different load
- Try to leave edge lanes without samples, in case of a bad run. If empty lanes, load empty lanes with loading buffer

We load ***10 ug*** total protein to immunoblot ***for p-HSL, HSL (82 molecular weight) and p-Perilipin A (pPKA-~ 62 molecular weight)***.
And load ***1 ug*** of total protein to immunoblot for ***Perilipin A(~ 62 molecular weight)***

(with 10ug, we get excess signal for the Perilipin A and the bands for each lane appear to merge with one another thus making it impossible to outline each band in a lane for density quantification)

UCP1 (~33 molecular weight): 5 or 10ug load

Beta- tubulin (~55 molecular weight): 10ug load

Electrophoresis:

- Run at **65 V for 3-3.5 hrs** or any other settings that best suits the user
- E.g. run at 120V for 1.5 h
- For UCP1 run for 45-60 min at 120V (smaller weight)

Transfer using semi-dry blot:

- Sit gels in Toubin's (Transfer) buffer for ~20min, separate from glass plate and remove excess gel (stacking gel)
- Label PVDF membranes (e.g. 'gel 1'), place in methanol for 10 sec, in dH2O for 10 sec and then transfer buffer for 20 min (Smooth surface up, positive charge)

- Place two filter papers soaked in Toubin's buffer on the transfer unit
- Over the filter papers, place the gel(s)
- Over the gel(s), place PVDF membrane (with smooth surface facing the gel)
- Finally, place two filter papers also soaked in Toubin's buffer over the membrane

Remove bubbles by running a tube over the filters

Transfer at ***constant voltage @ 5V for ~ 60min*** (optimization may require for protein content and size of the protein if transferring other proteins)

- Wash the membranes 2 X 10min in 1X TBS
- Dry membranes and **label ladders, lanes an gels & protein**
  (If you cannot block membrane on the same day as transfer then dry it completely before wrapping it in plastic bag and storing at room temperature or at 4 degree until blocking)

Part III: Immunoblotting:

(the following procedure can changed/adjusted to suit the user's need)

Day 1:

- Block for 2 hrs in 1XTBS included with 4% non-fat dry milk powder (w/v) and 0.3% Tween 20 with constant shaking (prepare fresh)
- Discard blocking solution
- After blocking, add primary antibody in 1XTBS included with 4% non-fat dry milk powder (w/v) and 0.3% Tween 20
  (reuse antibodies, make with 0.1% azide, store in fridge)
- Leave shaking gently at 4 degree, overnight (> 14h).

Day 2:

- Following day, wash 4X 5min in 1XTTBS buffer (membranes will stick to container when gently inverting and discarding solution)
- Incubate the membranes for 2 hours at room temperature in 1X TTBS supplemented with 0.2% non-fat dry milk powder and secondary antibody (GAR from BioRad, which is at 4 °C) at 1:1000 dilution, with gentle shaking
  Reuse 2nd antibody, make with 0.1% Sodium Azide

- At the end of the incubation, wash with TTBS 3X 10min

- Remove excess buffer from the membrane and add ECL substrate (Fridge) from the BioRad kit and incubate for 5 min at room temp.
- Remove the solution from the membrane by tapping on a kimwipe and cover the membrane with saran wrap or plastic bag to prevent it from drying.

- Finally, expose the membranes to whatever imaging method you please x-rays films or imaging camera apparatus (we use the latter)

Part III- Imaging:

Save the data in the hard drive under data folder in bartness lab sub-folder
Also copy data onto your flash drive or any other drives
There are directions for system in room on the wall

LAS 3000 Imager, Fujifilm, v. 1.1

Open software THEN turn on
System has to cool down to -30C

Acquiring image:

- When system is ready
- Adjust tray to position
  - 1 for 1 gel; 3 for 4 gels
- Exposure type: precision
- Auto exposure
- Senstitivity: standard
- Do not click 'image acquire and digitize'; leave off

Ladder:

Take ladder picture
Digitize
Epi
Set sensitivity to: normal
Pro
Precision
Focus and adjust brightness
Take picture
Save

Bands:

Take picture
Chemiluminescence
Set sensitivity to: normal
Pro
Precision
Focus and adjust brightness
Take picture
Save

Image analysis:

Open file in Multigauge
Play with image , if necessary
Select shape (object, square)
Adjust correct size and use same size for all bands, click copy for new object
Record 'AVG' (average after background substraction)

Part IV. Stripping Membranes

GM Biosciences
- -OneMinute Western Blot Stripping Buffer (GM6001, GM6002)
  - -follow manufacturer's protocol
  - -visualize blot before re-probing to make sure 1st Ab is stripped

Remove blot from pouch CAREFULLY
Rinse in TTBS, 1x, 10 min (can be increased if necessary)
1x Reprobe for > 2h
Dilute using dei H2O
Rinse 3x , 5 min each, TTBS

Block for 2h…(follow protocol)

Solutions:

1X TTBS

- 1M Tris pH 8.0 10 ml 10 mM final concentration
- 5M NaCl 30ml 150mM final concentration
- Tween 20 5 ml
- dH2O QS to 1000 ml

6X Loading buffer (mix, aliquot, store at -20 degrees)

- 1M Tris pH 6.8 5.4 ml rt = room temp
- Glycerol 7.2 ml rt
- SDS 1.54 g rt
- DTT 1.4 g -20 degrees
- 5% Bromophenol Blue 75 ul rt

Transfer buffer (a.k.a. Toubin's buffer)

- dH2O 400 ml
- Tris base 3.03g 25 mM final concentration
- Glycine 14.4 g 192 mM
- Methanol 200 ml 20%
- dH2O QS to 1000 ml

10X Running buffer

- dH2O 600 ml
- Tris base 30.3 g
- Glycine 144.0 g
- SDS 10.0 g
- dH2O QS to 1000 ml
- no need to pH; store at 4 degrees C; if ppt, warm to RT and mix before diluting

1X TBS

- dH2O 600 ml
- 1M Tris pH 8.0 10 ml
- 5M NaCl 30 ml
- dH2O QS to 1000 ml
- pH to 7.5