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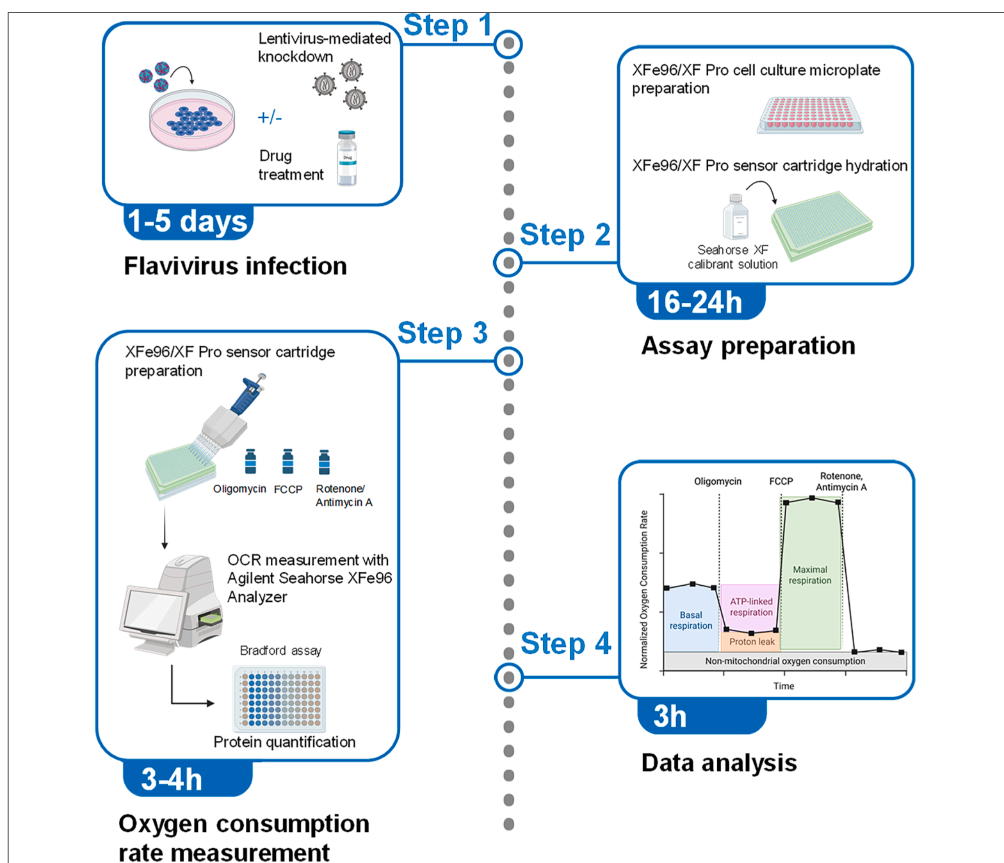
Protocol

Protocol to assess changes in mitochondrial respiration in living cultured cells infected with flaviviruses



Viviana Andrea Barragan Torres, Laurent Chatel-Chaix

viviana.barragan@inrs.ca (V.A.B.T.)
laurent.chatel-chaix@inrs.ca (L.C.-C.)

Highlights

Instructions for flavivirus infection and cell preparation before the assay

Procedure for measuring oxygen consumption rates using Seahorse technology

Guidance for the normalization of respiration data

Mitochondria are essential organelles involved in energy production, making them prime targets for flaviviruses, such as dengue and Zika viruses, to enhance viral replication. Here, we present a protocol to measure multiple respiratory parameters in living hepatocarcinoma cells infected with flaviviruses in combination with pharmacological treatments or genetic expression modulation. We detail steps for infecting cells with flaviviruses and measuring the oxygen consumption rates using Seahorse technology.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol to assess changes in mitochondrial respiration in living cultured cells infected with flaviviruses

Viviana Andrea Barragan Torres[1,2,*] and Laurent Chatel-Chaix[1,3,*]

[1]Centre Armand-Frappier Santé Biotechnologie, Institut National de la Recherche Scientifique, Laval, QC H7V 1B7, Canada

[2]Technical contact

[3]Lead contact

*Correspondence: viviana.barragan@inrs.ca (V.A.B.T.), laurent.chatel-chaix@inrs.ca (L.C.-C.)
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SUMMARY

Mitochondria are essential organelles involved in energy production, making them prime targets for flaviviruses, such as dengue and Zika viruses, to enhance viral replication. Here, we present a protocol to measure multiple respiratory parameters in living hepatocarcinoma cells infected with flaviviruses in combination with pharmacological treatments or genetic expression modulation. We detail steps for infecting cells with flaviviruses and measuring the oxygen consumption rates using Seahorse technology.

For complete details on the use and execution of this protocol, please refer to Freppel et al.[1]

BEFORE YOU BEGIN

The mitochondrion, often referred to as the powerhouse of the cell, is the cellular organelle responsible for most energy production through processes such as the Krebs cycle, oxidative phosphorylation, and β-oxidation. Beyond their metabolic and respiratory functions, mitochondria also regulate key processes such as apoptosis, cellular senescence, cell cycle control, and antiviral innate immune responses. Since each of these cellular functions can positively or negatively influence host cell infection, it is not surprising that mitochondria are often co-opted by viruses to maximize their intracellular viral replication (for a review, see[2]). Notably, infection with dengue virus (DENV) or Zika virus (ZIKV), two clinically relevant (ortho)flaviviruses, was shown to modulate mitochondrial respiration.[1,3,4]

Here, we present a detailed workflow for measuring multiple respiratory parameters in hepatocarcinoma cells infected with these flaviviruses in combination (or not) with pharmacological treatments or genetic expression modulation. This includes a step-by-step procedure for accurate measurements of the oxygen consumption rates using the Seahorse technology. Such a protocol is transposable to any virus or adherent cell line.

This protocol takes advantage of the Agilent Seahorse XFe96 extracellular flux analyzer, a specialized instrument for real-time monitoring of cellular respiration in living cells. It measures changes in extracellular oxygen (O_2) and proton (H^+) levels, notably providing insights into mitochondrial function. Since O_2 acts as the final electron acceptor at complex IV of the electron transport chain (ETC) in the mitochondria, the oxygen consumption rate (OCR) is a direct indicator of mitochondrial respiration. Here, we use the Seahorse XF Cell Mito Stress Test Kit from Agilent to assess mitochondrial respiratory capacity. This assay sequentially introduces ETC inhibitors to modulate OCR resulting from mitochondrial and non-mitochondrial sources. Oligomycin inhibits ATP synthase (Complex V) and helps measure ATP-linked respiration, while carbonyl cyanide-4-(trifluoromethoxy)

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phenylhydrazone (FCCP) acts as an uncoupler, collapsing the proton gradient and resulting in stimulating respiration to maximal oxygen consumption. Finally, the combined treatment with rotenone and antimycin A inhibits Complex I and Complex III and thus mitochondrial respiration completely, which results in detecting only non-mitochondrial OCR.[5]

Innovation

The strength of this approach relies on the possibility of measuring respiration in real time in living cells infected with flaviviruses under biosafety containment and in a time-effective manner. Of note, this protocol is complementary to a similar previously published protocol by Low and colleagues,[6] as it provides additional details about critical technical tips for reproducibility maximization, an extensive troubleshooting guide, and an alternative method to normalize the OCR data. In addition, the possibility to increase the throughput here by analyzing 96 samples simultaneously allows the inclusion of multiple treatment conditions and replicates within the same experiment.

