VPT5778 – 2022

Cell cycle and cytotoxicity staining protocol (adapted from Curr Protoc Mol Biol.; 111: 28.6.1–28.6.11, 2016. doi:10.1002/0471142727.mb2806s111

(Usually, the cytotoxicity assay we carried out using Annexin-V/PI solution, but how I can't not read at flow on Thursday (since it is not possible to maintain refrigerated) we will adapt to Trypan Blue staining protocol which is much less sensitive; but for cell cycle the cell can be fixed and kept in the freezer)

1. Plate cells in 6 well plates at $1x10^6$ cell density per well, in approximately 2ml of complete media (DMEM with 10% of SFB = D10) or starved condition (since serum starvation induced Cell Cycle synchronization); after harvest cells and after centrifugation cells resuspend cells in 10mL of D10 and count; divide 5mL of cells into 2 15ml tubes centrifuge again
2. After 24h incubation for cell adhesion, cells will be either starved with 1% SFB (2ml) and/or treated with Doxorrubicin (**Stock solutions of 1 mM doxorubicin hydrochloride and further dilutions (0.1 and 10 µM) were prepared in complete DMEM (D10);**
3. Each cell type will be exposed to 6 conditions: **(prepare 2 plates: plate 1 (Mel Cells); plate 2 (CF41 - Canine mammary cancer cell line),** as layout below:

Meln plate (canine lymph node metastatic melanoma cells) (from our Lab)

Complete medium D10	Control	Treated **1 µM**	Treated **10 µM**
Starved condition (D1=DMEM+1%SFB)	Control	Treated **1 µM**	Treated **10 µM**

CF41.Mg plate (canine mammary carcinoma cells) (from ATCC)

Complete médium D10	Control	Treated **1 µM**	Treated **10 µM**
Starved condition (D1=DMEM+1%SFB)	Control	Treated **1 µM**	Treated **10 µM**

4. After 24h incubation cells with DOXO the cells will be trypsinized starting transfer the supernatant to a new 15ml Falcon tube (because in the supernatant will have cell detached/dead) and add 2mL of PBS to wash serum; throw away the PBS and add 0,5mL of trypsin and wait 1 to 2mL until it is observed that the cells have detached from the bottom of the plate then add 2ml of D10; harvest the cells from well and transfer to the same 15 mL Falcon tube that you have already transferred the supernatant and …….

   ATTENTION: **before centrifuging the cells, remove a 10μL aliquot of cell and transfer to previously identified microtubes to performed Trypan blue staining/cell count at Neubauer chamber and centrifuge the rest of the cells. During count will**
5. Centrifuge cells 250xg for 5 min and proceed to the next step;
6. Previously prepare the fixative by filling tubes (15 mL Falcon tubes) with 4.5 ml of 70% ethanol. Keep tubes on ice;
7. After centrifuging the cells discard supernatant and suspend cells in 3 ml PBS in a centrifuge tube;
8. Centrifuge cells again for 5 min at 250 x g;
9. After centrifuging the cells discard supernatant thoroughly and resuspend cells in 0.5 ml PBS. It is very important to achieve a single cell suspension. Fixation of cells that are in aggregates while suspended in PBS stabilizes the aggregates, which are then impossible to disaggregate. It is essential, therefore, to make sure cells are in a single cell suspension prior to the time of mixing the cells with ethanol;
10. Add 4.5 ml pre-chilled 70% cold ethanol (−20°C) in a drop wise manner to the cell suspension while vortexing (use Vortex equipment).
11. Keep cell in fixative and cells suspended in ethanol can be stored at -20°C for several months to a year; keep cells in the freezer (-20°C) until labeling using the cytotoxicity kit (we will use on Monday);

12. On Monday (6/13/22): centrifuge the ethanol fixed cells 5 min at 300 x g and decant ethanol thoroughly (be careful not to lose your cells!)
13. Suspend the cell pellet in 5 ml PBS, wait 60 sec, and centrifuge 5 min at 200 x g;
14. Suspend the cell pellet according to protocol described by the manufacturer kit (FxCycle™ PI/RNase Staining Solution Catalog number: F10797) starting from the item 1.4 (pdf manual) that has been optimized for your cell type and concentration **(Use 300ul and not 500 μL);**
15. Keep at either 37°C for 15 min or 30 min at room temperature;

16. Bring cells in PI solution (analyze the samples without washing) to the flow lab for analysis.

If your lab doesn't have a kit mentioned above, you can prepare the PI solution (propidium iodide):

For 10ml solution:

1. 8,89 ml PBS
2. 1 ml PI [200 ug/ml]
3. 100 ul RNAse A [20 mg/ml]
4. 10 ul triton X-100

For 5 ml solution:

1. 4,445 ml PBS
2. 0,5 ml PI [200 ug/ml]
3. 50 ul RNAse A [20 mg/ml]
4. 5 ul triton X-100