10/15/16 – Quynh Nguyen

Sample lysate prep for JURKATS (~ 10-20M cells/ml)

Wash cell w cold PBS twice

Add Lysis buffer + Protein inhibitor

Centrifuge in cold + Save supernatant

centrifuge for 4-5 min @1000 rpm

discard supernatant; add 3ml of cold PBS + mix

centrifuge for 4 min @ 1000 rpm

add 3ml of cold PBS + mix again

[take 10 uL sample + dye (ratio 1:1 or 1:5) -> count!

Avg. #cell/4box * dilution factor * 10000 = xyz Mcell/ml

Total #cell in sample = xyz * 3 (ml)]

centrifuge for 4 min @ 1000 rpm

add protease inhibitor (1X) to lysis buffer

[~ 100 – 150uL/ 10M – 15M cell/ml)

add Native lysis buffer + mix + incubate on ice for 15' (mix every 5 mins)

after incubation, centrifuge in cold room (4C) for 15min @ 13000rpm

save supernatant (store @ -20C if needed)

[*use BCA to check for concentration immediately and note on tube]*

[if protein is frozen, thaw at RT, then centrifuge at 15000 rpm for 2 mins to get rid of debris. In pure protein, no pallet should be seen]

Reduce & denature loading sample

Boil water first (*it takes forever!!)*

loading buffer = 90uL 4X Laemmli loading buffer + 10 uL of BME (*in the chem hood)*

add mixed loading buffer to protein sample (1:3) [ex: 12uL protein + 4uL of load)

[*you can dilute protein if needed. Sample as follow for 24uL rxn volume:*

5uL protein + 13 uL water + 6 uL loading (w BME)]

[generally, for WB:

- cell lysate: use min 30ug protein

- pure protein: use max 1ug]

boil sample for 2-3 min -> then allow to cool

{ *loading buffer include: SDS -> neg. charge; DTT -> reduce bridges ; glycogen -> increase density}*

prepare buffer +dye for empty wells (needs 20uL/ well => dilute 4X Laemmli with water to 1X, by ratio 1 dye : 4 water (or running buffer))

SSD Page (separate by weight)

prep running buffer 1X TRIS-GLYCINE (1L)

1L = 100mL 10X buffer (stock **with SDS**) + 900 mL water

remove gel from package: peel off tape at the bottom + remove comb on top

place gel cassette into assembly (short side inward)

(if only 1 gel is run, used buffer damp on other side)

fill buffer inside cassette -> wait & check for leakage

pipet ladder: 4uL; pipet dye buffer in empty wells

pipet samples in wells

fill the rest of chamber w buffer

close chamber, connect supply

run @ 100V for 10 -15 mins (low setting)

then run 200V for ~ 30 mins (hi setting)

***meanwhile,**

make transfer buffer:

2L = 1400 mL dd water + 400 mL methanol + 200mL (10X) TRIS-GLYCINE

Wash sponges in water to get rid of residue salt

Soak sponges in transfer buffer

Cut membrane according to filter paper

(*cut at corner to mark side of membrane)*

Prepare 3 weight boats for membrane (containing respectively from bottom up: methanol, water, &transfer buffer)

Prepare 2 boats for gel (containing respectively water & transfer buffer)

***2 minutes before gel is done:**

!!! Do not touch membrane with hand. Handle with tweezer only.

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Soak membrane in methanol for 20s (flip sides in between); then in water for 30s

Leave membrane in transfer buffer)

turn off; remove assembly + pour out buffer

remove gel cassette

(must clean container carefully to get rid of SDS!)

Membrane transfer

Assembly order:

Cassette 1-6sponges 2-5 filter paper

3gel 4nitrocellulose membrane

remove gel (lining arrow =on the key with the arrow on the cassette)

wash gel in water for 15 sec (submerge the side with gel under water to easily remove from case)

soak gel in transfer buffer for 10 mins

***3 min before gel done soaking:**

soak filter paper in trans. buffer w the sponges

assemble sandwich into box ☺

(*can add filter paper under the gel in the buffer boat to easily adjust gel on membrane*

No bubble !

Always: gel on black side of cassette and box)

fill with transfer buffer; add stir bars

run in cold room at 100V for 2h30'

Note:

black side of cassette down n buffer

avoid moving membrane once put on gel

Immunoblotting (antibody binding specificity)

Add SDS gel stain to blue box

Add TBS-T to black box

open cassette; black side in blotting buffer

remove layers; add gel to stain

add membrane in TBST (wash x2 for 2', then 1')

add membrane to blocking buffer (Ody. in 4C) for 1-2hrs or overnight -> antibody won't stick on membrane

(blocking can be reused for next time)

wash for 5 mins with TBS-T buffer

***meanwhile, make primary antibody (20mL/membrane** = 10mL blocking + 10 mL TBS-T + 10uL antibody**)**

*(1°antib. is in -20C; usually need **1ug/mL** antib.)*

add primary antibody (stick the protein)

leave overnight rocking in cold room

(alternatively, leave at RT for 4-5 hours)

pour & save 1° antib. in a conical tube stored at 4C

wash x3 (10 mins each) with 15mLTBS-T

*** **meanwhile, make 2nd antib** (20mL/memb):

= 10 mL blocking + 10mL TBS-T + 1.5 uL antib

Mix well (*2°antib. is in 4C fridge & **light sensitive)***

add 2nd antibody (specific for primary antibody, has a reporter enzyme); incubate 50' rocking at RT

wash x3 (10' each) with 15mL TBS-T

wash last time with 20mL TBS (no Tween = no detergent)

Detection

Pick membrane from box and rest protein face down of glass surface (the ladder or the cut corner: on right); Close the top of machine

Wait and adjust brightness, etc if needed > Export

Washing procedure 1: after transfer,

→ TBS-T x 2 (2', then 1') → blocking (1-2hr)

→ TBS-T (5') → 1° antib (overnight)

→ TBS-T x 3 (10' ea.) → 2° antib (50')

→ TBS-T x3 (10' ea.) → TBS → scan

Or washing procedure 2: after 1° antib. :

→ TBS-T x3 (50mL – 10' ea.) → TBS x1 (50mL – 5')

→ 2° antib for 45' (15mL blocking + 15mL TBS-T + 2^0 antib)

→ TBS-T x3 (10' ea.) →TBS x1 (5') → scan