Institutional permissions

All the procedures described below were performed with the approval of the institutional biosafety committee ("Comité de biosécurité"; certificate number: #2016–12) of Institut National de la Recherche Scientifique (INRS).

To perform this protocol involving risk group 2-belonging DENV and ZIKV, the users must have obtained prior approval from their institutional biosafety committee and a proper biosafety certificate according to the national guidelines and regulations. This includes approval for the use of living infectious material with the Seahorse analyzer, which must be installed in a biosafety level 2 laboratory.

Flavivirus infection

Timing: 2–5 days

1. Seed 2×10^6 Huh7.5 cells in a 10 cm tissue culture dish in a final volume of 10 mL of supplemented DMEM culture medium.
2. The next day, prepare the virus solution at the desired multiplicity of infection (MOI) in 4 mL of supplemented DMEM.

Note: The MOI corresponds to the number of infectious virus particles per target cell during the infection and is calculated using the following formula based on known viral titer:

$$\text{MOI} = (\text{Viral titer} \times \text{Volume of virus stock added}) / \text{Number of cells}$$

Note: For flavivirus infection studies, optimal conditions for Zika virus (ZIKV) strain H/PF/2013 include a MOI of 5–10, with an incubation period of 48 hours post-infection (hpi). Similarly, for dengue virus serotype 2 (DENV2) strain 16681s, a MOI range of 1-2 with an incubation period of 48 hpi represents ideal infection conditions.

⚠ CRITICAL: An uninfected control condition must be included since it is the reference for normal respiration. It is important to ensure that the (infected) cells will be viable on the day of OCR measurements and that the infection rate is around 90%–100%. The percentage of infection can change depending on the used cell line and virus strain. Optimal MOI and time of infection can be determined through prior optimization experiments using confocal microscopy of viral protein-labeled infected cells.[1]

Optional: Cells knocked down for a gene of interest or overexpressing it via transfection or lentivirus transduction can also be used. In that case, we recommend to transfect/transduce the cells two days before flavivirus infection to maximize gene expression modulation.

3. Infect the cells (related to troubleshooting problem 5).
   a. Remove the culture medium by aspiration.
   b. Add 4 mL of the diluted virus suspension prepared in step 2.
   c. Incubate for 2 hours at 37°C in an incubator with 5% CO_2.
   d. Remove the viral solution by aspiration.
   e. Add 10 mL of supplemented DMEM medium.
   f. Incubate at 37°C/5% CO_2 until the desired time point.

Optional: After infection, cells may be treated with a drug of interest to be tested (*e.g.*, for its potential antiviral activity) although, as described below in step 5, we recommend treating cells directly in the Seahorse XFe96/XF Pro Cell Culture Microplates to avoid further trypsinization.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
ZIKV H/PF/2013	European Virus Archive Global	001v-EVA1545
DENV serotype 2 16681s	Produced with a reverse genetics system[7]	N/A
Critical commercial assays		
Seahorse XF Cell Mito Stress Test Kit	Agilent	103015-100
Seahorse XF DMEM medium, pH 7.4, 500 mL.	Agilent	103575-100
Seahorse XF 1 M glucose solution, 50 mL	Agilent	103577-100
Seahorse XF 100 mM pyruvate solution, 50 mL	Agilent	103578-100
Seahorse XF 200 mM glutamine solution, 50 mL	Agilent	103579-100
Seahorse XFe96/XF Pro FluxPak, containing: 1 500 mL bottle of calibrant 18 XFe96/XF Pro cell culture microplates 18 XFe96/XF Pro sensor cartridges	Agilent	103792-100 https://www.agilent.com/store/en_US/Prod-103792-100/103792-100
Experimental models: Cell lines		
Human hepatocarcinoma Huh7.5 cell line	Gift from Patrick Labonte (INRS)	N/A
Software and algorithms		
Wave	Agilent version 2.6.2	https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
GraphPad	Prism 8 GraphPad version 8.0.1	https://www.graphpad.com/features
Excel	Microsoft Office 365 suite	https://www.microsoft.com/en-us/microsoft-365/download-office
Other		
Seahorse XFe96 analyzer	Agilent	N/A
Spark multimode microplate reader	Tecan	N/A
Motic AE31 Elite phase contrast inverted microscope	Microscope Central	SKU: 1100100200861
Heracell VIOS 160i CO_2 incubator, 165 L	Thermo Scientific	51033559
Bright-Line hemacytometer	MilliporeSigma	Z359629

MATERIALS AND EQUIPMENT

Cell culture medium: Supplemented DMEM		
Reagent	Final concentration	Amount
Dulbecco's modified Eagle medium (DMEM)	N/A	440 mL
Fetal bovine serum (FBS)	10%	50 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Penicillin / Streptomycin (10,000 U/mL)	100 U/mL	5 mL
MEM non-essential amino acids (100X)	1X	5 mL
Total	N/A	500 mL
Note: Storage conditions: 4°C, up to 4 weeks.		

- Phosphate-buffered saline (PBS), cell culture grade.

  Note: Storage conditions: 4°C.

- Trypsin 0.25%- EDTA, cell culture grade.

  Note: Storage conditions: −20°C for long periods; when thawed, 4°C, up to 3 weeks.

Cell lysis buffer		
Reagent	Final concentration	Amount
NaCl 4 M	150 mM	7.5 mL
Tris 1 M pH 7.8	50 mM	10 mL
Triton X-100 20%	0.5%	5 mL
ddH_2O	N/A	177.5 mL
Total	N/A	200 mL
Note: Storage Conditions: 4°C, at least 6 months.		

STEP-BY-STEP METHOD DETAILS

Preparation of the XFe96/XF Pro cell culture microplate

Timing: 2–3 h

This section outlines the necessary steps for seeding the infected cells into the XFe96/XF Pro cell culture microplate following trypsinization.

⚠ CRITICAL: Verify the condition of the cells with a light microscope before proceeding with the experiment. Assess cell morphology and confluence, and document any signs of compromised viability (*e.g.*, virus-induced cytopathic effects), which may impact respiration.

1. Remove the medium from culture dishes by gentle aspiration.
   a. Wash cells twice with sterile cell culture-grade PBS.
   b. Add 4–5 mL of trypsin-EDTA to completely cover the cells.
   c. Incubate at 37°C for 2 min.
   d. Remove the trypsin-EDTA by aspiration.
   e. Tap the culture dish firmly to detach the cells.
2. Resuspend the cells in 2–5 mL of supplemented DMEM medium depending on cell confluence.

   Note: Avoid diluting the cells in large volumes, as this may make it difficult to achieve the desired cell concentration later during seeding.

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⚠ CRITICAL: Resuspend the cells by pipetting up and down several times to prevent clumping and obtain a single-cell suspension.

3. Determine the cell concentration of the solution using a hemocytometer.
4. Prepare a cell solution at 2.5 × 10^5 cells/mL by diluting the cells in supplemented DMEM and seed 180 μL per well in the XFe96/XF Pro cell culture microplate.

Note: Prepare as many wells as possible per condition to maximize the number of replicates. Generally, we prepare a minimum of 5 replicates. However, ensure that at least four wells contain only cell-free medium. They will serve as blank controls.

