SMN2 5'ss library analysis with LRS

Obtained lib1 of SMN2 5'ss libs with 285 5'ss variants with 200 unique barcodes per variant (b20E7). Grew up a maxiprep and obtained plasmid. Concentration:

Set up a 6 well plate for seeding cells. Will be using the specs outlined in thermo's protocol (amounts listed per well):

https://www.thermofisher.com/us/en/home/references/protocols/cell-culture/transfection-protocol/lipofectamine-2000.html

- 4ug of library (Mandy did 6ug for her experiments)
- 10ul Lipofectamine 2000
- Cell density is critical since high plasmid+Lipofectamine amounts can be toxic to cells. The higher the cell density the better. Recommended amount is 0.5-2x10^5 cells per 500ul of growth media. In 6 well plates, we use 2mL total so 1x10^5 cells to 8x10^5 cells needed per well. Mandy seeded 1x10^6 cells per well so let's stick with the amount she used

Seeding cells

1. Remove plate from 37C incubator and wash with 10mL PBS
2. Aspirate off all liquid
3. Add 1mL of 1x Trypsin if using 10cm plate, or 3mL 1x Trypsin if using 15cm plate
4. Put in 37C incubator for 5min. We want almost all the cells to detach from the bottom of the plate
5. Remove plate from incubator and add 9mL media for 10cm plate of 17mL media for 15cm plate. Mix well by pipetting up and down several times to collect all cells
6. Transfer cells to a clean glass bottle or 50mL conical (depending on how much cells+media are present) and pipet up and down once again several times. **It's critical to break up clumps of cells so that we have an accurate count of cells for seeding**
7. Take 10ul of the cell suspension and transfer into 1.5ml eppi tube. I make 3 tubes to do 3 separate counts to get a better idea of how many cells are present

8. Take tubes to the cell counting station and turn on the CytoSmart cell counting machine. Follow instructions for username and password as mentioned on tablet. Pick the 1:1 trypan blue:cell setting if already not done on the machine
9. Add 10ul of Trypan blue from the top (it gets clunky at the bottom and that interferes with the reading) and flick tube. Do this before every reading since Trypan blue will kill cells
10. Adjust the glass coverslip on the slide, tilt the pipette and eject the sample into the space between the coverslip and the slide
11. Put under camera and the cells will be visible on the tablet screen. The cells should be visible such that the outer rim of the cells are in focus. Adjust if necessary. Click on "Count" and the machine will generate the numbers. Do the second reading to make sure that it is within range of the second. If not, take the 3rd reading. Take average of the readings
12. Record the number of live cells present. Record the viability as well. Cell viability >90% is desirable
13. Count number of cells needed per well as follows: (1x10^6 cells needed/well) / ('x' cells/mL) = 'y' mL/well
14. Since the total amount of cells and media in each well will be 2mL for a 6-well plate:

    2mL- 'y' mL cells/well = (2-y) mL media per well. When seeding more than one well, make MasterMix (MM) of cells and media for the number of wells needed. Add 10% extra, so it will be for 2.2 wells

    media: (2-y)mL/well x 2.2 wells; cells: y mL/well x 2.2 wells. Make this MM in a new bottle/tube
15. Mix media and cells well and and using reverse pipetting, use the P1000 to pick up 1mL of mixture and add to a well on the 6-well plate, tilting to the side and eject liquid. Repeat for the other 1mL for each well
16. Mix well by shaking plate gently sideways and up and down a few times. Take plate to microscope to see what the cells look like. They should be floating and evenly distributed, and preferably not present in clumps, since we want to grow a monolayer of cells
17. Put plate in 37C incubator for the next 16-18 hours

Transfection

1. Warm up Opti-MEM and Lipofectamine 2000 to room temperature. It shouldn't take too long
2. Take plate out and look at the cells. The cells should be evenly spread out across the well in a monolayer, >90% confluency is desirable. For our experiments, 95% confluency works quite well and doesn't end up killing cells after 48h. After visualizing, put cells back in the incubator until needed in step 8 below
3. We will prepare our DNA-lipid mixes in 500ul Opti-Mem/well. Prepare everything in a MM as before, adding an additional 10%. Since we're doing two wells, it will be 2.2 wells for our calculations
4. Pipet 250ul x 2.2ul= 550ul Opti-MEM into a 1.5mL eppi
5. We will be using 10ul Lipofectamine 2000 per well so 10x2.2= 22ul Lipofectamin2000. Add this amount to the Opti-MEM aliquoted above. Mix 2-3 times and let sit on bench for ~5min
6. In a separate 1.5mL eppi, add the same amount of Opti-MEM as above
7. Since we'll be adding 4ug of the library per well, adjust again for 2.2 wells, so the amount needed is 8.8ug. 8.8ug/ 'concentration on maxiprep)= 'x' ul of DNA goes into the second 1.5mL eppi. Mix well and add the whole Opti-DNA mix to the Opti-Lipo mix above. Mix again several times to ensure even distribution and then let sit for 20min for the DNA-lipid complexes to form
8. Bring the 6-well plate out of the incubator and using reverse pipetting, add 500ul of the Opti-DNA-Lipo mix to each well. Can be added directly on top of the media
9. Shake plate gently sideways and up and down a few times to distribute the mixture across the media
10. Put back in incubator and record time at which the transfection was done. After 48h, the cells will be harvested

Cell harvesting

1. After 48h, visualize cells under microscope. There will be plenty of dead cells floating but there should be a confluent layer of cells underneath
2. Aspirate off media
3. Add 1mL of PBS to each well
4. Aspirate
5. Add 1mL of TriZol to each well

6. Let sit for a few mins for the TriZol to dissolve the cells and peel them off the plate
7. Pipet up and down several times to collect the cells from the plate and put into 1.5mL eppis
8. Cells can be frozen at -20C at this point until needed further