Western Blotting

I. Reagent Preparation

0.5M EDTA solution:
0.5/1000*250=0.125 mole
0.125 mole * 292.24g/mole =36.53 g
Dissolve 36.53g EDTA powder in 250ml MilliQ water

Sucrose Homogenization Buffer for Lysis:
10.953g sucrose
1ml 0.5M HEPES buffer(500ul if original concentration 1M)
0.2ml 0.5M EDTA
Add MilliQ water to 100ml Total Volume
→Filter the insoluble component away
Caution: add to 80ml first to dissolve sucrose. After sucrose totally dissolved, then add to 100ml. Sucrose will change the volume!

Lysis Buffer:
1pill protease inhibitor + 1 pill phosphatase inhibitor →10ml Homogenization Buffer
Caution: Stored in 4C no more than 7 days OR aliquot and store in -20C

0.2% TBST buffer:
500ml TBS(Tris-Buffer-Saline)
1ml Tween20

1000ml Transfer buffer:
100ml 10x Tris-Glycine
200ml methanol
Add MilliQ water to 1000ml Total Volume
Caution: Cool down overnight in 4C or coldroom before use

500ml Running buffer:
50ml 10x Running buffer
Add MilliQ water to 500ml Total Volume

Coomasie Blue Dye:
2% Brilliant Blue R (2g)
40% Methanol (40ml)
10% Acetic Acid (10ml)
Add MilliQ water to 100ml Total Volume

Destain Solution:
10% Methanol (100ml)

5% Acetic Acid (50ml)
Add MilliQ water to 1000ml Total Volume

II. Sampling

- Label the EP tube with sample name and date
- Wash cells with PBS(to wash away dead cells and debris)

Caution: Use PBS special for cell culture! PBS gently flows along the wall instead of directly hit cells!

- Remove PBS
- Add 200ul lysis buffer/well(6-well plate)

Caution: After adding lysis buffer, put plates on ice

- Use scraper to get all cells off
- Collect them into EP tube
- Sonicate each sample 10 times

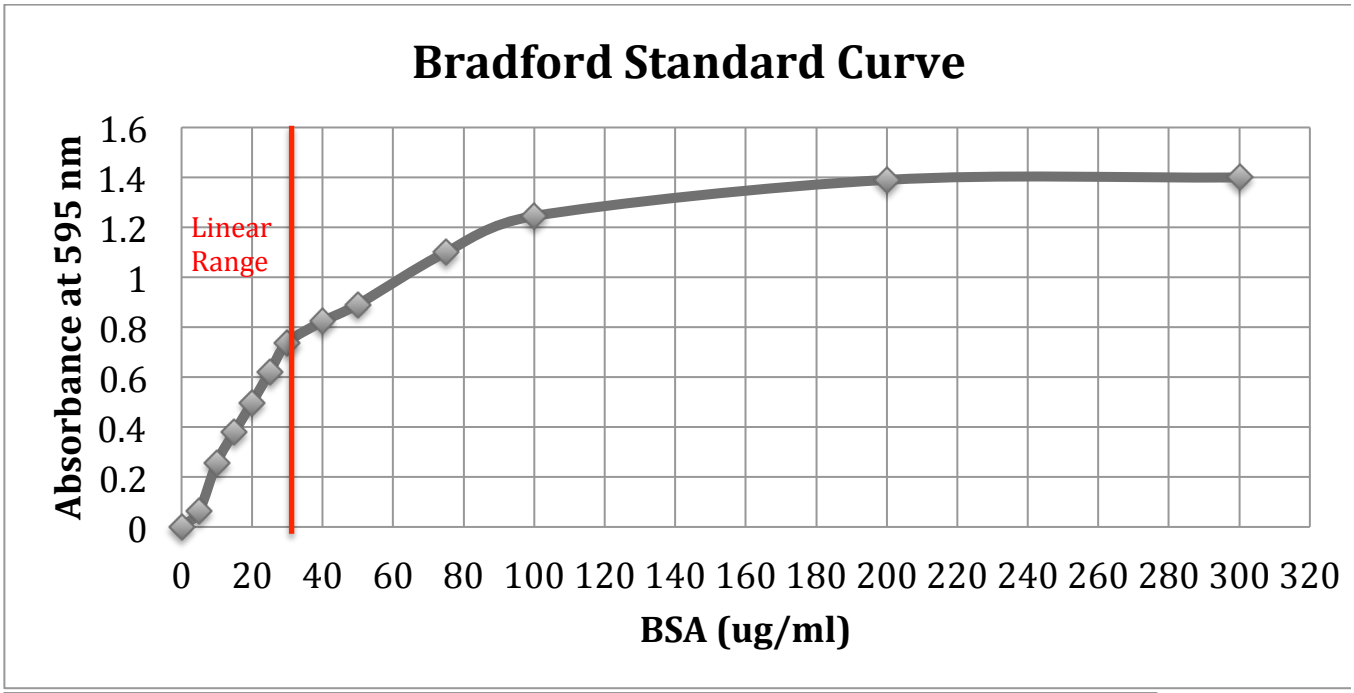
Caution: if sample is still sticky, sonicate 15 times

