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We use this protocol and it's working

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Mito and Glycolysis Stress Tests for Enteroendocrine Cells - Hutu-80

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ABSTRACT

The Seahorse XFp Extracellular Flux Analyzer is designed to compare two different cultures or treatments. Culture cells as usual in either cell culture dishes or flasks as preferred. It is best to compare two cultures of a similar passage number, cell density, time that the cells have been in culture and time since the medium was changed as all this influences mitochondrial respiration. Culture cells until they are almost/just confluent. Cell should have been fed recently before use.

MATERIALS

Materials for the culturing of fibroblasts:
Cell culture plates or flasks, standard cell culture medium, PBS, trypsin solution or accutase, laminar flow hood, pipets, tubes, centrifuge, CO_2 incubator, microscope, etc.

Materials for humidified incubation of cell cultures without CO_2:
A 37°C incubator (no CO_2). A large plastic box with a lid and a smaller plastic box without a lid. Put some tap water in the large box. Put the small box into the large box and close with the lid. Put the boxes in the incubator.

XFp Extracellular Flux Analyzer and XFp mini-plate carriers (Agilent, S7852A).
XFp FluxPak (Agilent, 103022-100).
This consists or a box with 12 XFp Cell Culture Mini-Plates and a box with 12 XFp Sensor Cartridges, and includes XF Calibrant. The XFp Cell Culture Mini-Plates can also be bought separately (103025-100) but the XFp Sensor Cartridges cannot be purchased on their own.

XF Calibrant (Agilent; 100-mL bottle, 103059-100; 500-mL bottle, 100840-000).
Seahorse XF DMEM medium, pH 7.4, 500 mL (Agilent; 103575-100).
Store at 4°C.

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Wave Controler 2.3 programme and Microsoft Excel macro for data analyses
Download at Seahorse website: http://www.agilent.com/en-us/products/cell-analysis-(seahorse)/software-download-for-seahorse-xfp-analyzer

Cell counting slides, *e.g.* C-Chip (DHC-NO1) Neubauer Improved Disposable Haemocytometer (Incyto, NanoEnTek; FisherScientific)

Trypan blue stain, 0.4% (Life Technologies (Gibco), 15250-061)
Store at room temperature.

BioTek Cytation-1 Cell Imaging Multimode Reader, Agilent

d-(+)-Glucose (Merck/Sigma, G7021-1KG)
Prepare a 2.5 M glucose stock by dissolving 4.5 g of glucose in a total volume of 10 ml of water. (Warm carefully to help to dissolve). Sterilise by passing through a 0.2-mm filter and store in a sterile 50-ml tube at room temperature.

100 mM Sodium pyruvate (Merck/Sigma, S8636)
Store at 4°C.

200 mM l- glutamine (Merck/Sigma, G7513)
Store at -20°C. Thaw out shortly before use in a water bath (vortex briefly when solution is thawed out). Freeze immediately after use because glutamine breaks down at room temperature.

2-Deoxyglucose (2DG; Merck/Sigma, D8375-5G)
Prepare a 500 mM 2-deoxyglucose solution by dissolving 82.1 mg of 2-deoxyglucose per 1 ml of Seahorse XF DMEM medium, pH 7.4, sterile in the laminar flow hood. Store in a plastic bijou at -20˚C.

20 mM Hoechst 33342 (ThermoFisher)
Store at 4°C.

Oligomycin A (Merck/Sigma, 75351-5MG; toxic, use gloves; store powder at -20°C).
Make a 10 mM oligomycin A stock in ethanol by adding 633 ml of ethanol to the vail. Vortex briefly to dissolve. Store stock at -20°C. Place on ice when the stock is used.

FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Merck/Sigma, C2920-10MG; toxic, use gloves; store powder at -20°C).
Make a 40 mM FCCP stock in ethanol by adding 984 ml ethanol to the vail. Vortex briefly to dissolve. Dilute to 10 mM FCCP with ethanol: in a 1.5-ml screw-top tube add 750 ml ethanol and 250 ml 40 mM FCCP. Store both stocks at -20°C. Place on ice when the stock is used.