⚠ CRITICAL: Distribute conditions horizontally across the plate (columns 1 to 12). This minimizes the impact of errors that may occur during cartridge preparation (which follows vertical orientation rows A to H).

5. Incubate at 37c-5% CO_2 for 16–24 h.

⚠ CRITICAL: Do not leave any wells empty in the XFe96/XF Pro cell culture microplate. Fill all unused wells with medium. They can serve as additional blank wells. This will prevent evaporation and drying of the surrounding wells.

Note: We do not recommend carrying out the flavivirus infection of cells directly in the 96-well XFe96/XF Pro cell culture microplate (instead of in a 10-cm dish) since this generally results in very high variability in infection rate across replicates. However, in other applications such as drug testing, it can be appropriate to treat the cells already seeded in the XFe96/XF Pro cell culture microplate (when no infection is intended). This aims to avoid the trypsinization step since some drugs may cause cellular stress and/or impact cell adherence during reseeding.

If both infection and drug treatment are required, we suggest infecting the cells in a 10 cm culture dish. At 2 hours post-infection, wash and trypsinize the cells, and seed them into an XFe96/XF Pro Cell Culture Microplate (generally at 25,000-50,000 cells/well). Four hours after seeding, remove the culture medium and replace it with fresh medium containing the desired concentration of the test compound.

Hydration of the XFe96/XF Pro sensor cartridge

⏲ Timing: 16–24 h

This section outlines the procedure for properly preparing the XFe96/XF Pro sensor cartridge the day before the assay in parallel to the cell culture/treatment, to ensure accurate sensor performance during the assay.

Note: Hydrating the XFe96/XF Pro sensor cartridge membranes one day before the assay is essential to ensure adequate calibration of the sensor cartridge and prevent the calibration error on the day of the experiment.

6. Unpack the XFe96/XF Pro sensor cartridge kit, which includes a clear 96-well utility plate, a green sensor cartridge, a pink calibration plate adaptor, and a transparent lid (Figure 1A).
7. Carefully remove the green sensor cartridge from the clear 96-well utility plate. Place it upside down, ensuring that the white membranes do not touch any surface (Figure 1B).
8. Add 200 μL per well of Seahorse XF Calibrant Solution in the wells of the clear 96-well utility plate while avoiding bubbles (Figure 1C).

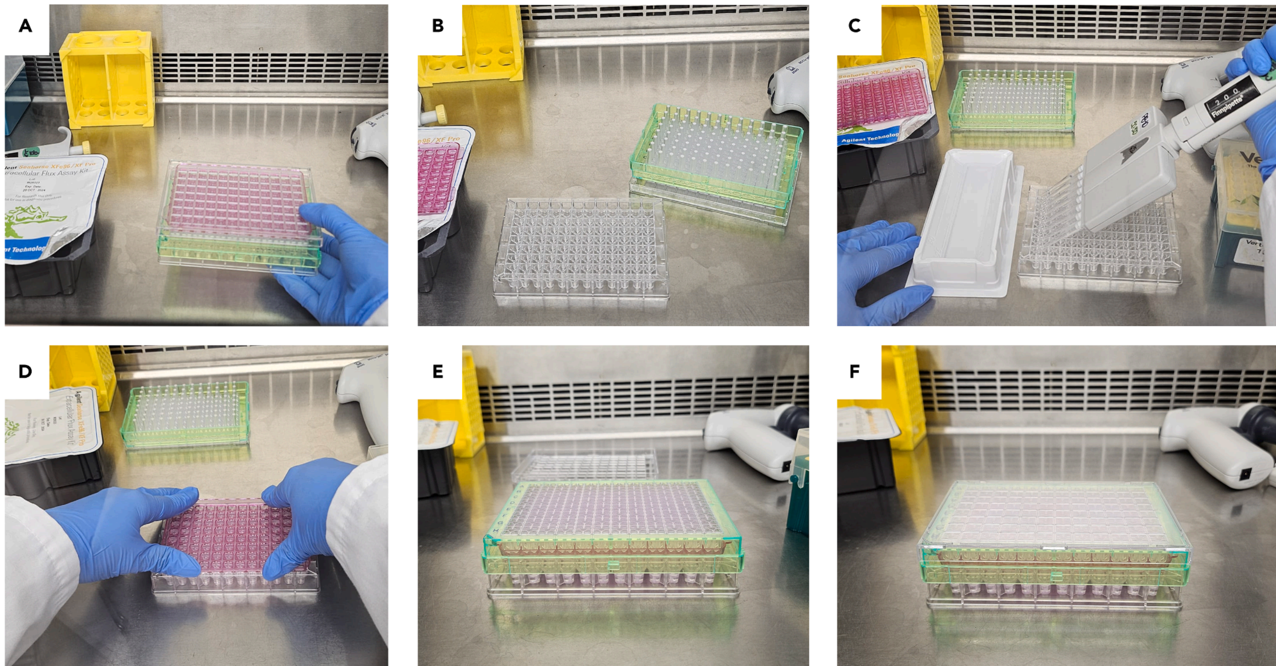

Figure 1. Sequential images illustrating the hydration process of the XFe96/XF Pro sensor cartridge
(A) Different parts of the sensor cartridge.
(B) Correct sensor positioning to prevent biosensor membrane damage.
(C) Gentle addition of 200 μL of Seahorse XF Calibrant Solution into the transparent microplate.
(D) Proper adaptor positioning.
(E) Assembly of the sensor cartridge with the calibrant solution.
(F) Fully assembled sensor cartridge, ready for hydration for at least 16 hours.

9. Attach the pink calibration plate adaptor on top of the clear 96-well utility plate until you hear a "click" sound, confirming that it is properly in place (Figure 1D).
10. Place the green sensor cartridge back on top so that the white membranes are fully immersed in the calibrant solution (Figure 1E).
11. Finally, place the transparent lid on top (Figure 1F).
12. Incubate the assembled cartridge for 16–24 h at 37°C in a non-CO_2 incubator.

Note: Place a disposable reservoir containing sterile distilled water inside the incubator to prevent evaporation.

Preparation of the Seahorse XF DMEM assay medium

Timing: 10 min

This section describes the steps for preparing the Seahorse XF DMEM assay medium to ensure optimal assay performance.

Note: From this section onward, all subsequent steps are performed on the day of the OCR measurements.

13. Transfer the volume of XF DMEM Assay Medium required for the assay into a sterile bottle.

Note: In general, 100 mL is sufficient for one XFe96/XF Pro cell culture microplate.

14. Add the XF supplements to reach the following final concentrations:

    XF Glucose Solution: 10 mM.

    XF Pyruvate Solution: 1 mM.

    XF L-Glutamine Solution: 2 mM.

15. Incubate the supplemented XF DMEM Assay Medium at 37°C until ready for use.

Note: For drug testing experiments, the drug or vehicle must be added to the supplemented Seahorse XF DMEM assay medium and be present throughout the assay.

Preparation of the XFe96/XF Pro cell culture microplate for OCR measurements

Timing: 45 min

This section outlines the steps required to prepare the XFe96/XF Pro cell culture microplate to ensure accurate and reliable OCR measurements.

16. Using a multichannel pipette, remove 160 μL of the cultured medium in the XFe96/XF Pro cell culture microplate, thus leaving 20 μL in each well.
17. Wash the XFe96/XF Pro cell culture microplate with 200 μL of supplemented Seahorse XF DMEM assay medium (pH 7.4), including the wells without cells (blanks).