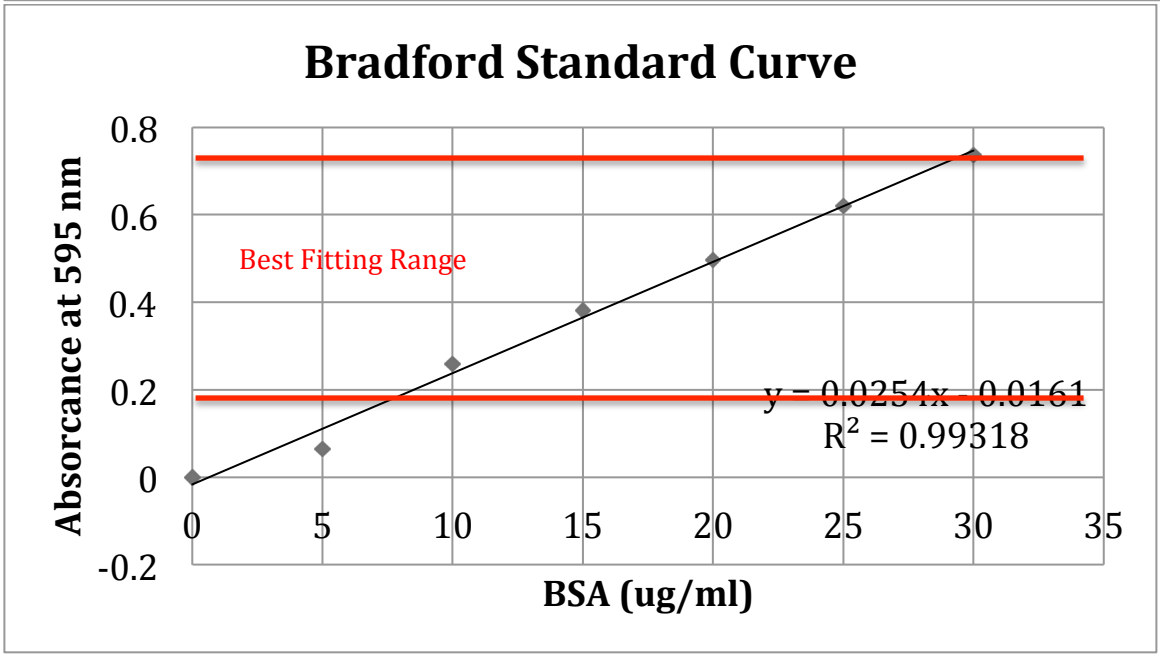
- Store at -80C

III. Bradford Assay

Principle: Binding between protein and Coomassie dye→Absorbance shift at 595nm

Shortcoming*:*
(1) Detergent (SDS and Triton) affects the binding
(2) Most readily bind to arginyl and lysyl residues, leading to variation when quantifying specific protein
(3) dye does not bind to peptide smaller than 3000Da
*(4) Short linear range (**Absorbance range from 0.2 to 0.7 is the best fitting range**)*





Stock BSA conc=1ug/ul
BSA volume=[Standard Point Conc(ug/ml)/1000]*200ul/1(ug/ul)

Standard curve:
Each Standard Point repeat 3 times

Standard Conc(ug/ml)	1ug/ul BSA volume(ul)	Bradford Reagent volume(ul)	ddH2O volume(ul)
0	0	40	160
5	1	40	159
10	2	40	158

15	3	40	157
20	4	40	156
25	5	40	155
30	6	40	154
49	8	40	152

(1)Add ddH20 to each well
(2)Add BSA to each well

Sample measurement:
Each Sample repeats 3 times
(1)Add 100ul ddH2O to each well
(2)Add 5ul protein sample to each well
(3) Make master mix of diluted Bradford reagent(40ul Bradford reagent + 55ul ddH2O)*N (N depends on how many sample you have, for example with 10 samples, N=10 *3 +1)
(4) Add 95ul **master mix** to each well **of Protein Sample**
(5) Add 40ul **undiluted** Bradford reagent to each well **of Standard Curve**
(6) Mix each well, **don't reuse any tips**
(7) Measure the absorbance at 595nm
(8) Calculate and make the sample for western blotting (40ug protein/40ul)
For example: x ul protein sample + (20-x)ul lysis buffer + 20ul Laemmle sample buffer= 40ul total volume
Cation: 50ul betaME/950ul laemmli sample buffer need to be added before use!
(9) Thermo Mixer at 37C 300rpm for 10min
(10) Stored at -20
(11) Before SDS-PAGE, thaw the samples at RT and then thermo mix at 37C for 5min

