Sive Lab Protocol
March 2007

Western Blotting

Prepare samples:

For zebrafish embryos:

Option 1:
Take live embryos and put into 1.5 ml tube with E3.
Centrifuge gently for 1-2 minutes
-yolk lipids will rise to the top
-remove supernatant and add sample buffer to embryos and homogenize.

Decide if you need protease/phosphatase inhibitors and add accordingly.

Option 2:
Freeze samples on dry ice/EtOH or in liquid nitrogen.
Thaw and add lysis buffer with protease inhibitors added. (lysis buffer should be cold)

TrisHCl pH 7.4	1ml
EDTA 0.5M	0.4ml
NaCl 5M	3ml
Brij 96 10%	8.75ml
NP40 10%	1.25ml

Bring up to 100 ml with H2O

Add protease inhibitors and keep on ice after this point

Leupeptin 1:1000
Aprotinin 1:1000
PMSF 1:1000

Add to tissue and homogenize.
After samples are in solution centrifuge in cold for 10 minutes at full speed
Remove the supernatants which contain your sample and keep on ice.

Perform protein assay if desired.
Add 2X SDS sample buffer and proceed.

SDS sample buffer (From Zebrafish book)

0.63 ml 1MTris-HCL, pH6.8
1.0 ml glycerol
0.5 ml Beta-mercaptoethanol
1.75 ml 20% SDS
6.12 ml H20
(10 ml total)

add 0..25% bromophenol blue
-store at -20 in aliquots

Pour gel

Set up Biorad (or desired) gel apparatus.
Be sure glass is sealed to prevent leakage.
-pour separating gel (see recipe for desired percentage) using a glass pipet along edge to prevent bubbles from forming in the gel.
-Gently overlay with EtOH and allow to solidify.
-Rinse with H2O and pour stacking gel.
-Insert desired comb size into stacking gel (be careful of bubbles) and allow to solidify.
-remove combs, set up running apparatus, and fill with running buffer.

Gel Loading/Running

-Take prepared samples including marker proteins
-heat prior to use (heating will prevent/remove precipitates caused by SDS)
-Load samples into well using gel loading tips.
-Set voltage low for the first 20 minutes ~60 Volts, until samples are through the stacking gel layer and lined up evenly at the edge of the separating gel.
-Turn up Voltage to 90-100Volts. Watch sample dye from and stop gel from running once the dye from is at the desired distance through the gel.

Transfer set up

-Before setting up transfer sandwich cut 3MM paper and PVDF to size of gel.
-Wet PVDF in MeOH, rinse with H20 and soak in transfer buffer.

Soak everything in transfer buffer before putting into sandwich.
Make transfer sandwich as follows
-Sponge
-2 pieces 3MM Paper
-Gel(s)
-PVDF
-2 pieces 3MM Paper
-Sponge

Put sandwich into transfer box, set up voltage box, fill with buffer to completely cover membranes, and run (in the correct direction!!)

Transfer for 1 hour at 100 Volts, or 70-80 for 2 hours, or lower to transfer overnight. (Best to do transfer in the cold room to prevent heating of samples.)

After transfer take apart sandwich and wash PVDF membrane 2X in TBST
-cut corner of PVDF membrane to remember orientation of gel/samples

Blocking and primary antibody incubation

-block in 5% skim milk in TBST for 1 hour at room temperature (or in block desired for best results with your antibody)

-incubate in plastic seal-a-meal bags in primary antibody at desired concentration.
- -seal 3 edges,
- -add ~3ml of fresh block solution with primary antibody added
- -add membrane
- -seal without bubbles!

-leave overnight at 4 degrees on shaker

Staining Gel in Coomassie Blue

-carefully put gel into stain for 2 hours on shaker
-destain overnight (see recipes for stain)

Secondary Antibody incubation

After overnight incubation wash 3X in 1XTBST for 30 minutes each
Incubate in the desired concentration of secondary antibody at room temperature in seal-a-meal bag for 2 hours
Wash 3X in 1XTBST for 30 minutes each

Developing Western

-Set up seal-a-meal bag for chemiluminescence reaction with ECL.
-add 1 ml of solution 1.
-add 1 ml of solution 2 and mix well.
-incubate membrane in solution for 1 minute.
-remove and place membrane between two sheet protectors and seal to keep blot moist and flat without bubbles.
-put into film container and add film in dark room.
-expose blot for required amount of time/check initially after 3 minutes and expose accordingly.
-develop in machine on 3^{rd} or 5^{th} floor.
-put blot back in 1XTBST to store in seal-a-meal bag at 4 degrees for stripping and/or reprobing experiments.

Western Recipes

For two THICK 12 % mini-gels

12 ml	30% acrylamide/bis
7.5 ml	4Xtris/SDS pH 8.8
10.5 ml	H2O
100 µl	10% APS
20 µl	TEMED

For Stacking gel

1.95 ml	30% acrylamide/bis
3.75 ml	4Xtris/SDS pH 6.8
9.15 ml	H2O
150 µl	10% APS
30 µl	TEMED

Block in 0.25% gelatin in TBST

200 ml TBST with 0.5 g gelatin
microwave to dissolve gelatin and cool, should be clear

10X Running buffer

30 g	Tris
144 g	glycine
10 g	SDS

Do not need to pH, should be about 8
Up to 1 liter

Recipe for 10X TBST (for 1 liter)

100 mM Tris Cl pH 7.5	1M Tris	121.1 g
150 mM NaCl	1.5M NaCl	87.66 g
0.1% Tween 20	1% Tween	10 ml

For three liters of 10X TBST

363.3 g	Tris Cl
262.98 g	NaCl
30 ml	Tween 20

up to 3 liters and pH to 7.5 (will take about 100 ml of 10M HCl)

1X BCA lysis buffer with phosphotase inhibitors

25 mM	Tris pH 8.0	250 µl of 1M solution
2 mM	EDTA pH 8.0	20 µl of 0.5 M solution
10%	glycerol	1 ml
1%	Triton-X 1000	100 µl
2 mM	Na3VO4	100 µl of 0.2 M solution
20 mM	NaF	100 µl of 2 M solution
		Up to 10 ml with H2O

5X BCA lysis buffer with phosphotase inhibitors

25 mM	Tris pH 8.0	1.25 ml of 1M solution
2 mM	EDTA pH 8.0	100 µl of 0.5 M solution
10%	glycerol	5 ml
1%	Triton-X 1000	500 µl
2 mM	Na3VO4	500 µl of 0.2 M solution
20 mM	NaF	500 µl of 2 M solution
		Up to 10 ml with H2O

2X Sample Buffer for Protein Gels

25 ml	4X Tris Cl/SDS pH 6.8
20 ml	glycerol
2.5 ml	β-mercaptoethanol (weigh on good balance)
50 mg	bromophenol blue
4 g	SDS

Up to 100 ml with H2O (52.5 ml)

4X TrisCl/SDS

6.05 g Tris base in 40 m (pH 6.8)
Filter 0.45 micron
H20 to 100 ml
0.4g SDS

4XTrisCl/SDS
91g Tris base 300ml (pH8.8)
H2O to 500ml total
Filter 0.45 micron
2g SDS

Coomassie Blue Stain
200ml acetic acid
1800 ml H2O
0.5g Coomassie blue G-250
Mix 1 hr and fliter (No. 1 Whatman paper)
Store at room temp
(final = 0.025% Coomassie blue with 10% acetic acid)

1X Transfer Buffer (4 liters)
12g Tris
57.6g glycine
800ml MeOH
H20 up to 4 liters