WESTERN PROCEDURE

EXTRACTIQN:
Using DLD cell, 6-well plate, 90-100% confluent
1. Aspirate media off cells and wash with I-IBSS w/25mM HEPES, pH 7.4
2. Aspirate HBSS off cell is, add different drugs
3. After each time point, aspirate drug off the cell
4. Wash with PBS, aspirate PBS off the cell
5. Add l00ul of lysis buffer (lcc lysis buffer with l0ul pmsf added prior to use, keep on ice)
6. Scrape cells, transfer to eppendorf tube (keep on ice)
7. Centrifuge at 10,000 rpm, 1 min, at 4°C
8. Transfer the supernatant to new eppendorf tube, sore at -70°C until analysis

PROTEIN DETERMINATION:
1 Add 200u1Bio-Rad reagent to a 5cc test tube
2. Add 795ul H_2O and mix
3. Add 5ul sample and mix
4. Transfer to a spec. micro-cuvette and rend a 595nm
5. Calculate protein conc. using WESPROT program on Hector's iBM
(This program will calc. protein conc. (ug/ul), in sample, and [volume needed to load between 25ug to 5Oug protein onto a get)

SAMPLE TREATMENT:
1. Transfer appropriate vol. of sample (vol. to yield 40ug protein) to an epp. tube.
2. Add 1-l0ul H2O to sample to dilute to volume of l0ul.
3. Add l0ul of2X treatment buffer (prepare by adding 10% BME prior to use) to yield a total sample volume of 20ul.
(eg. 6u sample yields 40ug protein, 4u1 H2O added to sample for total vol. of l0ul, add 10uI 2X treatment buffer, total volume 20u1)
4. Puncture lip of eppendorf tube containing sample
5. Boil for 3 min in boiling water

GEL ELECTROPHORESIS: (USE GLOVES FOR ALL STEPS)
Using the NOVEX Mini-Gel and Transfer apparatus.
Use a NOVEX 8-16% gradient gel, 1mm thick, 10 wells
I. Open package and empty buffer from gels
2. Pull tape off gels, do not touch side of gel with holes.
3. Wash wells with tank buffer.
4. Assemble gels in gel box. (see NOVEX manual)
5. Fill inner chamber with tank buffer and check for leaks.
6. Fill outer chamber with tank buffer.
7. Use Low and High M.W. markers (Bio-Rad Kaleidoscope STD #161-0324)
8. Add 7u1 marker to well
9. Add 20u1 sample to wells
10. Run gel far 100 120 min room temp. at volts. (time can very depending on dye front migration

TRANSFER: (USE OLOVES FOR ALL STEPS)
1. Al [end of electrophoresis, remove top of chamber and rinse.
2. Rinse gel and tank
3. Remove gel from cassette.
4. Get the transfer membranes ready for use. (NOVEX #LC200 0.45uM, Sec NOVEX manual)
5. Equal gel, NC paper, and Filter in transfer buffer for l0 min using orbital shaker.
6. Soak sponges in transfer buffer.
7. Load gel transfer sandwich as per directions. (LOAD WET)
8. Assemble gel box.
9. Fill inner chamber with transfer buffer.
10. Fill outer chamber with D.I. WATER.
11. Run transfer for 2hr for two blots(for one blots), room temp. at 30 volts.

STAINING BLOT:
At end of transfer, remove NC blot.
Stain with Ponceau Red in pipet box for 10-l5sec.
Pour stain back in bottle.
Rinse blots with dist. H20 until background is gone.
Check for even blotting of all samples.

BLOCKING:
For blocking all non-specific protein binding sites.
Use Blotto (TBS, plus 5% milk and 0.05% twecn-20)
Block for 30min at room temp. on orbital shaker using I0cc sol'n for each blot.
Rinse out blotto with TBS

INCUBATION W1TFI PRIMARY ANTIJ3ODY:
IkB-@/MAD-3(C-15), the amino terminal, from Santa Cruz Biotechnology, Cat. # sc-203
IkB-b(C-20), the carboxy terminus, from Santa Cruz Biotechnology, Cat. # sc-945
Dilute primary antibody 1/100 into Blotto incubate the blots with primary antibody for 45 mins, at room temperature.
Wash two times for 7 minutes witli TTBS (TBS plus 0.05% Tween-20)))