IV. Making Gel

Amount for 4 gel

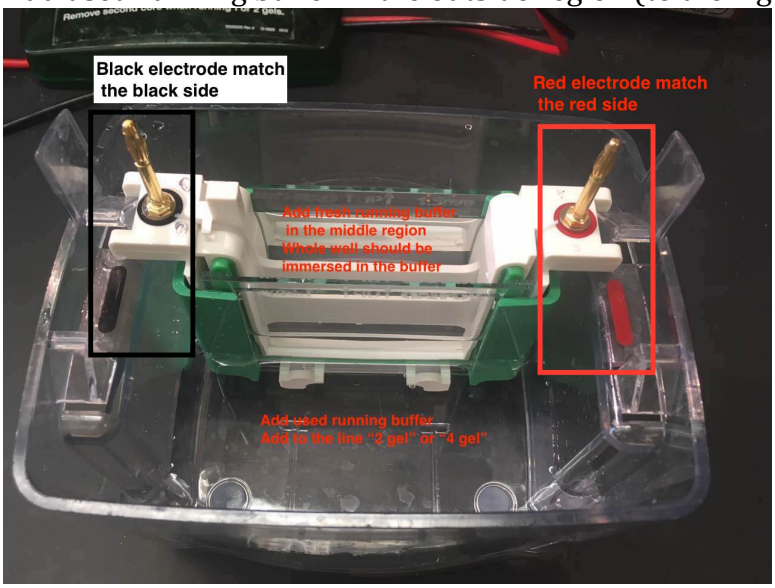
10% Lower Gel			Stacker (Upper Gel)	
Protogel	12.5ml		Protogel	2.6ml
1.5M Tris(pH8.8)	9.375ml		1.5M Tris(pH6.8)	2.5ml
20%SDS	187.5ml		20%SDS	200ul
H2O	15.4ml		H2O	14.5ml
10%APS	375ul		10%APS	200ul
TEMED	37.5ul		TEMED	20ul

Make lower gel first
Add isopropanol quickly
Caution: Avoid any bubbles, especially between lower gel and upper gel!
Wait for around 15min until the solidification of the lower gel
Clean the isopropanol out
Make upper gel

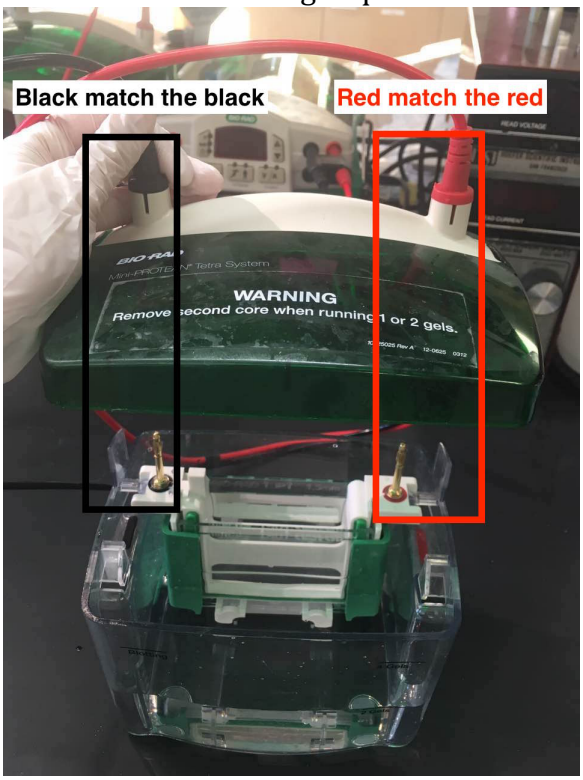
Insert comb
If the gels are not used right now, immerse in running buffer, store at 4C

V. SDS-PAGE

Assemble the equipment
Add **new** running buffer in the middle region between two gel
Add used running buffer in the outside region (to the 2 gel or 4 gel line)



Load sample and ladder in an **unsymmetrical** arrangement
Write done the loading sequence in the notebook



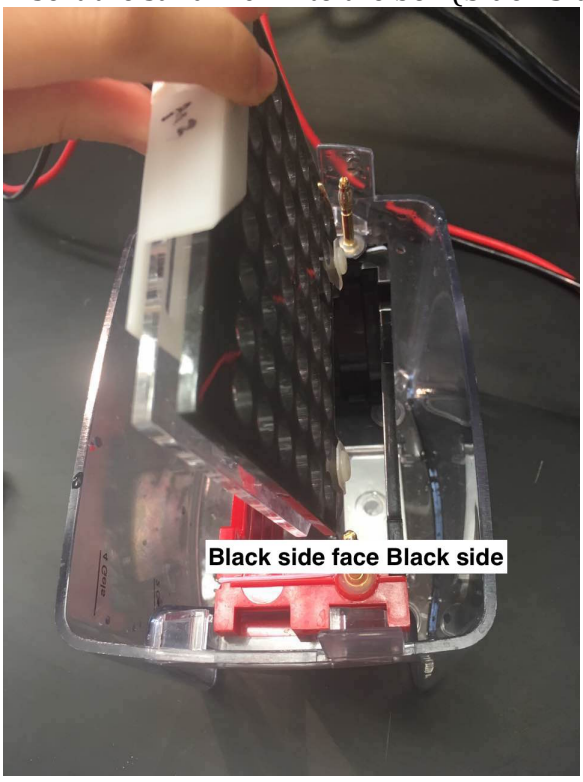
80mV 20min
130mV until the blue line running out of gel

