Immoblization of Non-Adherent Cells and XF Assay

This protocol covers those cells grown in suspension that do not naturally settle to bottom of well in a plate under gravity, thus requiring centrifugation to settle down at the bottom of the well.

Assay Medium: standard assay medium (DMEM or KHB)
Other Materials: XF24 Tissue Culture Plate coated with BD Cell-Tak Cell Adhesive
Benchtop centrifuge with swing-bucket rotor equipped with plate carriers, such as the Eppendorf Centrifuge 5810R.

I. Prepation of Cell-Tak Coated Plates

I.1. Reagents
- I.1.1. BD Cell-Tak Cell and Tissue Adhesive (BD Biosciences, CN 354240)
- I.1.2. $NaHCO_3$ (Sigma, S5761)
- I.1.3. Tissue Culture Grade Sterile Water (Invitrogen, CN 15230)

I.2. Preparation
- I.2.1. Dissolve 420 mg $NaHCO_3$ in 50 mL TC water and pH to 8.0 (should be very close) for 0.1 M solution. Filter-sterilize; use or store at 4°C.
- I.2.2. Follow Cell-Tak coating protocol provided with Cell-Tak.
  - I.2.2.1. Use a Cell-Tak density of 3.5 ug/cm2.
  - I.2.2.2. The area for one XF well is 0.32 cm2.
- I.2.3. 50 uL neutralized Cell-Tak solution should be applied per well.
- I.2.4. For one XF24 plate, prepare about 1.5 mL Cell-Tak solution.
- I.2.5. For example, if Cell-Tak stock density is 2 mg/mL, add 17 uL Cell-Tak stock to 1.50 mL 0.1 M $NaHCO_3$, mix, and pipette immediately onto XF plate, 50 uL/well.
- I.2.6. Let sit in hood about 20 minutes, then siphon off solution, add 200 uL TC water and siphon off to wash off bicarbonate, and let sit in hood for 20 minutes to dry with lids open.
- I.2.7. Use or save for (1 week) in 4°C refrigerator with rim wrapped in parafilm to avoid condensation.
- I.2.8. Let plates stored at 4°C warm up to room temperature for about 20 minutes in the hood before seeding. Do not warm at 37°C, Cell-Tak will lose activity.

II. Seeding Cells in Cell-Tak coated plates for XF assay.

Note: Optimal cell density is cell-line specific. Cell number titration will be necessary to determine optimal cell density for assays. In this protocol, example numbers are given in parenthesis for Jurkat cells seeded at 150,000/well.

- II.1. Warm assay medium in a 37°C water bath.
- II.2. Determine cell density of cell culture to be assayed using Vicell or hemocytometer.
- II.3. For two XF plates, transfer appropriate volume of cell suspension from the growth vessel to a 50 mL conical tube. To calculate the amount of cells needed, multiply the desired number of cells per well times 50 (for example, 150,000 cells per well x 50 = 7.5 million cells).
- II.4. Centrifuge cells down at room temperature, 1200 rpm, 5 minutes.
- II.5. While cells are centrifuged, pipette 100 uL assay medium into temperature control wells of two room-temperature Cell-Tak coated XF24 Tissue Culture plates.
- II.6. Remove supernatant and gently flick the bottom of the tube with finger to loosen the pellet.
- II.7. Resuspend cells in 5 mL warm assay medium to make a cell suspension (1.5 million cells/mL for Jurkat cells.)
- II.8. Change centrifuge settings to settings used for seeding: set centrifuge to slow acceleration (4 on a scale of 9 for Eppendorf 5810R) and zero braking.
- II.9. Transfer the cell suspension to a tissue culture reservior.

II.10. With a Biohit multipipettor, pipette 100 uL cell suspension to the side of each well, excepting temperature controls. (For Jurkat cells, final cell density is at 150,000/well.)

II.11. Centrifuge the cells down to the bottom of the well as follows:

II.11.1. Transfer plates to centrifuge plate carriers immediately after seeding. Orient plates symmetrically (e.g. so that column 1 of each plate points in to the center of the rotor), balancing the rotor.

II.11.2. Spin centrifuge up to 450 rpm (about 40g) and hit stop as soon as it reaches 450; let centrifuge spin slowly to a stop (remember, no braking).

II.11.3. Reverse the orientation of the plates on the plate carriers (now column 1 should point out to the rim of the centrifuge.)

II.11.4. Spin centrifuge up to 650 rpm (about 80g) and stop; again, let centrifuge spin slowly to a stop without braking.

II.12. Transfer plates to a 37°C incubator NOT supplemented with CO_2 and let sit for 25-30 minutes. Most of the cells should be stably adhered to the culture surface in 25-30 minutes.
Note: they will be morphologically indistinguishable from cells settled on an uncoated XF plate.
Note: sensor cartridge calibration should be started at this time on the XF Analyzer to streamline the assay process.

II.13. After 25-30 minutes incubation, slowly and gently add 500 uL warm assay medium to the top of each well along the side of the wall. Use manual P1000 pipettor only and add medium carefully and gradually to avoid disturbing cells.

II.14. Observe the cells under the microscope to check that cells are not disturbed.

II.15. Return to cell plates to incubator for 15-25 minutes.

II.16. After 15-25 minutes, cell plates are ready for assay. Total time following centrifugation should be no greater than one hour for best results.

II.17. Place cell plate on XF24 Analyzer (when calibration finishes).

II.18. Proceed following XF assay protocol.

II.19. This protocol specifies the full-plate seeding of two XF plates. If only one plate is desired at a time, a dummy plate can be made for rotor balance by adding 100 uL water to each well and cell quantity and suspension volume may be halved. However, the seeding of two plates is encouraged when beginning work with a cell line for additional practice with step 16 (addition of medium without disrupting cells). If there are not enough cells to seed one full plate, adjust resuspension volume to maintain appropriate concentration, seed preferentially into center wells (rows B and C, columns 2 through 5) and fill all remaining empty wells with 100uL assay medium before centrifugation.