**LM042-2: Bleo-tx A549 (HNE and Mitotracker)**

I forgot to bleo-tx the A549 cells in LM042-1. Oops

Day 1: coat coverslips and plate cells; low sera

1. Pipet 10uL RTC and 2490uL PBS into a tube. Vortex to mix.
2. Place coverslips in wells of a 24-well plate. Cover coverslips with collagen mixture.
3. Incubate 2h RT.
4. Wash 2x with PBS.
5. Plate 35k A549 cells/well.

Day 2: treatment

1. Prepare +bleo media in a 1.5mL tube:
   1. *[Bleo stock] = 3mg/mL. final conc = 50ug/mL. 3ug/uL\*x = 0.05ug/uL\*1000uL x = 16.66uL bleo*
   2. **16.66uL 3ug/uL bleo + 983uL media**
2. Pipet 750uL –bleo media onto isotype, 2oOnly, and untx wells. Pipet 750uL +bleo media onto +bleo well.
3. Incubate 37C 72h.

Day 5, fixing, blocking, and primary

Resuspend Mitotracker immediately before use in 94.1uL DMSO if applicable.

Dilute 1uL 1mM MT in 99uL media to make 1:100 dilution.

1. Prepare media for Mitotracker treatment:

+MT media: 2mL media + 10uL 1:100 MT dilution

1. Aspirate media from wells. Pipet 750uL +MT media onto –bleo and +bleo wells and 750uL –MT media onto isotype and 2oOnly.
2. Incubate 37C for 30 minutes.
3. Quick wash with PBS x 3
4. Fix in of 3% paraformaldehyde (in PBS) for 30 min at room temp - 1ml/well (938uL 16%PFA + 4.062mL PBS)
5. Quick wash x 3 with PBS

(Can leave at 4 degrees overnight if needed)

1. Permeablize with 0.2% Triton X-100 diluted in PBS for 5 minutes at room temp – 1mL per well
2. Wash x 3 for 5 minutes each with PBS
3. Make enough blocking solution for 750uL per coverslip (need this for blocking, primary, and secondary) and to make isotype
   1. 10% donkey or goat serum (whichever is the animal the secondary antibody is made in)
   2. 0.1% Triton
   3. 1%BSA
   4. PBS
4. Put 250uL of blocking solution onto each coverslip
5. Leave for 10 minutes at RT
6. While blocking, make primary antibody and isotype control diluted in blocking solution and centrifuge at 14000rmp x 5 minutes at 4 degrees
   1. Ab
   2. isotype
7. Put 250uL drops of primary antibody onto coverslips
8. Place in cold room overnight (or one hour at RT)

Day 3: secondary

1. Wash x 3 with PBS for 5 min each
2. Phalloidin staining
   1. Use 5uL/coverslip of phalloidin diluted in 200uL/coverslips of PBS +1% BSA (
   2. Before adding the PBS/BSA, dry the phalloidin under nitrogen (in glass tube). Remember to close the sash on the hood or brian will be upset
   3. Put 200uL drops of phalloidin solution onto coverslips
   4. Leave covered with foil for 20 minutes (to avoid bleaching)
   5. Aspirate phalloidin sol’n
   6. Wash with PBS x 3 for 5 minutes each
3. Make secondary antibody in 1:100 dilution in blocking solution (make enough for 250uL/coverslip). Centrifuge at 14000rpm for 5 minutes at 4 degrees
4. Place 250uL of antibody onto each coverslip
5. Incubate for 1 hour at room temp covered with foil
6. Wash 3 x5 min
7. After the last wash get a beaker with Mq H2O
8. Keep in mind which side cells are on and dip the coverslip into the water, then wipe off excess on rim
9. Use Kimwipe to dry the cell-free side of the coverslip
10. Place 10uL (no bubbles) of mounting medium +DAPI onto labeled slides
11. Place coverslip cell side down onto mounting medium
12. Keep slides covered and place in cold room

**Antibody info**

Primary antibodies:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

Secondary antibodies:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Dilution:

|  |  |  |
| --- | --- | --- |
| **color** | **fluorophore** | **target** |
| blue |  |  |
| green |  |  |
| orange |  |  |
| red |  |  |