PROTOCOL – ANDREW CLARK (PALUCH LAB) **Protocol #: 5**

Western Blot (Big Gels) **13.Sept.2009**

Day 1 (AM/EarlyPM):

1. Prepare plates for pouring gel:
    a. Get glass plates for pouring gels, large binder clips, silicon tubing, plastic spacers and gel plate holder
    b. Wash plates (the side that will face the gel) first with 70% EtOH, then with ddH_20
    c. Wash silicon tubing and spacers with 70% EtOH
    d. Lay square plate down on bench (on paper towel to protect), find the center of the silicon tubing and lay across the plate, ~5cm from bottom
    e. Lay spacers down near the edges, orthogonal to the silicon tubing
    f. Place the glass plate with "tabs" on top of the square plate with tubing and spacers, and hold at the bottom with a binder clip
    g. Pull silicon tubing around the spacers, so that it makes a "U" shape between the glass plates
    h. Hold both sides with two binder clips each

2. Pour resolving and stacking gels
    a. Make mixtures for resolving gels and stacking gels WITHOUT TEMED AND APS!!
    b. Recipes:
        i. Gel Mixtures:

Resolving gel - 25 ml

Gel Percentage	6	7,5	8	10
Sol A (µl)	4992,9	6241,1	6657,1	8321,4
Sol B (µl)	6250,0	6250,0	6250,0	6250,0
Water (µl)	13328,6	12080,4	11664,3	10000,0
TEMED (µl)	71,4	71,4	71,4	71,4
APS (µl)	357,1	357,1	357,1	357,1
SUM (µl)	25000,0	25000,0	25000,0	25000,0
DESIRED VOLUME (µl)	25000,0	25000,0	25000,0	25000,0

Stacking - 10ml

Sol A (µl)	1500
Sol C (µl)	2500
Water (µl)	5400
TEMED (µl)	100
APS (µl)	500
SUM (µl)	10000
DESIRED VOLUME (µl)	10000

ii. Solutions:

Solution A (30% w/v acrylamide solution; 100mL; store 4º in dark):

29%	acrylamide	29.0g
1%	N,N'-methylene-bisacrylamide	1.0g
	ddH_2O	to 100mL

**Note: can use pre-made solution:
Severn Biotech Ltd. (Product # 20-2100-10)
30% Acrylamide + 35.7:1 bis-Acrylamide

Solution B (1.5 M Tris-HCl buffer + 0.4% SDS; 100mL; store at RT):

1.5M Tris	18.2g
0.4% SDS	0.40g
ddH_2O	to 100mL
	pH 8.8

Solution C (0.5 M Tris-HCl buffer + 0.4% SDS; 100mL; store at RT):

0.5M Tris	6.1g
0.4% SDS	0.40g
ddH_2O	to 100mL
	pH 6.8

APS (ammonium persulfate; 10% w/v; make up fresh):

10%	APS	0.09g
	ddH_2O	900µl

TEMED (Tetramethylethylenediamine):
99% pure from Sigma

c. Very quickly, add APS and TEMED to resolving gel mixture and pour immediately between glass plates
d. Also very quickly, as soon as gel is poured, use a P1000 micropipetter to drip ddH_2O on top of gel, to get rid of bubbles and to ensure that the resolving gel is straight on top (*Note: this is not necessary [and can ruin] for high percentage [10%] gels)
e. Leave undisturbed for 1h at RT
f. Gently rinse the top of the gel with ddH_2O
g. Very quickly, add APS and TEMED to stacking gel mixture and pour immediately between glass plates (*Note: the stacking gel will polymerize much faster than the resolving gel, so be quick!)
h. Immediately after pouring the stacking gel, insert the comb in the top of the gel (the top edge of the comb should be flush with the top of the square glass plate, otherwise it is very difficult to get the comb out later).

i. Leave undisturbed for 1h at RT

DAY 1 (Late PM):

1. Remove binder clips and silicon tubing from around the gel
2. Assemble gel apparatus (ring stand with two reservoirs) (*Make sure that bottom reservoir has "feet" toward back and top reservoir has cut-out side toward front)
3. Fill reservoirs with running buffer (need about 1L for each gel):

		10x
25mM	Tris	30g
0.5%	SDS	10g
192mM	Glycine	144g
	ddH_2O	to 1L

4. Put gel (still between glass plates with spacers) on gel apparatus and remove bubbles at bottom of gel by tilting at an angle until the bubbles run off of the sides
5. Affix gel to top reservoir with one binder clip on each side
6. Using a hypodermic needle/syringe, straighten and clean wells
7. Load gel (*Note: leave one lane between ladder and samples and fill with sample buffer)
8. Run gel:
    a. 40V at Constant Voltage for 18h (for a ~70kDa protein in a 7.5% gel)

DAY 2:

1. Transfer gel to membrane
    a. Cut 2 pieces of (thin, Whatman) filter paper to the same size as sponges (better to be a little bit smaller)
    b. Cut PVDF membrane to the same size as filter paper, remove blue backing and soak in MeOH for 2min
    c. Rinse membrane with water 3x and then equilibrate in blotting buffer for 10min
    d. Once gel has finished running, disassemble apparatus and gently transfer gel to box containing cold blotting buffer (4º)
    e. Equilibrate in blotting buffer for several minutes
    f. Wet filter paper and 2 sponges with blotting buffer and put everything together in "sandwich" in the following order:
        i. Side of sandwich case
        ii. Sponge
        iii. Filter paper
        iv. Gel
        v. Membrane (be sure there are no bubbles between membrane and gel)

vi. Filter paper
vii. Sponge (after this, roll a 50ml Falcon tube over the sandwich to push out any remaining bubbles)
viii. Other side of sandwich case
ix. Mark this side of the sandwich to denote that it is the "membrane" side

g. Close sandwich case and slide into transfer box (make sure to remember which side is the membrane side)
h. Fill transfer box with blotting buffer (will take about 3L; this buffer can be re-used several times)
i. Run transfer at 50V constant voltage for 15h in cold room (*Note: the membrane side should face the positive [red] lead!!)

DAY 3:

1. Disassemble transfer apparatus and put membrane in a box containing blotting buffer
2. Perform rest of Western Blot as in Western_Blot protocol

Acknowledgements:

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