Antimycin A (Sigma, A8674-100MG; toxic, use gloves; store powder at -20°C). Make a 10 mM stock: weight out 5.4 mg of antimycin A in a 1.5-ml screw-top tube. Add 1 ml of ethanol. Vortex briefly to dissolve. Wrap tube in aluminium foil because antimycin A is light sensitive. Store stock at -20°C. Place on ice when the stock is used.

Rotenone (Sigma, R8875-1G; toxic, use gloves; store powder at room temperature). Make a 10 mM stock: weight out 4 mg of rotenone in a 1.5-ml screw-top tube. Add 1 ml of ethanol. Vortex extensively (for minutes) to dissolve. Make sure that the rotenone is fully dissolved. Wrap tube in aluminium foil because rotenone is light sensitive. Store stock at -20°C. Place on ice when the stock is used.

Day 1 - Harvesting, Counting and Diluting

5m

1 Harvest two cell cultures flasks - work in a laminar flow hood.

1.1 Aspirate off medium; rinse each flask with 5 mL of PBS; aspirate off PBS; add 1 mL of accuitase solution to each flask; incubate 00:05:00 at 37 °C; add 9 mL of medium to the plate; transfer resuspended cells to a 50-ml centrifuge falcon. 5m

2 Pellet cells by centrifugation 500 x g, Room temperature, 00:05:00 5m

3 While centrifuge is spinning, pipet 10 µL of Trypan blue solution in two 0.5-ml tubes placed in a rack.

4 When centrifugation of cell suspension is finished, aspirate off supernatants.

5 Re-suspend cell pellets thoroughly in 5 mL of medium, , by pipetting up and down.

6 Pipet under sterile conditions 10 µL of the cell suspension into two 0.5-ml tubes with Trypan blue one for each condition.

7 Load 10 µL of each cell suspension in Trypan blue into the slots of a counting slide.

8 Under a microscope, count the number of cells in 5 of the 9 large squares, average the counts and write down the cell number. (Each large square has a volume of 0.1 ul).

9 Calculate the number of cells per ul:

Number of cells per ul = [(number of cells in 5 large squares)/5] x dilution factor x 10
The dilution factor is 2 (1:1 dilution with Trypan blue solution).
Thus: Number of cells per ml (cell density=CD) = number of cells in 5 large squares x 4

10 The wells of the XFp Cell Culture Mini-Plate need to be seeded with 20,000 enetoendocrine (EEC) cells in 80 µL medium. Thus, a cell density of (20,000/80=) 250 cells/ul is required.

Use the equation below to calculate the amount of medium that needs to be added to the cell suspension in the 50-mL centrifuge falcon to obtain the correct density for seeding of the XFp Cell Culture Mini-Plate.

$$B = [(CD/250) \times A] - A$$

A = the total volume of the cell suspension in the 50 mL centrifuge falcon (in ml; see step 5)

B = the volume to be added to the 50-mL centrifuge falcon to obtain the correct cell density for seeding of the XFp Cell Culture Mini-Plate (in ml)

CD = the cell density determined with the counting slide (in cells/ml; see step 9)

11 To the 50-mL falcon with the cells add the calculated amount of medium to obtain a cell density of 250 cells per ul

12 Add 400 µL of the XF Calibrant to each moat of the XFp Cell Culture Miniplate. Once this is done you can place the lid back on and place this in a CO_2 incubator Overnight .

13 Re-assemble the XFp Extracellular Flux plate and place in a humidified 37 °C incubator (no CO) Overnight

14 Then in wells A and H of the XFp Cell Culture Miniplates add 80 µL of your cultuing media containing no cells - these are use to calibrate the plate.

15 In wells B-D for condition 1 (usually the control group) and wells E-G for condition 2 add 80 µL of the media now containing the resuspended cell. After seeding the remaining cells can be used to seed new dishes or flasks for further culturing.

