Cell Mito Stress Test for Monocyte Mitochondrial Function

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Abstract

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Materials

Cell-Tak 354240 by Corning

Seahorse XFp FluxPak 103022-100 by Agilent Technologies

Seahorse XFp Cell Mito Stress Test 103010-100 by Agilent Technologies

Seahorse Base Medium DMEM 102353-100 by Agilent Technologies

100 mM Sodium Pyruvate S8636 by Sigma

200 mM L-Glutamine G7513 by Sigma

45% D-( )-Glucose G8769 by Sigma

0.1 M Sodium Hydroxide Solution 71395 by Sigma

✓ 0.1 M Sodium Bicarbonate Buffer, pH 8.0, Sterile by Contributed by users

✓ Pipettes and P1000, P200, P10 tips by Contributed by users

Protocol

Step 1.

Day Prior to Assay:

-Turn on XFp analyzer to warm up overnight

-Hydrate XFp sensor cartridge

Add 200 µl XF calibration solution (included with FluxPak) to each

well. Add 400 µl sterile PBS or H2O to each moat. Incubate overnight at 37degC in non-CO2 incubator.

Step 2.

Materials to Prepare:

Seahorse DMEM Media:

Add 50 µl pyruvate, 50 µl L-glutamine, 20 µl glucose to 5 ml Seahorse Base Medium. Sterile filter before use.

Cell-Tak coated XFp plate.

Prepare Cell-Tak. Add Cell-Tak and sodium hydroxide to 0.1 M sodium bicarbonate so that each well will receive 0.56 µg Cell-Tak, and sodium hydroxide concentration will be 0.63 mM. Cell-Tak concentration varies by batch, so calculations for each new batch will need to be performed. Add 25 µl Cell-Tak solution to each well. Plates can be stored at room temperature (at least 20 minutes) until use (for a few hours). Plates can also be prepped prior to assay day by incubating plates in Cell-Tak (at least 20 minutes), then aspirating remaining solution, air drying, and storing at 4degC until use.

Monocytes

Monocytes should be prepared as directed in the monocyte isolation protocol (https://dx.doi.org/10.17504/protocols.io.kwtcxen). Isolated monocytes should be diluted to a concentration of 3×10^6 cells / ml in prepared Seahorse DMEM media prior to use in the assay. Use monocytes immediately.

Step 3.
Aspirate Cell-Tak solution if not already done.

Step 4.

Add 50 µl medium to wells A and H and 50 µl cells to wells B-G. Samples are generally run in duplicate or triplicate on each plate. Cell number is 1.5×10^5 cells per well.

Step 5.
Place plate in the carrier and place in centrifuge. Spin 300×g for 1 minute without break.

Step 6.
Add 130 µl assay medium to each well A-H (final volume 180 µl).

Step 7.

Incubate plate at 37degC in non-CO2 incubator for 1 hour.

Step 8.

While plate is incubating, perform steps 7 through 11

Step 9.

Prepare preliminary drug dilutions (mix by pipetting up and down)

- 50 µM oligomycin (blue cap) - add 252 µl medium

- 50 µM FCCP (yellow cap) - add 288 µl medium

- 25 µM Rotenone/Antimycin A (red cap) - add 216 µl medium

Step 10.

Prepare final drug dilutions

- 10 µM oligomycin - 120 µl of 50 µM oligomycin in 480 µl medium

- 20 µM FCCP - 120 µl of 50 µM FCCP in 180 µl medium

- 5 µM rotenone/antimycin A - 60 µl of 25 µM rot/AA in 240 µl medium

Step 11.
Remove sensor cartridge from incubator and remove and reinsert sensors briefly to clear air bubbles.

Step 12.

Fill cartridge:

- Port A (all wells): 20 µl oligomycin (1 µM final concentration)

- Port B (all wells): 22 µl oligomycin (2 µM final concentration) (note 1)

- Port C (all wells): 25 µl FCCP (2 µM final concentration)

- Port D (all wells): 27 µl rotenone/antimycin A (0.5 µM final concentration)

NOTES
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(1) We do two separate injections of oligomycin, although one seems to work (although less consistently). If one injection is desired, prepare oligomycin so that final concentration after the first injection is 2 µM.

Step 13.

Select Cell Mito Stress Test program (note 2) on Seahorse XFp and calibrate sensor cartridge (remove lid).

NOTES
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If two oligomycin injections are used, a custom program will need to be run.

Step 14.
After 1 hour cell incubation, remove utility plate from XFp and insert cell plate (remove lid).

Step 15.
Run Cell Mito Stress Test. 3 measurements per injection (including basal) is sufficient.

Step 16.
After run is completed, image each well by photomicroscopy or collect and isolate protein from each well to normalize cell numbers.

Step 17.

Data Analysis

Data can be analyzed in the following manner. Averages or mean/max for each condition can be used.

Basal Respiration: (unstimulated OCR) - (rotenone_antimycin A OCR)

ATP-Linked Respiration: (unstimulated OCR) - (oligomycin OCR)

Respiratory Capacity (Maximal Respiration): (FCCP OCR) - (rotenone_antimycin A OCR)

Spare Capacity: (FCCP OCR) - (unstimulated OCR)

Proton Leak: (oligomycin OCR) - (rotenone_antimycin A OCR)

Non-Mitochondrial Respiration: (rotenone_antimycin A OCR)

Calculations are depicted in Figure 1.

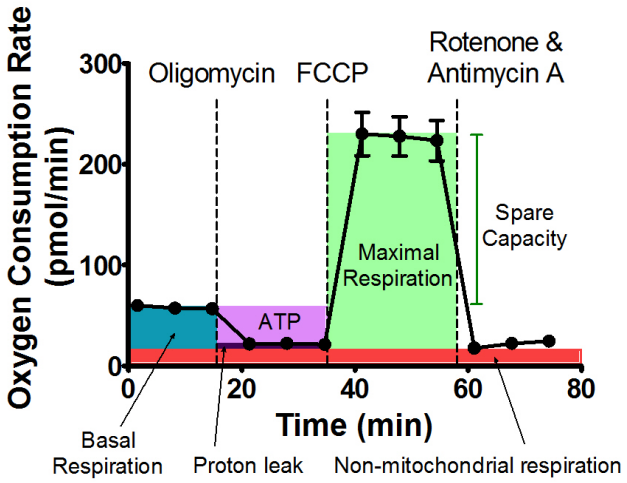

Figure 1: Cell Mito Stress Test calculations