⚠ CRITICAL: Be very gentle during the wash to prevent cell detachment.

18. Using a multichannel pipette, remove 200 μL from each well, leaving 20 μL to prevent the cells from drying out.
19. Add 160 μL of supplemented Seahorse XF DMEM assay medium (pH 7.4) to bring the final volume to 180 μL in each well.

Note: Before proceeding, examine the cell monolayer under a light microscope to assess cell morphology and overall condition. Look for any signs of cell detachment or stress, as these factors may affect the accuracy of the OCR measurements.

20. Incubate the XFe96/XF Pro cell culture microplate for 1 hour at 37°C in a non-CO_2 incubator to degasify the medium and ensure accurate OCR measurements.

Preparation of the XFe96/XF Pro sensor cartridge with ETC inhibitors

Timing: 30–45 min

This section details the necessary steps for preparing the XFe96/XF Pro sensor cartridge using the ETC inhibitors of the Seahorse XF Cell Mito Stress Test Kit to ensure optimal performance during the assay.

Note: For drug treatment tests, neither the drug nor the vehicle should be added at this step. Oligomycin, FCCP, and the rotenone/antimycin A mixture must be diluted exclusively in the XF DMEM assay medium.

21. Use the gray plastic key (included in the kit) to open the tubes containing lyophilized oligomycin, FCCP, and the rotenone/antimycin A mixture (Figure 2A).

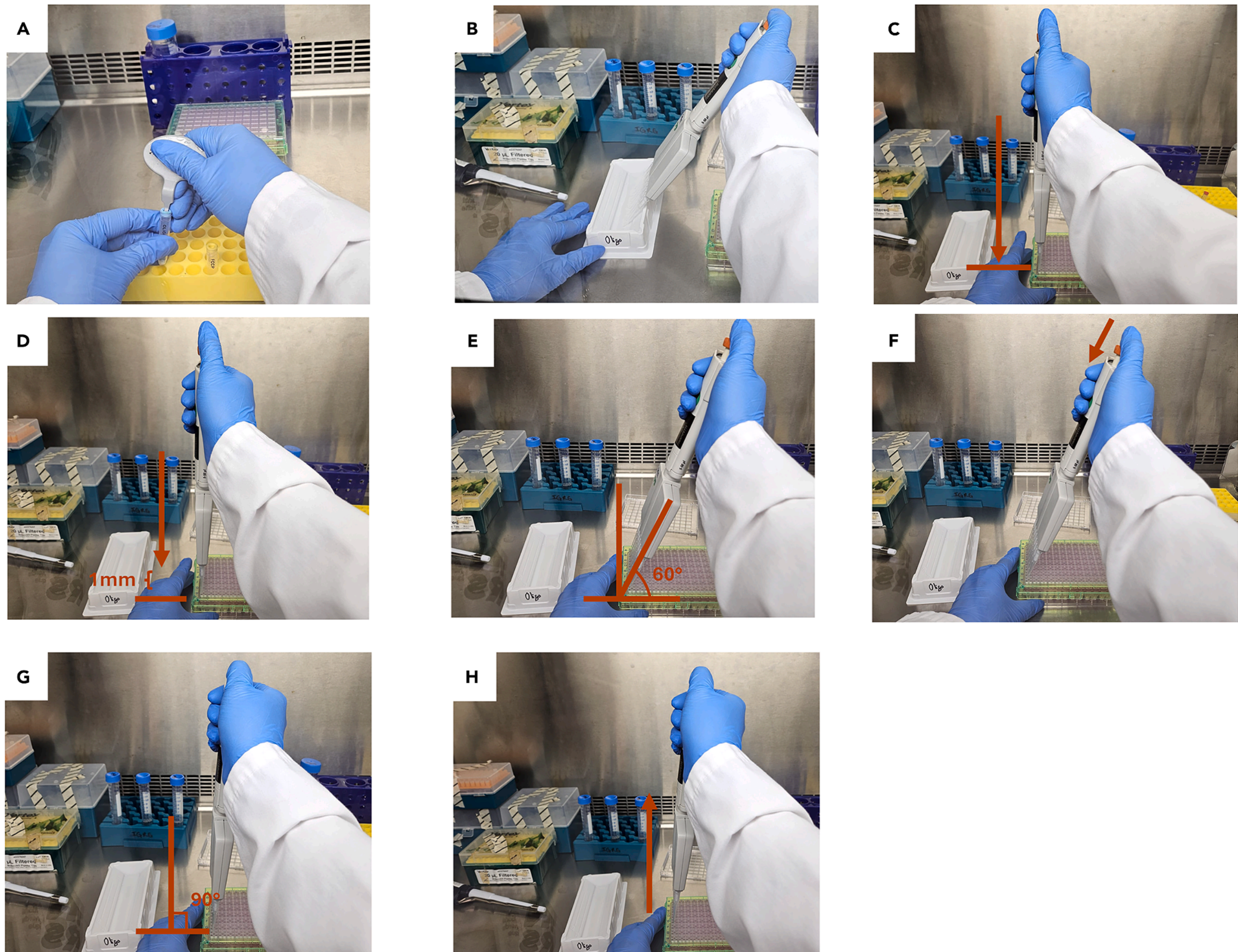

Figure 2. Mito Stress Test drug loading procedure for the XFe96/XF Pro sensor cartridge
(A) Opening of the drug vials.
(B) Pipetting the drugs.
(C) Positioning the tips at the bottom of the well.
(D) Lifting the tips by 1 mm.
(E) Tilting the tips at a 60° angle.
(F) Releasing the contents.
(G) Returning the tips to a 90° angle.
(H) Removing the tips from the cartridge.

22. Prepare stock solutions at the following concentrations:
    a. Oligomycin: Dissolve the drug with 630 μL of supplemented Seahorse XF DMEM assay medium to achieve a final stock concentration of 100 μM.
    b. FCCP: Dissolve the drug with 720 μL of supplemented Seahorse XF DMEM assay medium to achieve a final stock concentration of 100 μM.
    c. Rotenone/antimycin A: Dissolve the mixture with 540 μL of supplemented Seahorse XF DMEM assay medium to achieve a final stock concentration of 50 μM.
23. Prepare a 1:10 dilution of these stock solutions in a total volume of 4 mL by adding 400 μL of the stock solution to 3.6 mL of supplemented Seahorse XF DMEM assay medium (related to troubleshooting problem 3).
24. Load the cartridge ports with the diluted compounds using a multichannel pipette.

⚠ CRITICAL: It is important to do this gently to avoid the premature injection of the drugs through the biosensor-containing membrane. The loaded drop must encompass the whole port diameter, as the drugs are delivered to the cells under pressure during subsequent OCR measurements (Methods video S1).

a. Pipette the following volumes into each port (Figure 2B):
   Port A: Oligomycin: 20 µL.
   Port B: FCCP: 22 µL.
   Port C: Rotenone/antimycin A: 25 µL.

Note: If a loading mistake is made and the compounds are added to the wrong ports, assess the number of affected wells and maintain a pattern that preserves the highest number of usable wells. The port identification configuration can be adjusted in step 28.