16 In the XFp Extracellular Flux plate remove the top green section of the cassette and place upside down without touching the sensors (light sensitive) fill each moat with 400 µL of the XF Calibrant and then each well with 200 µL of the XF Calibrant.

17 Switch on the Seahorse XFp Extracellular Flux Analyzer with the switch at the back of the machine. It is important to switch on the machine ≥5 h before use. In practice, switch the machine on the day before you use it. The machine is designed to be switched on over long periods of time, so there is no need to switch it off if it is not used for 1–2 days.

18 Step 18 includes a Step case.
Preparation of mito stress test assay medium
Preparation of glycolysis stress test assay medium

Day 2 - Preparation of Assay Medium

5m

step case

Preparation of mito stress test assay medium

Prepare under sterile conditions in laminar flow hood.
As we routinely perform two glycolysis stress tests on one day, the volumes given below are sufficient for two experiments. If only one experiment is carried out, half the volumes.

19 Pipet 20 mL of Seahorse XF DMEM medium, pH 7.4, in a 20-ml tube.

19.1 Add to the tube:

- 80 µL of [M] 2.5 Molarity (m) d -(+)-glucose
- 200 µL of [M] 100 millimolar (mM) sodium pyruvate
- 200 µL of [M] 200 millimolar (mM) l-glutamine

Mix by vortexing

The XF DMEM medium, pH 7.4, now contains [M] 10 millimolar (mM) d -(+)-glucose, [M] 1 millimolar (mM) sodium pyruvate and [M] 2 millimolar (mM) l-glutamine. This medium is called mito stress test assay medium.

20 Warm mito stress test assay medium in a 37 °C water bath.

Once warmed up, the mito stress test assay medium can be kept at room temperature. The medium can be used for one day. It is not necessary to keep the medium sterile, as it is used within one day.

Day 2 - Replacing of standard medium with assay medium

1h

21 There is no need to work sterile in the laminar flow hood. It is easier to do this on the lab bench.

Retrieve the XFp cell manipulate(s) Fromm the tissue culture incubator. You may wish to keep the miniplate(s) in the Carrier Tray for ease of handling.

22 Look at the cells under the microscope to:

- Confirm cell health, morphology and purity (no contamination).
- Ensure that the cells are adhered, and appear as a consistent monolayer.
- Make sure the background cells (A and H) contain no cells.

23 Wash the cells with assay medium:

Remove all but 20 µL of the culture medium from each well. The small amount of medium is left to keep the cells from drying out.

23.1 Gently add 200 µL of assay medium, then remove the same amount.

23.2 Repeat step 24.1.

23.3 Add 155 µL to a total volume of 175 µL per well.

24 Observe the assay wells under a microscope to ensure that the cells were not washed away.

25 Place the plate in a humidified 37 °C incubator **without** CO_2 for 01:00:00 prior to the assay. 1h

Day 2 - Preparation oligomycin A, FCCP and rotenone + ant...

26 Take the [M] 10 millimolar (mM) oligomycin A, [M] 10 millimolar (mM) FCCP, [M] 10 millimolar (mM) rotenone and 10 mM antimycin stock solutions out of the -20 °C freezer and put on ice. Also take the [M] 20 millimolar (mM) Hoechst 33342 stock solution out of the fridge and put on ice.

27 Pipette 1 mL of mito stress test assay medium each in three 1.5-ml tubes and place in a rack.

28 Label the tubes with “Oligo”, “FCCP” and “Rot + AA”.

29 Pipette 5 µL of [M] 10 millimolar (mM) oligomycin A into the tube labelled “Oligo” to make a [M] 50 micromolar (µM) solution.

30 Pipette 5 µL of [M] 10 millimolar (mM) FCCP into the tube labelled “FCCP” to make a [M] 50 micromolar (µM) solution.

31 Pipette 5 µL of [M] 10 millimolar (mM) rotenone and 5 µL of [M] 10 millimolar (mM) antimycin A into the tube labelled “Rot + AA” to make a [M] 50 micromolar (µM) + [M] 50 micromolar (µM) solution.