Total time: around 1.5 hr

VI. Transfer

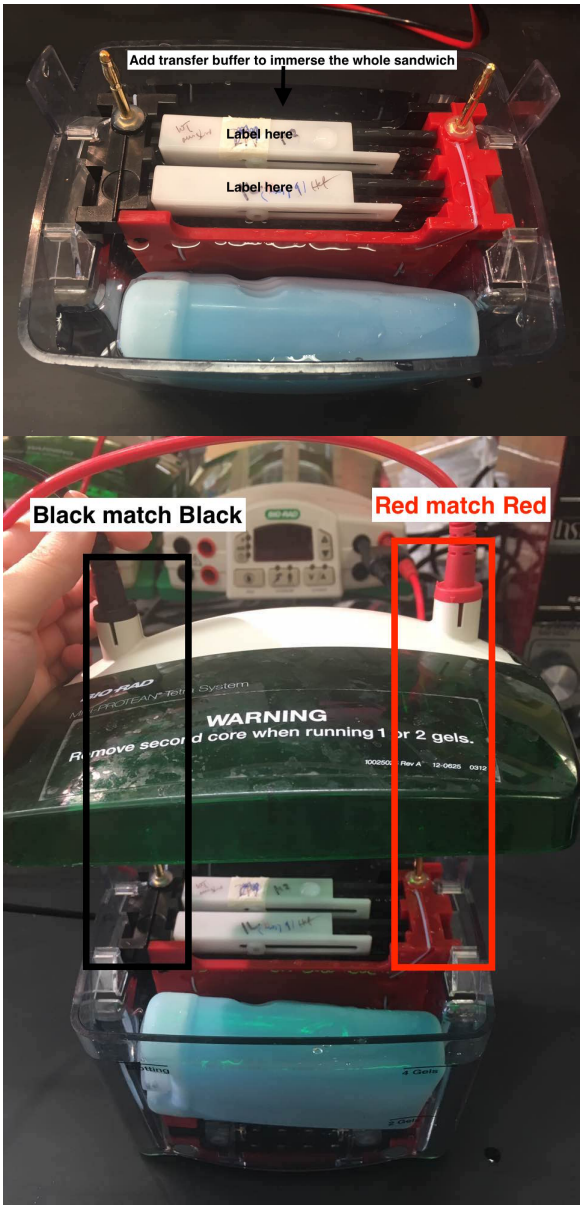
Use running water to wash away the remaining SDS in the gel
Immerse the black sponge, filter paper, nitrocellulose membrane and gel into transfer buffer for at least 5min
Arrange them in the following order:
(Black side)Sponge-filter paper-gel-nitrocellulose membrane-filter paper-Sponge(Transparent side)

Caution: Avoid bubbles!
Insert the sandwich into the box (**black side face black side**)



Add **fresh** transfer buffer

Put an ice block inside



Transfer at 100mV for 1hour

VI. Blocking and incubation with primary and secondary antibody

Stain with Ponceau S for 10 min
Continuously wash with MilliQ water for 3 times
Image the total protein amount
Add blocking buffer

10ml Blocking buffer:

- 5ml commercial blocking buffer
- 5ml 0.2%TBST

5ml for each membrane

blocking for 1hour

1:2000 anti-HA antibody (5ul) + 1:5000 anti-GAPDH antibody(2ul) diluted in 10ml Blocking buffer
5ml for each membrane
Incubate overnight

Wash with 0.2%TBST 3 times each 10min
1:10000 anti-mouse antibody (1ul) diluted in 10ml Blocking buffer
5ml for each membrane
Incubate at RT for 2 hours

Wash with 0.2%TBST 3 times each 10min
Image the membrane by Licor machine

VII. Coomasie Blue Staining

Stain with Coomasie Blue dye for 30min
Wash with new Destain Solution every 30 min
Before leave, add new Destain Solution and let it wash overnight
Continuously wash for two or three days for clear bands