⚠ CRITICAL: Do not inject air bubbles into the ports. If air bubbles are present in the pipette tips, change them before proceeding.

b. Insert the tips into the ports, ensuring they touch the bottom by maintaining the pipette in a vertical orientation (Figure 2C).
c. Lift the tips approximately 1 mm above the bottom (Figure 2D).
d. Tilt the pipette at a 60° angle (Figure 2E).
e. Dispense the solution gently into the ports (Figure 2F).
f. Return the tips to a 90° angle (Figure 2G).
g. Remove the tips from the ports vertically (Figure 2H).
h. Repeat for the remaining ports and drugs.
i. Keep the loaded cartridge at 20°C–24°C and proceed with step 25.

Optional: Each Mito Stress Test Kit includes a drug loading adaptor. To use it, place the adaptor on top of the desired port and dispense the drug by inserting the tips through the adaptor holes. However, in this protocol, we recommend not using the adaptor, as it is easier to monitor the correct placement of the drugs and to maintain the proper order of port loading without it.

Setting up the Seahorse analyzer

⏲ Timing: 15–20 min

This section outlines the steps required to configure and measure the OCR using the Seahorse Analyzer.

25. Switch on the Seahorse XFe96 Analyzer.
26. On the Seahorse controller screen, open the Wave software.
27. In the Wave dashboard, select the "XF Cell Mito Stress Test" template (Figure 3A).
28. The template indicates the sequence of injections and the port order (Figure 3B). Do not modify the injection strategies, as they are pre-configured for the assay.

Note: The Seahorse Mito Stress Test typically consists of three baseline OCR measurement cycles, followed by the sequential injection of mitochondrial stressors. First, oligomycin is injected, and OCR is measured over three additional cycles. Next, FCCP is added, followed by three cycles of OCR measurement. Finally, a combination of rotenone and antimycin A is injected, with OCR recorded during the last three cycles.

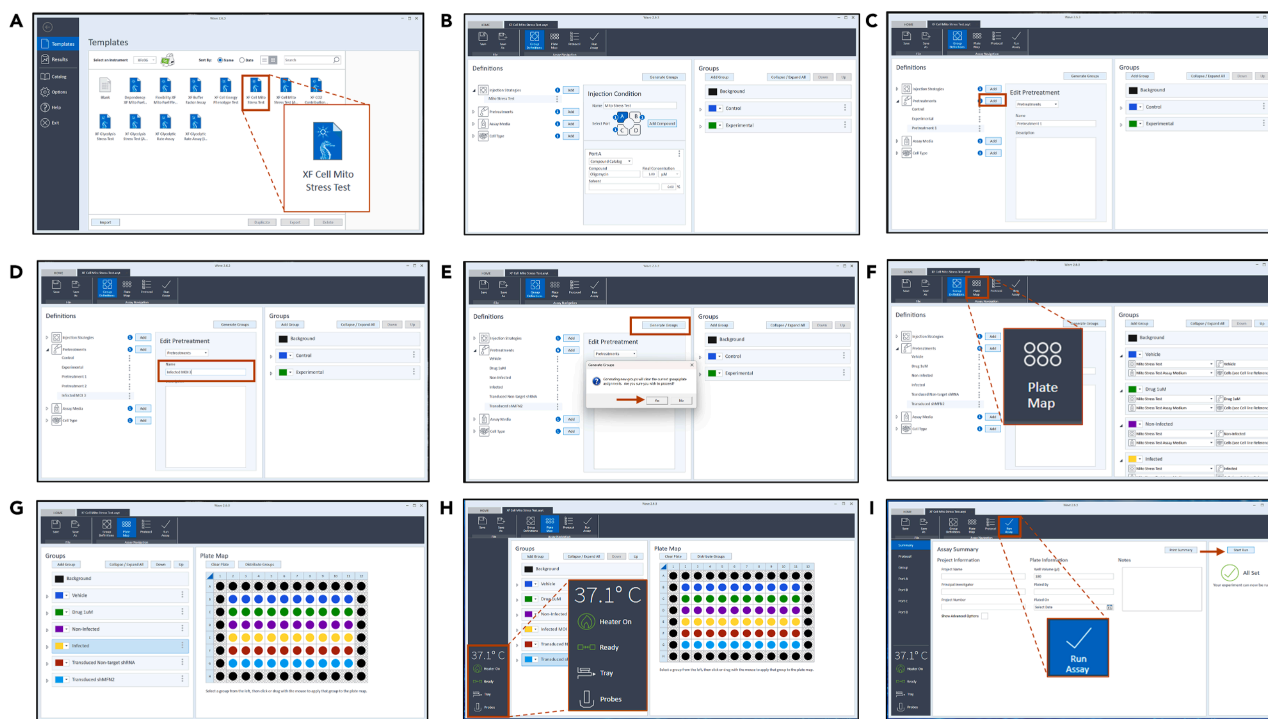

Figure 3. Experiment setup in the Seahorse XFe96 analyzer
(A and B) Configuration of the parameters of the assay in Wave software.
(C–G) Definition and assignment of the experimental groups (pre-treatments).
(H) Verification of system connections.
(I) Project saving and starting of the assay.

Note: If any changes were made in step 24 while loading the cartridge with oligomycin, FCCP, and rotenone/antimycin A in ports A, B, and C, the configuration should be adjusted accordingly during this step.

29. Enter your different experimental conditions in the pretreatment section (e.g., infected, non-infected…).
    a. If necessary, click "Add" to include more conditions (Figure 3C).

Note: The software includes predetermined names for pretreatments (e.g., "Pretreatment 1"). You can modify these names to better describe your conditions (e.g., "Infected MOI 3") (Figure 3D).

Optional: The assay medium and cell type can be specified during the experiment setup.

30. Once the groups are set up, select "Generate Groups" (Figure 3E).

Note: A small window will appear requesting confirmation. Click "Yes."

31. Verify that the newly generated groups appear on the screen's right side, then click "Plate Map" (Figure 3F).
32. Identify the wells based on what was seeded in the Seahorse culture microplate by dragging them with the mouse from the list on the left to the plate map on the right (Figure 3G).

Note: The black wells named "Background" refer to the blank wells that contain only cell-free Seahorse XF DMEM assay medium.

33. Verify that the communication between the controller and the Seahorse XFe96 Analyzer is established, and that the temperature has reached 37°C. (Figure 3H).

Note: This generally takes 15–20 minutes after switching the analyzer on. "Ready" should be displayed in the bottom left corner of the window.

Optional: The temperature setting can be adjusted if the cell line of study requires a different temperature. If the selected temperature is different from 37°C, the calibration process will take about 30 additional minutes (related to step 37).

34. Click "Run Assay" and enter the relevant experiment information. (Figure 3I).
35. Click "Start Run" and save the project.

Note: The experiment will start running immediately. A message prompting you to load the cartridge will appear on the screen, and the tray will open automatically.

Running the OCR measurement assay

Timing: 2 h

This section outlines the steps to run the assay using the Agilent Seahorse XFe96 Analyzer once the experiment is set up.

36. Place the XFe96/XF Pro sensor cartridge on the tray **after removal** of the transparent lid (Figure 4A).
37. Click "I'm Ready" on the confirmation message on the screen (Figure 4B). The tray will close.

Note: The Agilent Seahorse XFe96 Analyzer will begin calibration. Wait until the O_2 and pH are verified, and a new message appears requesting you to load the cell plate (Figure 4C). If a calibration error message displays, refer to troubleshooting problem 1.

38. Click "Open Tray". The tray will open.

Note: The cartridge will stay inside the analyzer, and only the clear 96-well utility plate with the calibrant solution and the pink adaptor will go out of it. Remove them from the tray.

