ELISA protocol 20160601 TY

Seed cells to a filter plate

1. Prepare a filter plate and a separate plate for collecting 'leaked out' media.
2. Cover the plate with a film and expose wells-in-use/get a lid for the plates.
3. Prepare cells at $1x10^6$ cells/mL (log phase between $0.65\text{-}0.85x10^5$ cells/mL.)
4. Seed 750,000 cells in 750uL to the filter plate.
5. Treat cells (see table) in the filter plate and incubate at 37C for 2 hours.
6. Meanwhile, prepare lysis buffer, peptides, and the neutravidin plate.\
7. Lysis buffer: PhosphoSafe + 4mM EDTA + 1X protease inhibitor.

Treatments/exp groups
Cells+inhibitor+biosensor
Cells+inhibitor
Cells+biosensor
Cells only

Prepare the neutravidin plate (capacity 15 pmol)

1. Add 100uL TBS-T + 1% BSA to each well that will receive lysate
2. Be sure to have systemic positive control (100uL TBS-T + 1% BSA + 5uL of 1mM phospho-peptides)and negative control (100uL TBS-T + 1% BSA + 5uL of 1mM unphospho-peptides)

Treat cells with peptide biosensors

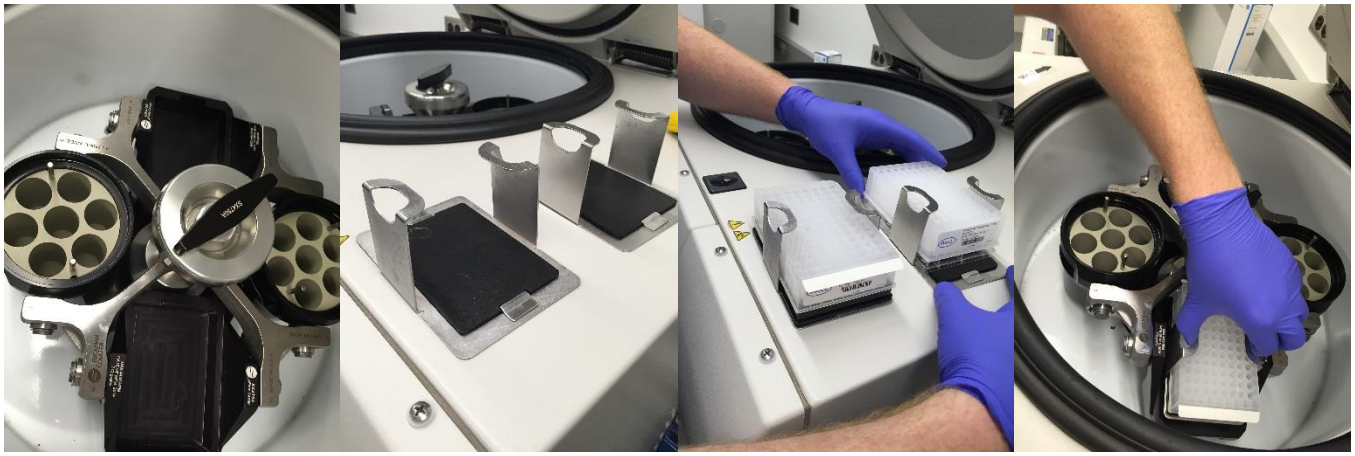
1. Prepare biosensor at 40uM, add 50uL to get final concentration of 2.5uM
2. Place the plate back to the shaker in the incubator and shake for 5 minutes.
3. Set up plates and the manifold in the hood as shown below.
4. Place the filter plate on the vacuum manifold.
5. Turn on vacuum to remove media. Subsequently, vent and release the plate (see video 1.)
6. Stack the filter plate on the neutravidin plate.
7. Add lysis buffer as shown in the video 2.

Lyse cells on a shaker (Video 3)

1. Weight the filter plate and the neutravidin plate together on the scale.
2. Use a rubber band to hold a deep well plate and a regular plate together to make a balance for shaking. Use a rubber band to hold the filter plate and the neutravidin plates together and shake for 5 min at speed 500. (Video 4)
3. Meanwhile, prepare another set of balance plate for centrifugation. Take another two plates and use a squeeze bottle to add some water until the weight matches the number that is obtained in step 1.

Collect lysate

1. Take the "filter plate + neutravidin plate" set and the centrifugation balance set to the cell culture room.
2. Use adaptors and centrifuge the plates at 1500 *xg* for 5 min.



Bind peptides to the neutravidin

1. Remove the filter plate and leave only the neutravidin plate.
2. Measure volume in the neutravidin plate. Usually 130-140uL is normal (100uL TBST + 50 uL lysis buffer)
3. Shake plate for 1 hour at speed 500.

Wash, add antibodies, and developing the plate

1. Wash plates with 200uL TBS-T, 3 times.
2. Between washes, flip and plate to dry (video 5)

3. Dilute 4G10 1:12,000 in TBS-T+1% BSA. Add 100uL to each well.
4. Shake 1 hour
5. Wash plates with 200uL TBS-T+1% BSA, 3 times.
6. Add anti-IgG HRP 100uL (1:1000 in TBS-T+1% BSA)
7. Shake 1 hr, meanwhile make developing buffer (Na2Po4 buffer 7.4 + AmplexRed+H2O2 see below.)
8. Wash plates with 200uL TBS-T+1% BSA, 3 times.
9. Wash plates with 50mM Na2PO4 phospho-buffer (pH7.4) twice. (This is a base solution used for equilibrate pH and remove detergent)
10. Add 100uL of developing buffer
11. Shake 30 minutes
12. Read plate using the AmplexRed ELISA protocol (excitation 532 emission 590)
13. Clean: bleach the vacuum manifold and chase with water

developing buffer	
Phospho-buffer: 50mM Na2PO4 pH 7.4	493.5uL
H2O2 (1:100 rom the 30% stock)	4uL
AmplexRed (10mM stock)	2.5 uL

Where to find the reagent & the plate?	
Neutravidin plate	4 degree shelf 2-4
BSA	4 degree 1-5 bottle, weight it out fresh every time
AmplexRed (10mM stock)	-20 box A 1-1 black tubes labeled with AR
Phosphor-safe	-80 III 1-1 box 1
Protease inhibitor 50x	-20 A box 2-1 (box big steve)
4G10	4 degree green-yellow hinged box
HRP-IgG	4 degree Shelf 2-1