32 Vortex the three tubes briefly.

33 Label three new 1.5-ml tubes with “Oligo 2”, “FCCP 2” and “Rot + AA + H”.

34 Pipette 408 µL of mito stress test assay medium in the tube labelled “oligo 2” and add 192 µL of [M] 50 micromolar (µM) oligomycin A.

35 Pipette 438 µL of mito stress test assay medium in the tube labelled “FCCP 2” and add 162 µL of [M] 50 micromolar (µM) FCCP.

36 Pipette 380 µL of mito stress test assay medium in the tube labelled "Rot + AA +H" and add 100 µL of [M] 50 micromolar (µM) rotenone + [M] 50 micromolar (µM) antimycin A. Then add 20 µL of [M] 20 millimolar (mM) Hoechst 33342 stock solution.

37 Vortex the three tubes briefly.

NB: The final concentrations in the wells of the XFp cell miniplate are: [M] 2 micromolar (µM) oligomycin A, [M] 1.5 micromolar (µM) FCCP, [M] 1 micromolar (µM) rotenone, [M] 1 micromolar (µM) antimycin A and [M] 66 micromolar (µM) Hoechst 33342.

38 Eliminate air bubbles from the XFp Extracellular Flux Cartridge/Plate.

38.1 Following the overnight incubation, remove the cartridge assembly from the incubator.

38.2 Lift the sensor cartridge completely out of the calibrant and utility plate.

38.3 Immediately return the sensor cartridge back onto the utility plate, submerging the sensors in calibrant. This step helps eliminate any bubbles that may form during overnight hydration.

Day 2 - Loading of the XFp Sensor Cartridge with oligomyci...

39 Orient the XFp Assay Cartridge: Place well labels (letters A-H) to the left. The triangular notch will be in the bottom left-hand corner.

40 Using a p200µL pipette, make sure the tip is securely fitted onto the pipette. Pipette 25 µL of the oligomycin A solution from the tube labelled "oligo 2" in all eight ports A.

41 Pipette 25 µL of the FCCP solution from the tube labelled “FCCP 2” in all eight ports B.

42 Pipette 25 µL of the rotenone + antimycin A + Hoechst 33342 solution from the tube labelled “rot + AA + H” in all eight ports C.

Port D remains empty.

Day 2 - Calibration and measurements with the XFp Extrace..

1h 40m

43 Touch the screen and Press “Start” on the screen of the XFp Extracellular Flux Analyzer.

44 Press “Cell Mito Stress Test”

45 Press “**>**” (bottom right of screen).

46 Repeat step 46.

47 Press “Start Assay”. After some time, the plate holder will come out of the machine.

48 Carefully transfer the cartridge/utility plate assembly to the plate holder of the XFp Analyzer according to the orientation as indicated on the screen.

IMPORTANT: Remove the plate lid before inserting the cartridge into the machine.

49 Press “Continue”. 20m

The calibration of the XFp Assay Cartridge will take ~ 00:20:00 . After the calibration is completed, the screen will indicate that the machine is ready to receive the XFp Cell Culture Miniplate.

50 Press “Continue”.

Take the XFp Cell Culture Miniplate from the 37 °C incubator and replace the utility plate with the XFp Cell Culture Miniplate as indicated on the screen.

IMPORTANT: Remove the plate lid before inserting the cartridge into the machine.

51 Press “Continue”. 1h 20m

Throw away utility plate. The equilibration and measurements will take ~ 01:20:00 .

During this time, you can replace the standard medium of a next plate with mito stress test assay medium and incubate the next plate for ≥1 h in the humidified 37 °C incubator (no CO_2).

52 About 5–10 min before the assay is finished, you can eliminate air bubbles from the next XFp Sensor Cartridge and load the Cartridge with oligomycin A, FCCP and rotenone + antimycin A + Hoechst 33342 for the mito stress.