39. After the one-hour incubation, remove the XFe96/XF Pro cell culture microplate from the incubator (37°C, 0% CO_2) and place it on the tray **without** the transparent lid (Figure 4D).
40. Click "Load cell plate" on the confirmation message on the screen.

Note: The tray will close, and inside the analyzer, the cartridge will be automatically deposited on the cell culture plate (Figure 4E).

41. The OCR measurement will begin. Wait for the confirmation message indicating the end of the experiment to appear on the screen.
42. Click "Eject" (Figure 4F). The tray will open. Remove the XFe96/XF Pro sensor cartridge with the XFe96/XF Pro cell culture microplate.
43. Cover it with the transparent lid and place it in the biosafety cabinet (class II, type A2).
44. Click "Done" on the confirmation message on the screen (Figure 4G). The tray will close.
45. Click "View results"
46. Click "Export". Select "Select XF Cell Mito Stress Test Report Generator" (Figure 4H).
47. Save the "XXX.asyr" file, which opens with Wave software.
48. Save the exported results file, which opens in Excel.

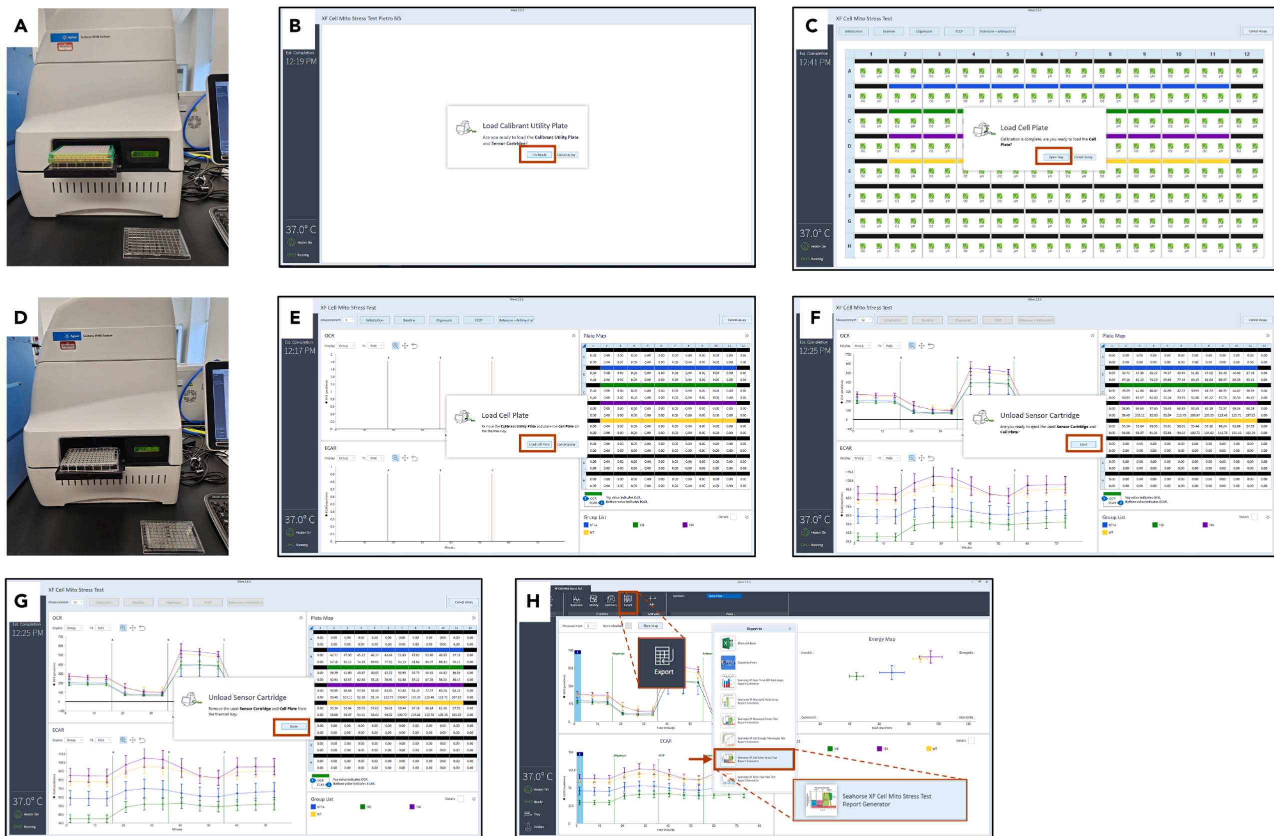

Figure 4. Initiating the assay with the Seahorse XFe96 analyzer
(A–C) Calibration of the XFe96/XF Pro sensor cartridge.
(D and E) Loading the XFe96/XF cell culture microplate.
(F–H) Finalizing the experiment and saving results.

Protein quantification

Timing: 1 h

This section details the steps required to extract proteins directly in the wells of the Seahorse XFe96/XF Pro cell culture microplate and measure total protein content using a standard Bradford assay. This process is essential for normalizing OCR values to protein content, ensuring that OCR values of the various conditions are compared for the same number of cells.

49. Perform the protein extraction directly in the wells of the XFe96/XF Pro cell culture microplate.
    a. With a multichannel pipette, remove completely the medium from the XFe96/XF Pro cell culture microplate, while ensuring not disturbing the cell monolayer during aspiration.
    b. Gently wash the cells twice with 50 μL PBS.
    c. Add 20 μL of cell lysis buffer to each well and pipette up and down to mix each well thoroughly using a multichannel pipette.

    Note: Avoid sample cross-contamination by changing the tips every time you move to the next row of wells.

    d. Incubate on ice for 30 minutes.

Note: If cells were infected, this step must be performed in a biosafety cabinet (BSC) class II, type A2 to ensure proper containment and safety of the users. After lysis, the subsequent steps can be performed in a biosafety level 1 laboratory.

e. Add 198 μL of 1x Bradford reagent to each well of a new 96-well transparent flat-bottom plate (reading plate).
f. After the lysis incubation time, pipette up and down the samples thoroughly in the XFe96/XF Pro cell culture microplate using a multichannel pipette.

Note: Avoid sample cross-contamination by changing the tips every time you move to the next row of wells.

g. Add 2 μL of each cell lysate to the 198 μL of 1x Bradford reagent in the 96-well reading plate to achieve a final volume of 200 μL per well.

Note: Be sure to include some blank wells with only the Bradford reagent and the cell lysis buffer. For that, the blank samples included in the Seahorse microplate can be used.

h. Gently mix by rocking the plate for 2 minutes and incubate for 3 minutes at 20°C–24°C until samples become homogeneously blue.

50. In parallel, prepare a protein standard curve.
    a. Prepare solutions of bovine serum albumin (BSA) at concentrations of 0, 0.1, 0.2, 0.5, and 1 mg/mL in cell lysis buffer.
    b. Add 198 μL of 1× Bradford reagent to each well of a transparent, non-cell culture-treated, flat-bottom 96-well plate (reading plate).
    c. Pipette 2 μL of each standard into the wells of the reading plate in triplicate to achieve a final volume of 200 μL per well.

