**LM044-11rep2-n: A549 3d bleo senescence induction with SA-B-Gal readout**

**3d bleo induced senescence which Alda-1 reversed in LM044-11. Now I need to repeat the experiment to build statistical significance. I’ll repeat this experiment 2x/week (plate Monday, tx Tuesday, collect and plate new rep Thursday, treat Friday, repeat ad infinitum) until statistical significance is achieved.**

**Day 0-3: plate cells, treat, incubate, harvest**

1. A549 cells were trypsinized, resuspended in 10% FBS DMEM, and plated 30k cells/well in 18 wells of a 24-well plate.
2. Cells were incubated overnight to adhere.
3. Prepare 3x 7mL aliquots of media, add compounds, and vortex to mix:

|  |  |  |
| --- | --- | --- |
| **Alda-1** |  |  |
| **conc uM** | **[stock] mM** | **vol stock uL** |
| 0 | 0 | 2.8 |
| 5 | 10 | 3.5 |
| 40 | 100 | 2.8 |
|  |  |  |

1. Divide each 7mL aliquot into a 3.25mL aliquot (total 6 aliquots).
2. Pipet 54.16uL of ultrapure water OR bleomycin (3U/mL stock, final conc = 50ug/mL) into each aliquot. Should end with three –bleo aliquots with 0, 5, and 40uM Alda-1 and three +bleo aliquots with 0, 5, and 40uM Alda-1. Vortex to mix.
3. Pipet 1mL media onto respective wells:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Alda-1 veh | Alda-1 5uM | Alda-1 40uM | **Alda-1 veh** | **Alda-1 5uM** | **Alda-1 40uM** |
| Alda-1 veh | Alda-1 5uM | Alda-1 40uM | **Alda-1 veh** | **Alda-1 5uM** | **Alda-1 40uM** |
| Alda-1 veh | Alda-1 5uM | Alda-1 40uM | **Alda-1 veh** | **Alda-1 5uM** | **Alda-1 40uM** |
|  |  |  |  |  |  |
| vehicle |  |  | **bleo** |  |  |

1. Incubate 72h.

**Day 3: SA-B-Gal harvest**

1. Dilute the 10X fixative solution with water.
   1. Make 15mL: 1.5mL 10x + 13.5mL H2O
2. Wash wells/plates with PBS.
3. Add 1mL 1x Fixative Solution to wells/plates.
4. Fix 10-15min at RT.
5. Rinse wells/plates 2x with 1X PBS. Leave at 4C until stain is performed.

**SA-B-Gal staining and WB**

**Solution preparation**

1. Resuspend the 10x staining solution by heating to 37C with agitation. Dilute the solution to 1x with H2O.
2. Dissolve 20mg X-gal in DMSO to make 20mg/mL stock. Store excess at -20C for 1 month. **Must prepare in polypropylene plastic or glass.**
3. B-gal staining solution: For each well to be stained, prepare the following:
   1. 930uL 1x staining solution
   2. 10uL 100x Solution A
   3. 10uL 100x Solution B
   4. 50uL 20mg/mL X-gal stock solution
   5. **pH the final solution. pH should be 5.9-6.1. DO NOT SKIP THIS!!**

**Staining procedure**

1. Aspirate PBS from each plate/well.
2. Add 0.5mL B-gal staining solution to each well. Seal plate with parafilm.
3. Incubate 37C at least O/N in dry incubator.
4. Check blue staining under a microscope.
5. Make a 1:600 dilution of DAPI.
6. Incubate cells in DAPI 5min RT in a dark drawer.
7. Wash 2x 5min in PBS.
8. Cover with 500uL PBS.

**Quantitation**

1. Measure DAPI intensity on a fluorometric plate reader in a 7x7 grid (ex 354 nm em 456 nm).
2. Measure SA-B-Gal stain absorbance on absorbance plate reader in the same 7x7 grid (max abs = 615nm)
3. Average readings for each well.
4. Divide each well’s SA-B-Gal signal by its DAPI signal to get well signal.