PROTOCOL FOR CELL ADHESION ASSAYS

Prepared by: Bin Hu

A. Pre-coating of multi-wells:

Usual substrates	Stock	Final concentration
• Fibronectin (bovine, Calbiochem)	1 mg/ml	5 µg/ml
• Laminin (human, Invitrogen)	1 mg/ml	5 µg/ml
• Collagen type-IV (bovine, Sigma)	1 mg/ml	5 µg/ml
• Poly-L-Lysine (75 to 300kDa, Sigma)	500 µg/ml	50 µg/ml
• Hyaluronic acid (bovine, Calbiochem)	1 mg/ml	200 µg/ml

1. Dilute all substrates from the stock concentration to the final concentration using 1XPBS (pH 7.4). Prepare only the amount of diluted substrate necessary for each experiment. DO NOT PREPARE SUBSTRATE IN A LARGE EXCESS (it cannot be stored once it is diluted). **DO NOT VORTEX THE SUBSTRATES BECAUSE THEY CAN PRECIPITATE! (mix gently…)**

2. Using a 48-well plate, add 200 µl of the desired substrates/well. Avoid bubbles or touching the bottom of the wells with tips.

3. Incubate inside the culture hood x 2h x room temp.

4. Remove the substrates by inverting the multiwell over a plastic reservoir and tapping gently. DO NOT USE GLASS PIPETTES TO REMOVE THE SUBSTRATES AND/OR WASH THE WELLS, THEY WILL SCRATCH THE SURFACE OF THE WELLS.

5. Wash the wells 2 x PBS (fill the wells with 500 µl PBS and empty the plate on the reservoir)

6. Add to each well 500 µl of blocking solution:
   Blocking sol 1: DMEM with 10% FBS (same as used for culture)
   Blocking sol 2 (alternative): 1% cell culture-treated serum albumin in 1X PBS

7. Incubate 30 min at 37C (if using Sol 1) or at room temp (if using Sol 2)

8. Wash the blocking solution once with PBS and fill each well with 200 µl of normal culture medium (**CM**) for the cells that are going to be tested ← Do this last step just before trypsinizing the cells, and keep the multiwell plates at 37C.

B. Cell counting and seeding (All cell manipulation steps in the culture hood!):

1. Resuspend cells with 0.025% trypsin/EDTA x 1-2 min. Stop enzyme activity with CM containing 10% serum and collect the cells in 15 ml conical tubes.

2. Centrifuge cells for 3-4 min at <300 g (position "1/2" in the clinical centrifuge)

3. Resuspend cells GENTLY in 1 ml of CM and add 2-4 ml CM to yield a final volume of 3-5 ml.

4. Pipette 15-20 µl cells into a hemocytometer and count the cells in the microscope at 10X

5. Adjust the cell density to 300,000 cells/ml (if it is already lower, re-centrifuge the cells and resuspend in a smaller volume). If cells from different treatments were cultured at different densities and yield very different initial cell counts (> 3 fold different), the margin of error will be too big to perform the experiment!

6. Count and adjust ALL THE SAMPLES quickly, keeping them at room temperature inside the hood. Every few minutes, rock or resuspend gently the cells so that they DON'T SETTLE OR AGGREGATE in the bottom of the conical tubes. Inspect the tubes visually all the time!!

7. Add equal cell numbers to the pre-coated wells (remember they have 200 µl of CM at this point). If you are applying different cell volumes for each treatment, compensate the total volume with fresh CM.

8. These cell densities have been optimized for glioma cell lines:

- U87MG: 30,000 cells/100 µl
- CNS-1: 60,000 cells/100 µl
- U251MG: 60,000 cells/100 µl
- U373MG: 50,000 cells/100 µl
- U118 MG: 50,000 cells/100 µl

9. Add a total volume of 300 µl of CM to the "blank" wells for each substrate. These wells will be used to calculate background staining.

10. Incubate the cells at 37C x 30 min.

C. Cell fixation and quantification (All steps can be performed at the lab bench:

1. Remove the CM and non-adhered cells by inverting the plates on a plastic reservoir. DON'T TAP THE MULTIWELL PLATES!

2. Wash the wells quickly 2 times with 300 µl of ice-cold PBS **containing 1mM $CaCl_2$ and 1 mM $MgCl_2$ (**also known as "PBS^{++}", prepare fresh from normal PBS for each experiment!)

3. Fix the cells using **300 µl of FREEZER-COLD (-20C) 100% METHANOL**. Add the methanol to the wells and let the plate at room temp for 10 min.

4. Wash the methanol three times with normal PBS (1 min each wash)

5. Add 100 µl of crystal violet solution to each well (0.5% w/v crystal violet in 20% ethanol). Gently shake the plates at room temperature x 10 minutes.

6. Remove excess of crystal violet by immersing the plates in large beakers (2 lt) filled with ddH_2O (three immersions of 1 min each, with gently twirls of the plates).

7. Recover crystal violet by adding 200 µl of 100 % methanol to each well. Gently shake the plates at room temperature x 15 minutes

8. Transfer 100 µl of the extracted crystal violet to flat-bottom 96-well plates. Measure absorbance at 590 nm.