Note: Include at least three blank wells containing only Bradford reagent and lysis buffer.

    d. Gently mix the plate and incubate for 5 minutes at 20°C–24°C to allow a blue color to develop.
51. Measure the optical density at 595 nm of both the BSA standards and Seahorse samples using the Spark Multimode Microplate Reader or any relevant and standard plate reader.
52. Generate a standard curve to calculate protein concentrations.
    a. Plot the absorbance values of the BSA standards against their known concentrations to create a standard curve.
    b. Calculate the linear regression equation from the standard curve.
    c. Use the regression formula to determine the protein concentrations of the Seahorse samples based on the measured optical density at 595 nm.

Note: When calculating protein concentrations, account for the dilution factor. Each sample is diluted 1:100 (i.e., 2 μL of sample in 198 μL of 1x Bradford reagent).

    d. Calculate the mean protein concentration for each condition using the protein concentration values from individual wells.

Detailed data analysis

Timing: 3 h

This section describes how to normalize the data based on protein concentration, convert them into fold changes for easy comparison between biological replicates, and perform statistical analysis.

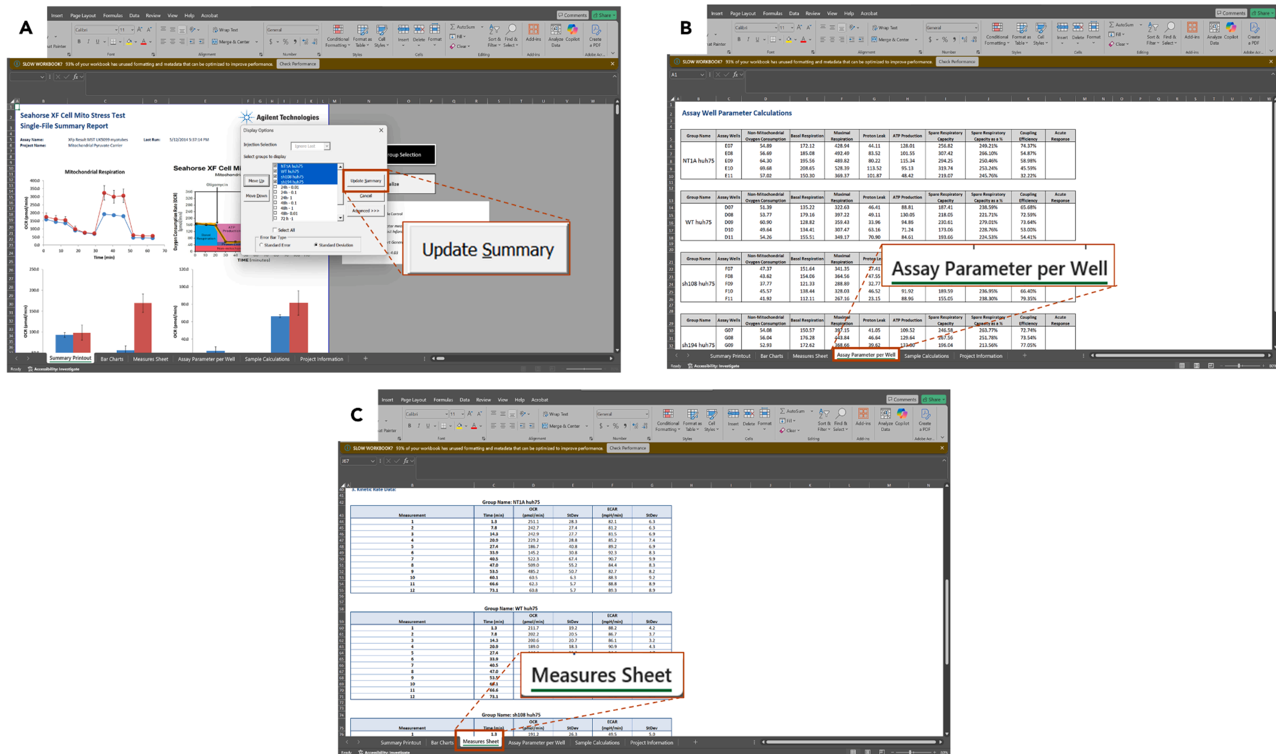

Figure 5. Retrieving data for analysis

(A) Displaying OCR groups for analysis.

(B and C) Retrieving values for analyzing individual respiration parameters (B) and generating OCR curves (C).

53. Open the exported XF Cell Mito Stress Test Report Generator file.
54. Enable content in Excel.
55. Select the groups you want to analyze and click "Update Summary" (Figure 5A).
56. Select the spreadsheet "Assay Parameter per Well" (Figure 5B).
57. Copy the measurement tables into a new Excel file.

Note: Normalizing and working with data in a new Excel file helps preserve the integrity of the original file.

58. Normalize the OCR values by protein concentration by dividing each OCR measurement by the mean protein concentration of the corresponding condition.

Note: Alternatively, OCR normalization can be made following nucleus staining with the DAPI dye in each well of the Seahorse XFe96/XF Pro cell culture microplate and counting the number of intact nuclei per well by automated fluorescence microscopy with an appropriate plate imager when available.

59. Calculate the fold values.
    a. Determine the average OCR for each parameter (*e.g.*, basal respiration, maximal respiration) in the control condition. If high variability is observed between wells of the same condition, refer to troubleshooting problem 2.
    b. To obtain fold changes, divide each OCR value in that parameter by the calculated average OCR of the reference control condition (*e.g.*, "uninfected cells"). The resulting value may be multiplied by 100 to express it as a percentage.
60. Use GraphPad to analyze the fold change values.

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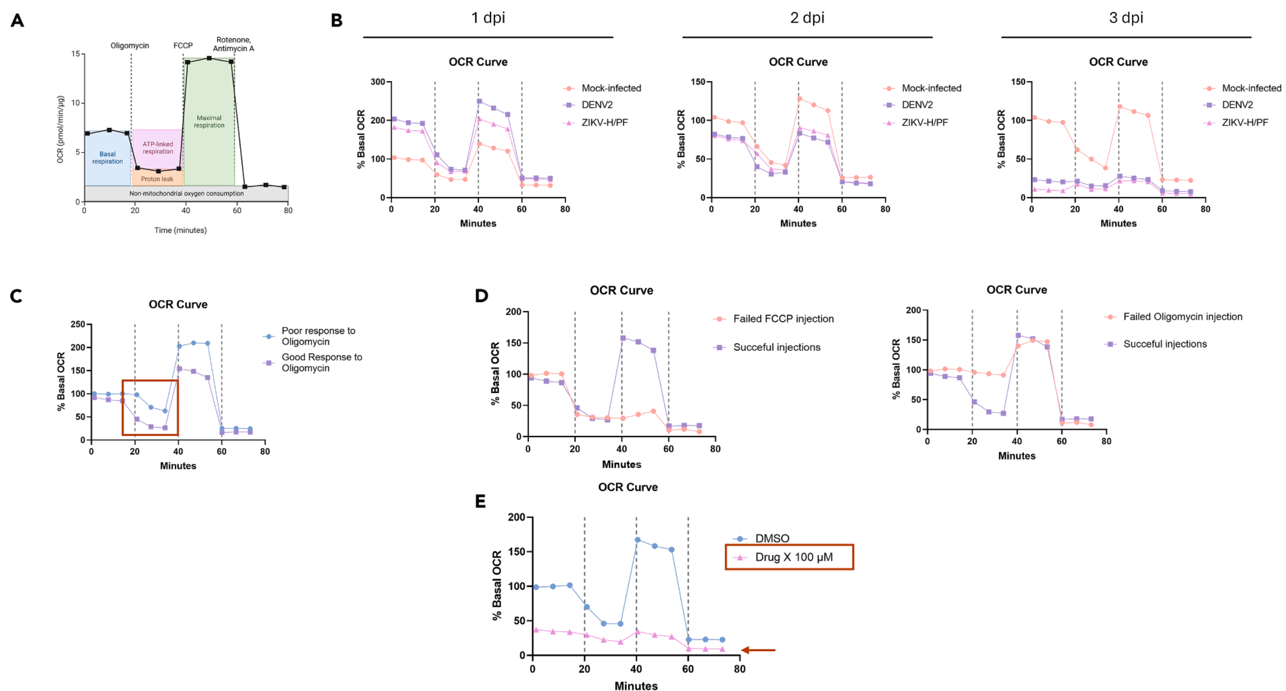