VIII. Troubleshooting with SDS-PAGE (Gel making, Loading, Pipetting)

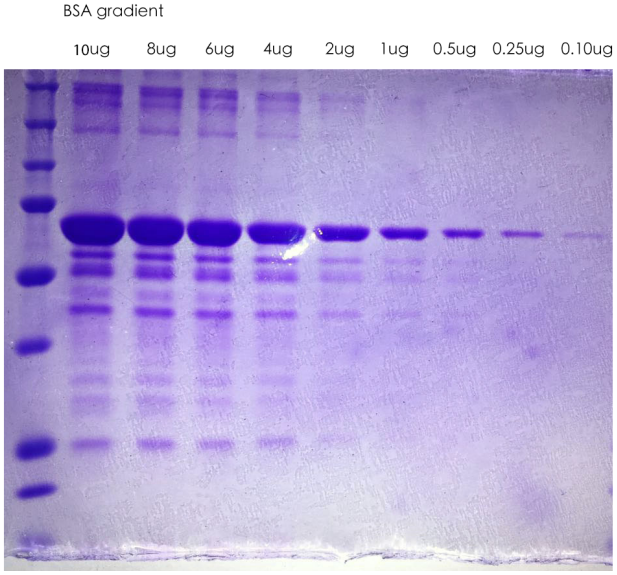
BSA amount for loading (ug)	4ug/ul BSA volume	MilliQ water volume	Laemil buffer(50ul betaME to 950ul laemil buffer)	Loading volume
10	5	5	10	10
8	4	6	10	10
6	3	7	10	10
4	2	8	10	10

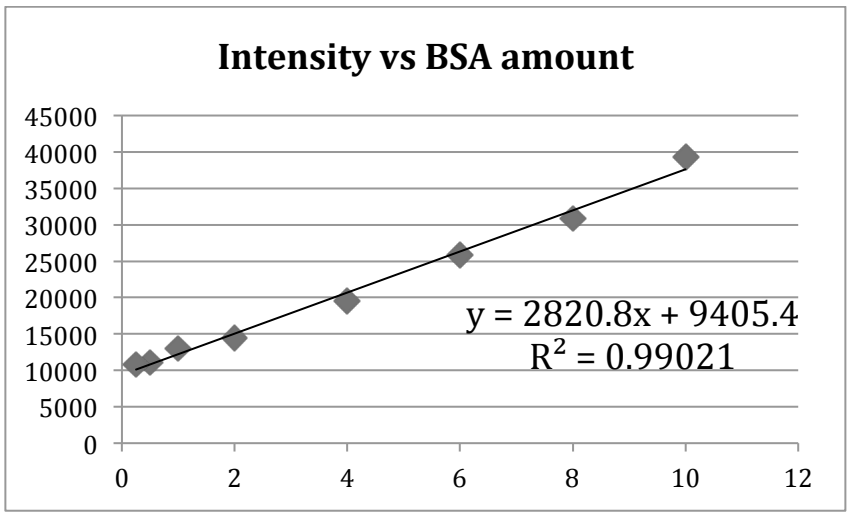
BSA amount for loading (ug)	0.4ug/ul BSA volume	MilliQ water volume	Laemil buffer(50ul betaME to 950ul laemil buffer)	Loading volume
2	10	0	10	10
1	5	5	10	10
0.5	2.5	7.5	10	10

BSA amount for loading (ug)	0.1ug/ul BSA volume	MilliQ water volume	Laemil buffer(50ul betaME to 950ul laemil buffer)	Loading volume
0.25	5	5	10	10
0.1	2	8	10	10

Thermo mixer at 95C for 10min
Spin down
Run SDS-PAGE
Coomasie Blue staining

Anticipated Outcome:





IX. Troubleshooting with Bradford Assay and Whole Procedure

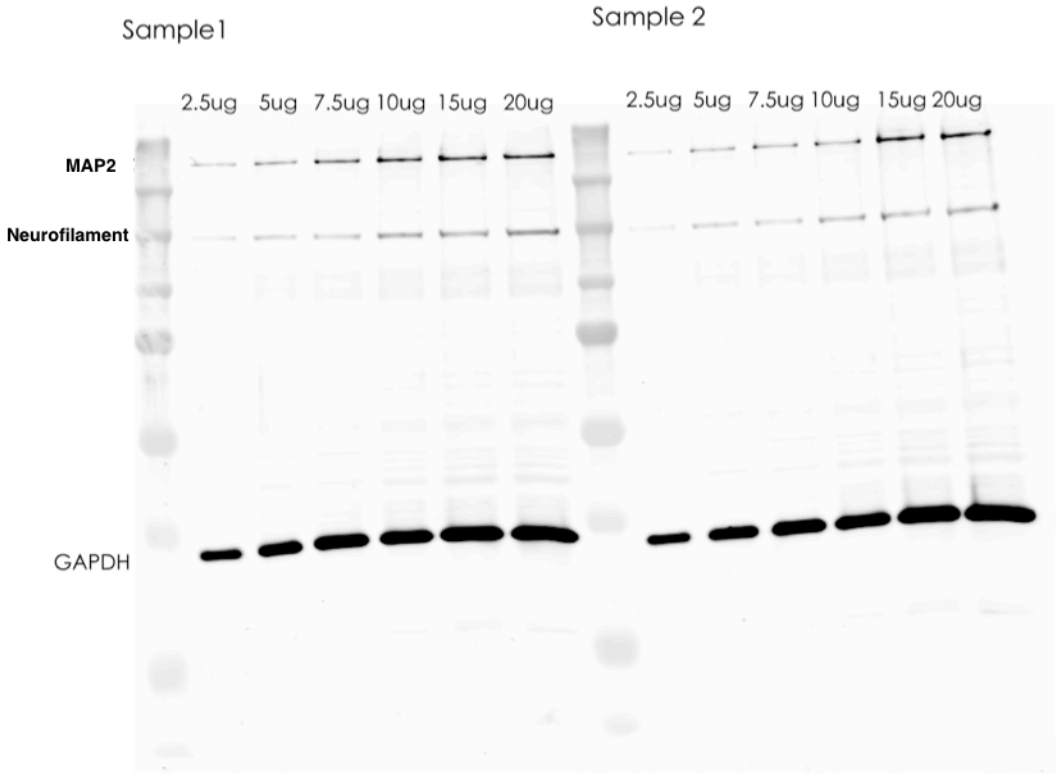
Use brain tissue sample 1 and 2 from Yousuf
Follow Bradford procedure to make 1ug/ul protein sample of total volume 80 ul

