IMMUNOBLOTTING William C. Earnshaw

7/5/12

SOLUTIONS

TRANSFER BUFFER
12.1 gm Sigma 7-9 (cheap tris)
57.7 gm Aldrich 98% glycine800 ml Methanol
3.2 liters H_20 --> remove 250 ml for wetting nitrocellulose
then + 18.75 ml 20% SDS

10x PTX

10x final concentration		stock soln	add/1 liter final
100 mM $NaPO_4$ pH 7.5		0.5 M	200 ml
1.5 M NaCl		5 M	300 ml
10 mM Na EGTA	0.1 M	100 ml	
2% triton X-100	10 %	200 ml	
(1 mM NaN3	10 %	6.5 ml)	

PTX/BSA

10 ml 10x PTX
13.3 ml 30% BSA (Miles Pentex - final concentration = 4 %)
H_2O to 100 ml

Gerace Buffer (GB)

5x final concentration	stock soln	add/500 ml final
250 mM Triethanolamine:HCl pH 7.4	1 M	125 ml
500 mM NaCl	5 M	50 ml
10 mM K-EDTA pH 7.4	0.1 M	50 ml
2.5 % Triton X-100	10 %	125 ml
0.5% SDS 20 %	12.5 ml	

^{125}I-protein A - or ECL detection kit

10x PBS:

95 mM $NaPO_4$ buffer pH 7.5	14.4 gm $Na_2HPO_4 \cdot 2H_20$ + 2.4 gm $NaH_2PO_4 \cdot 2H_20$
1.37 M NaCl	80 gm NaCl
27 mM KCl	2 gm KCl
	H_20 to 1 l

Note when making this that it is essential to use phosphate reagents with the proper hydration state. If other hydration states are all that is available, you must recalculate how much to add to give the proper molarity.

Tween-20: 20 % solution (v/v)

PBS-Tween:

10x PBS	100 ml
20 % Tween-20	5 ml
H_20	to 1 l

Milk: Safeway dried skimmed milk with added vitamins A & D (340 gm)

PROCEDURE

THE BLOT:
1. Run a gel containing the antigen of interest -> assemble the transfer apparatus.

2. Transfer the proteins from this gel to nitrocellulose paper (5 hr at 55 v/ 450 mA in transfer buffer - if your power supply produces a different current at this voltage, adjust conditions so that the total watt-hours are conserved).

3. Rinse the nitrocellulose nitrocellulose 3x with H_2O; stain with Ponceau S.
The stained membrane can be dried for extended periods if desired.

ANTIBODY INCUBATION - ECL:

place membrane in plastic container and wash as follows:

4a. Add 10 ml PBS-Tween + 0.5 gm dry milk -> agitate 1 hr @ RT. Other possible blocking agents to use include 10 % goat serum & 4 % BSA.

5a. Rinse 2x with 40 ml PBS-Tween

6a. Wash with 40 ml PBS-Tween on shaker -> 15min

7a. Wash with 30 ml PBS-Tween -> 2 x, 5 min. each on rocker

8a. Incubate with 1^o antibody in 10 ml PBS-Tween -> agitate 1 hr @ RT

9a. Wash with 40 ml PBS-Tween on shaker -> 15min

10a. Wash with 20 ml PBS-Tween -> 2 x, 5 min. each on rocker

DETECTION - ECL:

(11a.) If antibody incubation used another protocol (PTX:BSA, for example), wash with 40 ml PBS-
Tween -> 3 x, 2 min. each

12a. Incubate with 2^o antibody (HRP-conjugate 1:5000 - 1:10,000) in 25 ml PBS-Tween -> agitate 1 hr @ RT on rocker

13a. Wash with 40 ml PBS-Tween -> 4 x, 5 min. each on shaker

14a. for each blot mix 5ml each black and white ECL reagent

- TAKE TO DARKROOM (or 12-15 can be done in lab):
  membrane, film, forceps, cassette, timer, fluorescent marker strip
- IN DARKROOM:

15a. lay out 2 pieces of saran wrap

16a. on one piece place membrane (transferred side up)

17a. pipette on black/white mixture, covering blot completely -> incubate 1 min.

18a. With forceps transfer membrane to fresh saran wrap (dumping ECL solution on original saran
wrap), wrap and expose to film

ANTIBODY INCUBATION - protein A:

4b. Incubate membrane with PTX:BSA 20 minutes at room temp. with shaking. Other possible blocking agents to use include 10 % goat serum & 5 % dried skimmed milk.

5b. Add antibodies to desired concentration.

6b. Incubate 1 hr - overnight. For many antibodies, the longer you incubate after 3-4 hours, the greater problems you have with background.

DETECTION 1 - protein A:

7b. Wash 5 times for 3 minutes each with GB. Use enough volume so the the nitrocellulose moves back and forth in the container.

8b. Rinse 1x briefly with 1x PTX.

9b. Add a minimal amount of PTX:BSA.

10b. Incubate 5 minutes at room temp.

11b. Add ^{125}I-protein A to desired amount (typically 1:1,000-1:2,000).

12b. Incubate 30 minutes at room temp.

13b. Wash 5x for 3 minutes with GB.

14b. Dry thoroughly, mark with radioactive pen, and mount on stiff backing covered with plastic wrap.