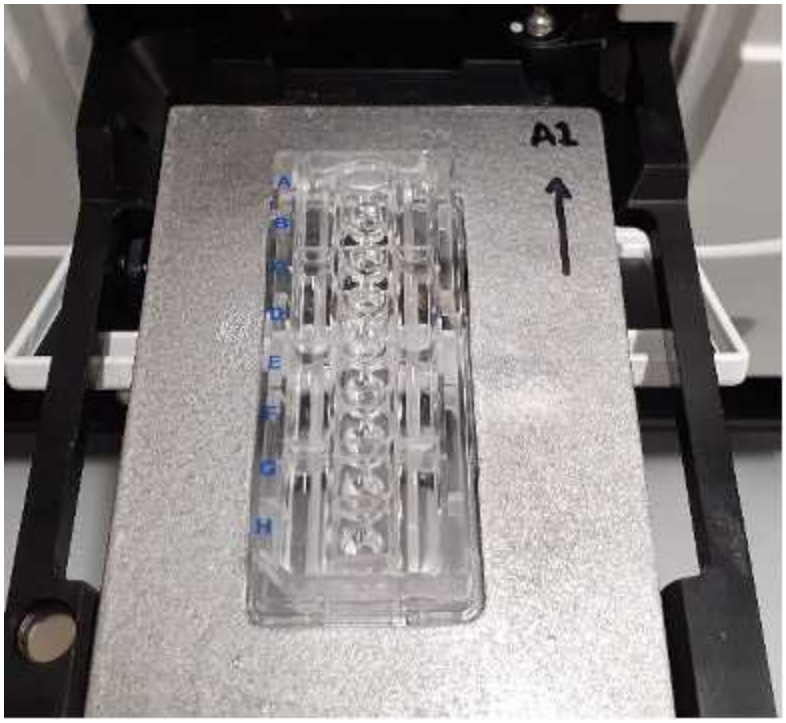
53 After the measurements have been taken, follow the instructions on the screen. This will ensure that the data are automatically stored on the memory stick under a distinctive name consisting of the date, a unique number and the type of test.

Day 2 - Determination of cell number with the BioTek Cytat... 10m

54 Switch on the BioTek Cytation-1 Cell Imaging Multimode Reader and computer - may require booking out in advance. Let the reader and computer start up (~ 00:05:00). 5m

55 Take the Miniplate out of the Seahorse machine.

56 Place the Miniplate in the metal adaptor (very snug fit) and place in the plate holder of the BioTek Cytation-1 Cell Imaging Multimode Reader with well A at the top (see picture).



57 Start the Gen5 programme by clicking on the icon on the screen.

58 Open the existing protocol “JWT 8 well seahorse DAPI only” in the Task Manager window. (Cancel saving “Experiment1”.)

59 Click “OK” when the plate is loaded in the plate holder to start the analysis. There should not be a lid on the miniplate but, on the screen, the box “Use lid” can be checked and let the machine record the images (~ 00:05:00). 5m

60 Save the Excel file to save the images.

61 Click on an image of a well to open the analyses window - in the Gen5 software.

62 Deselect the “SEAHORSE: bright Field” box, so that only the “SEAHORSE DAPI” box is selected. Then Click on “Analyse” (middle top of the screen).

63 Select “I want to edit an existing Image Analysis step”.

64 Click on “Cellular Analysis Cell Count” and click “OK”. Then click on “Options”.

65 Make changes so that most nuclei are counted. Here we checked the “Auto” box (top left of screen). Changed the min object size to 5um and the max object size to 40um, in the advanced setting we also changed the image smoothing strength to 1. Then click on “APPLY CHANGES”.

66 The cell count is given on the top right side of the screen. Close window (“X”, top right of the screen). Write down cell numbers for each well. Multiply the cell count by 10 to get the number of cells per well. This is used to normalise the data in the Wave Programme.