Load sample 1	2.5 ul	5 ul	7.5 ul	10 ul	15 ul	20 ul
Load sample 2	2.5 ul	5 ul	7.5 ul	10 ul	15 ul	20 ul

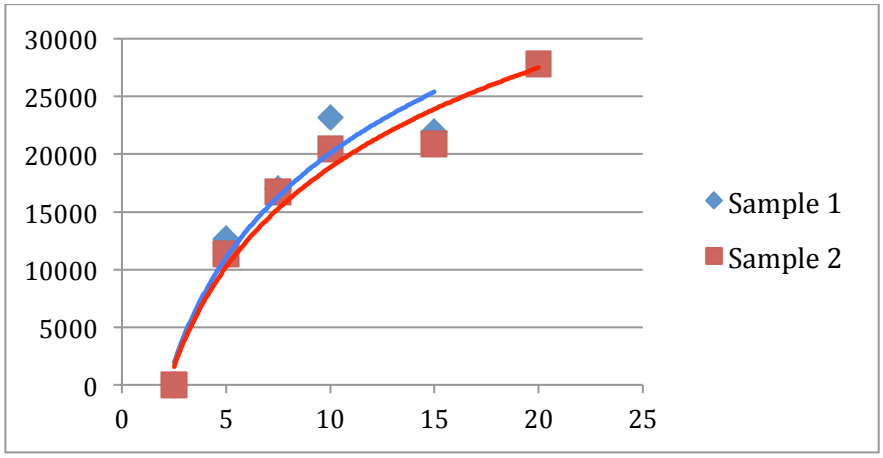
Continue with SDS-PAGE, Transfer, Ponceau S staining and blotting
Blot with primary antibody:
1:5000 anti-GAPDH Mouse 37kDa
1:1000 anti-MAP2 Rabbit 220kDa
1:2000 anti-Neurofilament 150 kDa

Blot with secondary antibody:
1:10000 anti-Rabbit 680
1:10000 anti-Mouse 680

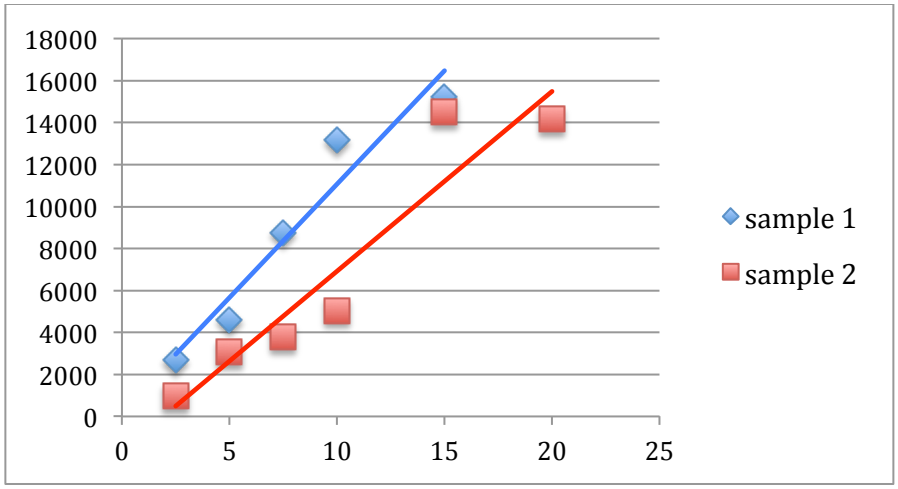
Anticipated Outcome:



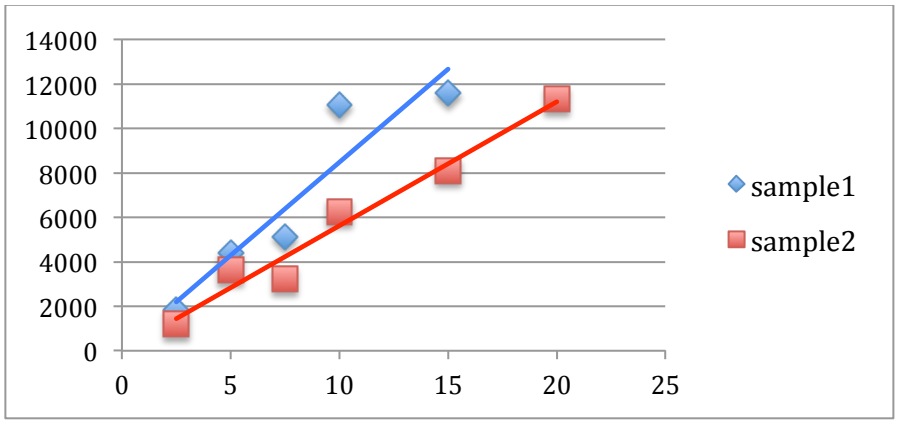
GAPDH:



MAP2:

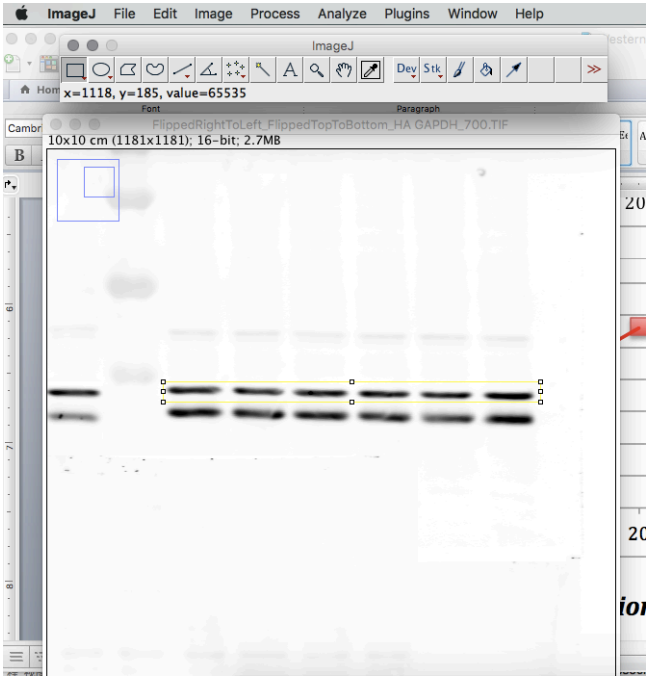


Neurofilament:

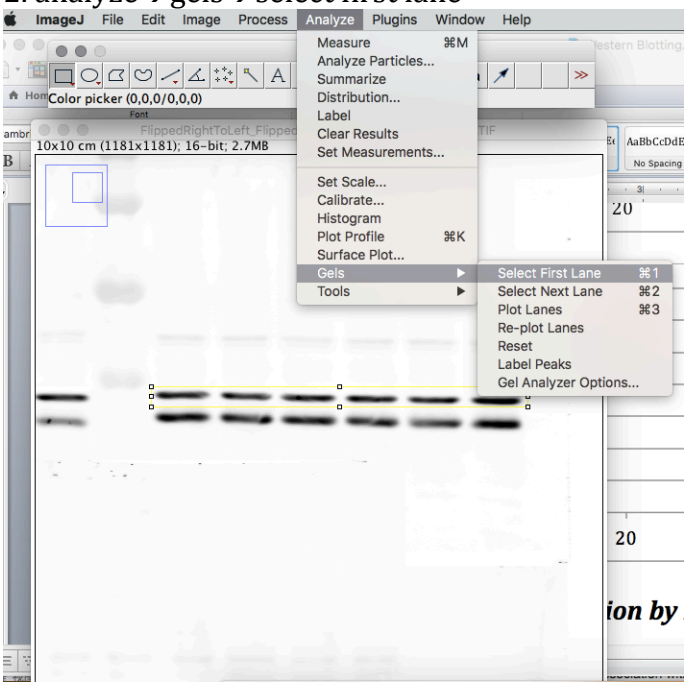


X. Western Blotting Quantification by Image J

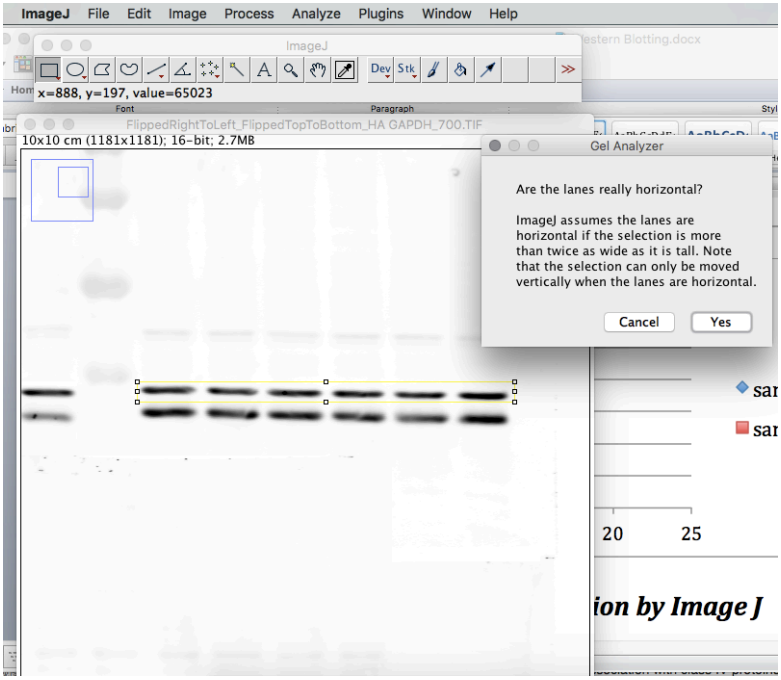
1. Select horizontal lane



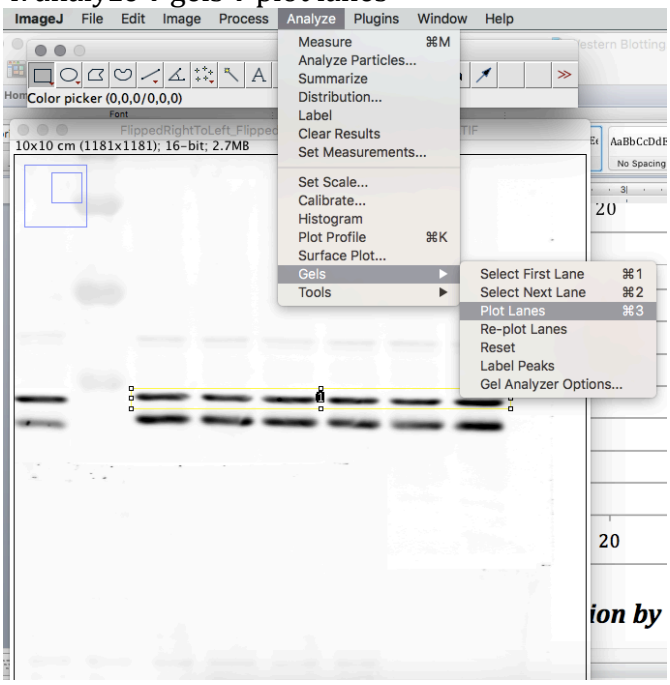
2. analyze→gels→select first lane



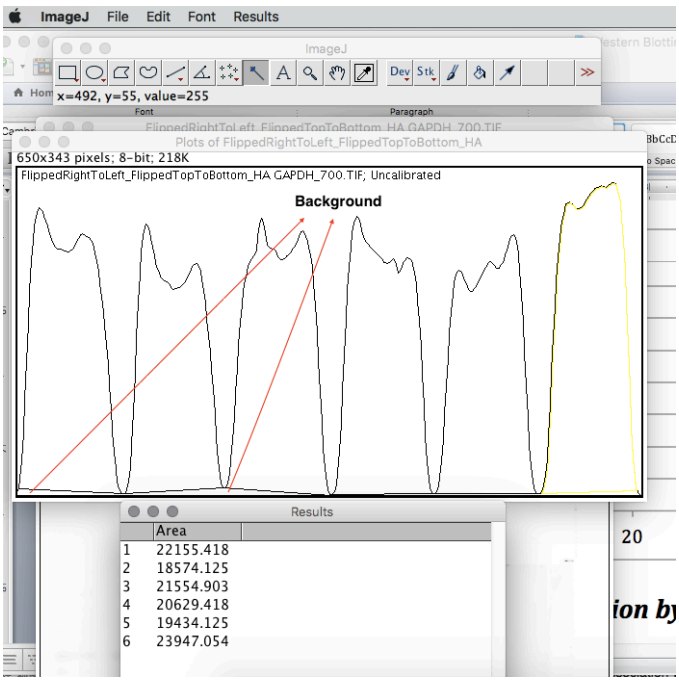
3. click yes



4. analyze→gels→plot lanes



5. Subtract background and use the magic rod to get value of intensity



6. Normalize the intensity with loading control

For example:

	HA Intensity by Image J	GAPDH intensity by Image J	Normalize factor	Noramalized Intensity
	x	y(i)	y(i)/y max	x/normalize factor
wt 0hr	19609.175	21893.589	0.912979692	21478.21596
wt 0.5hr	23694.125	22908.953	0.955321161	24802.26124
wt 1hr	23867.953	22190.196	0.925348435	25793.47638
wt 2hr	21002.296	23980.368	1	21002.296
wt 4hr	16366.418	21252.368	0.886240278	18467.24687
wt 6hr	17181.125	22384.296	0.933442556	18406.19424