1NCUBATION WITH SECONDABX ANTJ.BODY:
Anti-Rabbit IgG FI8 (Goat), peroxidase conjugated, from Cal. Biochem., Cat. #403 15
Dilute secondary antibody 1/10000 into Blotto, incubate the blots with secondary antibody Or 30 mins, at room temperature.
Wash three times for 5 minutes with TTBS and one time for 5 minutes with TBS

DEVELOPING:
After washing step of secondary antibody, the blot is ready for addition of developer.
Leave blot in container moist.
Using Amersharn ECL reagent (RPN 2109)
Mix lcc reagent I to 1cc reagent 2 prior to use.
Add 2cc mixed reagent on top of blot and mix gently by hand for 1min.
Wrap blot in saran wrap, place in cassette, bring 10 X-ray room and develop.
Developing time can vary from 5min to 10 min depending on antibody reactions and amount of protein on the blot.
Bands of high M.W. marker: green-81,000, magenta-134,000, violet-41,500

Transfer buffer

0.192 M Glycine, 0.025 M Tris ph 8.3, 20% MeOH
Do not pH.
Store in brown bottle at room temp.

Volume	Tris	Glycine	MeOH	H_2O
1.0 L	3.0 g	14.4 g	200 mls	to 1.0 L
2.0 l	14.4 g	28.8 g	400 mls	to 2.0 L
4.0 L	12.0 g	57.6 g	800 mls	to 4.0 L
8.0 L	24.0 g	115.2 g	1600 mls	to 8.0 L
20.0 L	75.0 g	360.0 g	-----	----
30L	9.0 g	43.2 g	600 mls	to 3.0 L

Tirs Buffered Saline (TBS)

0.02 M Tris, 0.5 M NaCl, pH 7.5
Make fresh for immunoblotting
pH to 7.5 with 6N HCl (approx 10 mls for 4 L)

0,5 l	1,21 g	14,6	
Volume	Tris	NaCl	H_2O
1.0 L	2.42 g	29.22 g	to 1.0 L
2.0 L	4,84 g	58.44 g	to 2.0 L
4.0 L	9.68 g	116.88 g	to 4.0 L

Tween-TBS (TTBS)
0.05% Tween in TBS

Tween-20	TBS volume
1.0 ml	2.0 L
0.5 ml	1.0 L
220 µl	0.44 l
0.25 ml	0.5 L
0.125 ml	0.25 L
0.0625 ml	0.125 L

Monomer Solution
Store in brown bottle
Store at 4 °C
Caution NEUROTOXIN!!!!

Acrylamide	Bis (N,N'_Methylene bis-acrylamide)	H_2O
58.4 g	1.6 g	to 200 mls
29.2 g	0.8 g	to 100 mls
116.8 g	3.2 g	to 400 mls

4X Running Gel Buffer
1.6 M Tris-HCl pH 8.8
Store at 4 °C

Tris – 36.3 g
H_2O – to 200 mls
pH – to 8.8 with 6 M HCl

4X Stacking Gel Buffer
0.5 M Tris-HCl pH 6.8

Tris – 3.0 g
H_2O – 50 mls
pH – 6.8 with 6 M HCl

Tank Buffer (1X) (0.025 M Tris pH 8.3, 0.192 M glycine, 0.1 % SDS)
Do not pH
Store at room temp

Volume	Tris	Glycine	SDS (10%)	H_2O
1.0 L	3.0 g	14.4 g	10 mls	to 1.0 L
2.0 L	6.0 g	28.8 g	20 mls	to 2.0 L
4.0 L	12.0 g	57.6 g	40 mls	to 4.0 L
10.0 L	30.0 g	144.0 g	100 mls	to 10.0 L
24.0 L	72.0 g	345.6 g	240 mls	to 24.0 L
24.0 L	144.0 g	691.2 g	480 mls	to 24.0 L

2X Treatment Buffer (0.125 M Tris-HCl pH 6.8, 4.0 % SDS, 20% glycerol, 10% 2-mecaptoethanol)

10.0 mls
Tris 2.5 mls stacking gel buffer
SDS 4.0 mls 10% solution
Glycerol 2.0 mls
H_2O 0.5 ml
And 10% BME before use