

## Anoikis Assay

### Prepare poly-HEMA plates :

For each 10 cm<sup>2</sup> tissue culture plate, you will need approximately 10 ml of 20 mg/ml poly-HEMA (Sigma #P3932). Weighing poly-HEMA directly into a sterile flask and using stirbars that have been autoclaved will help ensure the sterility of your poly-HEMA plates. Plates can be made in advance and stored, wrapped in parafilm, in the 4° fridge, until needed. Plates need to be made at least one day in advance, as they will need to dry overnight.

- 1.) Dissolve appropriate amount of poly-HEMA in 95% ethanol to equal 20 mg/ml poly-HEMA solution.
- 2.) Stir vigorously on a hot plate (set heat to setting 3 or 4) until dissolved, about 1 hour.
- 3.) In the tissue culture hood, pipette 5 ml of poly-HEMA solution into each 10 cm<sup>2</sup> plate.
- 4.) Let plates sit, partially covered with lid, until ethanol evaporates and the poly-HEMA has solidified (approximately 1-2 hours). Periodically rock plates by hand to help ensure even coating.
- 5.) Add remaining poly-HEMA solution to plates (this helps fill in any gaps in the first coat), and let sit as above. Plates can be left to dry overnight.

### Plating cells on poly-HEMA:

- 1.) Trypsinize cells and quench as usual.
- 2.) Count cells – the cell number needed per sample will depend on the cell type and what is going to be done with the cells. For example, if MCF10A cells will be harvested for protein, we generally plate  $2.5 \times 10^6$  cells in 10 ml ( $2.5 \times 10^5$  cells/ml).
- 3.) Wash plates 2x with 1X PBS to remove residual ethanol.
- 4.) Pipette appropriate number of cells/volume of media onto plate and incubate.
- 5.) Harvest cells as needed.

### **Assessment of Anoikis:**

In general, any assay used to normally examine apoptosis can be used to assess anoikis. For example, cells can be harvested for protein and PARP cleavage can be monitored by western blot. Alternatively, a quantitative assay such as the Cell Death Detection ELISA from Roche (catalog # 11544675001 ) can be used.

### **Using the Cell Death Detection ELISA to Monitor Anoikis: (modified from product insert)**

- 1.) Before you begin collecting your samples, start preparing the assay plate by coating the appropriate number of wells with working coating solution (coating solution plus anti-histone antibody). You will need two blank wells, and two wells/sample. The supplied coating buffer is 10X – this is diluted to 1X in water. Then the coating buffer is used to dilute the anti-histone antibody (1:10 dilution of antibody:coating solution). Use 100 µl per well, and incubate 1 hour RT. Cover the plate with the supplied adhesive tape.
- 2.) At the time that you want to assay the cells for anoikis, collect the media (containing the cells in suspension). Plates can be washed with 1X PBS to collect any cells that may have been left behind. It is recommended that an attached plate or flask is also harvested as a no-apoptosis control.
- 3.) Pellet cells and wash 1x with 1X PBS. \*\*\*Note: If cells are very clumpy, you can trypsinize the cell pellet to break them apart and quench in media. This will aide in counting the cells.\*\*\*
- 4.) Count cells. The number of cells needed for the assay should be determined empirically. For MCF10A cells,  $5 \times 10^4$  cells are used per sample.
- 5.) Retain appropriate number of cells for the assay, and harvest the remaining cells for protein (useful for monitoring molecular markers and proteins of interest).
- 6.) Pellet cells, wash once in 1X PBS, and lyse in 500 µl incubation buffer 30 minutes at RT.
- 7.) During this 30 minute incubation, wash the assay plate 3x with 300 µl 1X washing buffer. Washing buffer is supplied as a 10X stock (dilute in water) and needs to be warmed to RT before using.

- 8.) Add 200  $\mu$ l incubation buffer to each well of the assay plate and incubate, covered, 30 minutes at RT.
- 9.) Following the 30 minute lysis, spin samples at top speed for 10 minutes.
- 10.) Remove supernatant to a new tube, being very careful not to disturb the pellet.
- 11.) Dilute sample 1:10 in incubation buffer (use at least 25  $\mu$ l in 225  $\mu$ l incubation buffer).
- 12.) Wash plate 3x with 300  $\mu$ l 1X washing buffer.
- 13.) Pipette 100  $\mu$ l diluted sample into two wells. The “blank” wells get 100  $\mu$ l incubation buffer.
- 14.) Incubate covered, 90 minutes, RT.
- 15.) Wash cells 3x with 300  $\mu$ l 1X washing buffer.
- 16.) Pipette 100  $\mu$ l conjugate solution (anti-DNA-POD antibody diluted in incubation buffer) into each sample well. The blank wells get incubation buffer only.  
Conjugate solution is a 1:10 dilution of anti-DNA-POD antibody:incubation buffer.
- 17.) Incubate covered, 90 minutes, RT.
- 18.) Wash cells 3x with 300  $\mu$ l 1X washing buffer.
- 19.) Add 100  $\mu$ l substrate solution to each well (including blanks), and incubate, covered (with both adhesive tape and foil), 10-20 minutes RT on a shaker (250 rpm).  
Substrate solution is a substrate tablet dissolved in 5 ml of substrate buffer. It can be stored (wrapped in foil) at 4°C until needed, up to one month.
- 20.) Read the assay plate in a plate-reader at 405 nm and 490 nm (490 nm is a reference wavelength).
- 21.) Data is usually expressed as Absorbance 405 nm – 490 nm.

**Transfection of MCF10A Cells with siRNA for anoikis studies:**

- 1.) Plate cells the night before transfection at a density of  $6 \times 10^4$  cells/ml in 10  $\text{cm}^2$  dishes (10 ml volume =  $6 \times 10^5$  cells total).
- 2.) The following day, dilute oligofectamine. Use 30  $\mu$ l in 53  $\mu$ l optimem per 10  $\text{cm}^2$  dish.
- 3.) Dilute oligos: For each dish use 200 nM siRNA in 1 ml optimem per dish.
- 4.) Let sit 10 minutes RT.

- 5.) Add diluted oligofectamine to each diluted oligo. Use 83  $\mu$ l diluted oligofectamine per dish.
- 6.) Let sit 15 minutes RT.
- 7.) In the meantime, wash plates 2x with optimem.
- 8.) Add 3 ml optimem per dish to each transfection mix.
- 9.) Pipette 4 ml onto each dish
- 10.) Incubate 5 hours in 37°C incubator.
- 11.) Change media.
- 12.) After 36 hours the cells can be moved to poly-HEMA.