n3D Biosciences, Inc.

Optimization Procedure for Treating Supended Cells with Nanoshuttle-PL™.

We suggest to start with cells in media at a concentration of 4.8 million cells/mL (cell mixture).

Step 1: Prepare two 60 mm petri dishes under sterile conditions. Label petri-dishes as: Petri-dish A and Petri-dish B.

Step 2: Add 2.5 mL of cell mixture (4.8 million cells /ml) to each 60 mm petri-dish from Step 1 (total of 12 million cells per dish).

Step 3: Adding Nanoshuttle-PL™ solution:
- *Petri-dish A*: Add 600 uL of Nanoshuttle-PL™ solution to one of the petri-dishes, bringing the concentration of Nanoshuttle-PL™ solution to 1 uL per 20,000 cells.
- *Petri-sish B*: Add 400 uL of Nanoshuttle-PL™ solution the other petri-dish, bringing the concetration of Nanoshuttle-PL™ solution to 1 uL per 30,000 cells.

Step 4: Shake both petri-dishes 30 minutes (preferably at at 37°C) at 80 RPM on an orbital shaker.

Step 5: Transfer petri-dish A and petri-dish B from step 4 to two separate 15 mL conical tubes and centrifuge at 1,000 rpm for 1 minute.

Step 6: Add warm media to each 15 ml tube, bringing the cell concentration to 2 million/mL.

Step 7: Pipette the solution in both dishes vigorously to break up clumps and aggregated particles.

Step 8: Transfer the cell-media mixture from both petri-dishes to a 24-well plate (orient the plate so there are 4 rows and 6 columns) such that:
- Each of the 4 rows are populated with a different number of cells (400,000; 200,000; 100,000; and 50,000).
- The left side of the 24-well plate contains cells from petri-dish A and the right side of the plate contains cells from petri-dish B (so as to separate the different concentration levels of Nanoshuttle-PL™ solution).

Step 8: Place the magnetic driver on top of the 24-well plate, and put the plate in an incubator at 37°C.

Step 9: Evaluate levitation yield and culture morphology differences between cultures from petri-dish A and petri-dish B to decide which condition is optimum to your experiment.