

Cell Lysis/Western/IP

(Compiled by 6/95 by AJS from AB)

Fresh 1X Lysis	Final	5ml	10ml
2X Lysis		2.5 ml	5ml
NaCl	0.25M	0.25ml of 5M	0.5 ml of 5M
Na ₃ VO ₄	0.1mM	25l (ul) of 200 mM	5l (ul) of 200mM
NaF	50mM	0.5 ml of 0.5M	1ml of 0.5M
DTT	1mM	5l (ul) of 1mM	10l (ul) of 1mM
Aprotinin	3ug/ml	15l (ul) of 1 ug/ul	3l (ul) of 10 ug/ul
Pepstatin	2 ug/ml	10l (ul) of 1 ug/ul	20l (ul) of 1 ug/ul
Leupeptin	1 ug/ml	5l (ul) of 1 ug/ul	1l (ul) of 10 ug/ul
Okadaic		1:500	
PMSF		35l	70l
H ₂ O		1.75 ml	3.5ml

1. 1X Lysis Buffer -- 4°C
2. Cool eppys -- 4°C put 4x # of tubes needed
3. PBS -- 4°C
4. Stimulation -- 37°C 10' NGF=100ng/ml à first aspirate 5 ml!
- 5.
6. KCl=5 ml to 10 ml
7. Lyse -- on ICE TRAY Stop stimulation
- 8.
9. asp. Media/wash 2XPBS/ +500l lysis buffer
10. scrape into eppy
11. incubate 45' 4°C à Vortex several times during
12. spin 10 secs 4°C
13. divide sample: ~400 l for IP/ ~ 100 l for Western
14. IP -- 1° Ab => 4°C 1 hour rocking
- 15.
16. if 1° is not rab. à 2° Ab => 4°C 1 hour rocking (RaM 2l)
17. -- PrA/Seph 4°C 1 hour rocking 40l
18. -- Spin down 4°C 1'
19. -- Wash 3X: 2X Lysis Buffer (~500 l)/ 1X PBS
20. Sample Prep -- (a) IP: add (40 l) 2X Sample Buffer (1X V PrA/S)
- 21.
22. -- (b) W: add (50 l) 3X Sample Buffer
23. Boil 5'
24. Run Gel -- 100 V through Stacking
- 25.
26. 100 – 200 V after (in Separating)
27. Prepare Transfer -- make fresh TB: 1L= 200ml 5X TBb
- 28.
29. 200 ml Methanol
30. 600 ml H₂O
31. -- cut fresh nitrocellulose (11 X 17 cm) & Whatman soak in TB

32. -- 2 grids outside plate on Yellow Tape side
33. 1 grid in middle – SMOOTH SIDE TO GEL!
34. Cushions
35. pour in TB until just wet
36. put 1 Whatman over Gel à smooth out
37. place onto cushion
38. Nitrocellulose over gel
39. 2nd Whatman over Nitrocellulose à smooth out bubbles
40. 4 cushions on top
41. 2nd Grid – SMOOTH SIDE TO GEL!
42. Add more TB
43. 2nd metal plate
44. place in tank: liquid should cover gel area
45. Transfer
46. use black battery charger: 24V 45'
47. After Transfer
48. remove blot and place in container face up quick rinse TBST
49. Block
50. 3% BSA/TBST or 5% Milk/TBST 1 hour RT shaking
51. 1 °Ab
52. dilute in 20ml: 3% BSA/0.05% NaN₃/TBST
53. Wash
54. pour 1 ° Ab back (RE-USE!) quick rinse, then 3X5' rinse TBST
55. ° Ab
56. HRP_aM or HRP_aR (depending on 1° Ab) dilute 1:20000 (1.25 l in 25 ml): 3% BSA/TBST 1 hour RT shaking
57. Wash
58. quick rinse, then 3X5' rinse TBST
59. ECL
60. 5 ml DuPont NEN White + 5 ml Dark bottle place blot into solutin for 1' + agitation put onto Saran Wrap and smooth out bubbles put fluorescent dot markers on for orientation