Figure 6. Expected and examples of problematic OCR profiles
(A) Expected OCR curve with a well-defined response to the added ETC inhibitors.
(B) Representative OCR profiles following flavivirus infection at 1, 2 and 3 days post-infection (dpi) (unpublished data).
(C) The OCR curve shows poor response to oligomycin treatment.
(D) Potential outcome when the FCCP or oligomycin injection fails.
(E) Cells exhibit a quiescent phenotype, resulting in a flat OCR curve.

a. Create a separate data sheet for each parameter (*e.g.*, basal respiration).
b. Copy the fold change values into the corresponding data sheet.
c. Assess whether the data follows a normal distribution using the Shapiro-Wilk test.
d. Perform the appropriate statistical test (parametric (normal distribution for all conditions) vs. non-parametric (non-normal distribution in at least one condition) to determine significant differences among the conditions.

61. Generate the OCR profile curves.
a. In the exported XF Cell Mito Stress Test Report Generator file, select the spreadsheet "Measures Sheet" (Figure 5C).
b. Copy the measurement tables into a new Excel spreadsheet.
c. Normalize each OCR value by dividing it by the mean protein concentration of the corresponding condition.
d. Calculate the % basal OCR values by dividing each normalized OCR measurement by the average OCR of the first three measurements in the reference uninfected/untreated reference condition and multiplying by 100.
e. Copy the % basal OCR values into an XY GraphPad data sheet.
f. Plot the % basal OCR values(Y-axis) as a function of time in minutes (X-axis).

EXPECTED OUTCOMES

Clear and well-defined OCR curves can be obtained by following the procedure outlined above. A typical time-resolved OCR profile is shown in Figure 6A, which also illustrates how the various respiration parameters are defined. Based on our experience using the Seahorse XF Cell Mito Stress Test kit, a healthy, confluent monolayer of Huh7.5 cells typically exhibits basal respiration around 100 pmol/min and maximal respiration between 250 and 400 pmol/min before normalization. However, these values

may vary depending on the cell type, metabolic state, pretreatments, and assay conditions. Typically, DENV and ZIKV infection of Huh7.5 cells (and other cell types that we have tested) will result in increasing basal, maximal, and ATP-linked respiration at one day post-infection, while decreasing these parameters from two days post-infection (*i.e.*, the peak of replication in this cell line) onwards.[1] At 3 days post-infection, respiration in infected cells is almost completely shut down because of flavivirus-induced cytopathic effects. OCR profiles from a typical infection kinetic experiment are shown in Figure 6B.

LIMITATIONS

This protocol is designed to be performed on cells grown as a monolayer culture, which may not fully recapitulate the complex environment of an organ *in vivo*. In particular, the absence of tissue-specific architecture, extracellular matrix, and cellular interactions in culture can influence mitochondrial activity, making the results less reflective of physiological conditions. Additionally, the Seahorse Mito Stress Test relies on functional extracellular measurements, such as OCR and extracellular acidification rate (that can be used as an indirect readout of glycolysis), but it does not provide direct information on mitochondrial functions (apoptosis, innate immunity…), quality control (mitophagy…), morphology and physical contact-based communication with other organelles. These processes are critical for overall mitochondrial health and function and are all interconnected. As a response to stress, mitochondria may exhibit changes in their respiration efficiency, which might be only indirectly linked to the initial stimulus. Therefore, Seahorse should always be interpreted with caution in a broader cellular context via their complementation with other technical approaches (*e.g.*, imaging), assessing mitochondrial functions.

While the Seahorse Mito Stress Test provides valuable insight into mitochondrial respiration at a specific point in time, it primarily measures the acute mitochondrial response to the various injected compounds. Unfortunately, it does not account for long-term changes in mitochondrial dynamics or functions. As a result, the assay may not detect gradual adaptations of the cell to metabolic changes, mitochondrial biogenesis, or alterations in mitochondrial DNA over extended periods.

TROUBLESHOOTING

Problem 1

If an error message appears during step 37 mentioning a calibration error, it may be due to improper hydration of the Seahorse XFe96/XF Pro sensor cartridge.

Potential solution

To ensure proper hydration of the Seahorse XFe96/XF Pro sensor cartridge, this step must be performed for at least 16 hours, as outlined in step 12. When pipetting the calibrant, take care to avoid introducing air bubbles. If a calibration error message appears and the experiment must proceed the same day, you may use a new sensor cartridge and repeat the hydration process (steps 6 to 11), incubating it at 37°C in a non-CO_2 incubator for at least 4 hours. This workaround should be reserved for urgent situations. Whenever possible, prioritize hydration for at least 16 hours (generally overnight) to ensure optimal performance.

Problem 2

If there is high variability between wells of the same condition during step 59a, it may indicate a cell seeding consistency issue.

Potential solution

High variability between replicates is often caused by the presence of cell clumps. To prevent this, thoroughly trypsinize the cells during step 2. Before seeding into the XFe96/XF Pro cell culture microplate, use a light microscope to confirm that you have a homogenous single-cell suspension.

Table 1. Preparation of various Mito Stress Test drug concentrations for optimization

Drug	Stock concentration	Stock solution volume (μL)	Seahorse medium volume (μL)	Final concentration (μM)	Volume added to the port (μL)
Oligomycin (Port A)	100 μM	200	3800	5	20
		400	3600	10	
		600	3400	15	
FCCP (Port B)	100 μM	50	3950	1.25	22
		100	3900	2.5	
		200	3800	5	
		400	3600	10	
Rotenone/ antimycin A (Port C)	50 μM	400	3600	5	25

Problem 3

In step 61, the cells exhibit a low response to the sequentially injected ETC inhibitors, resulting in poorly defined OCR profiles. Figure 6C illustrates poor efficiency in oligomycin treatment.

Potential solution

- During steps 22 and 23, adjust the optimal concentration of the ETC inhibitors. Differences in the optimal ETC inhibitors concentration required for different cell lines may impact the generation of well-defined curves. The final compound concentration needed for maximal effect varies from one cell line to the other and can be influenced by the assay medium. Therefore, for each new cell line or assay medium, a titration experiment may be relevant to determine the appropriate concentration. This is of particular importance for FCCP treatment since excessive FCCP concentrations can reduce OCR responses. Here, we propose a range of concentrations for optimization tests (Table 1).

For more information, check the Agilent Seahorse XF Cell Mito Stress Test Kit user guide (available online: https://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Mito_Stress_Test_Kit_User_Guide.pdf).

- **Injection Problem:** If the concentrations of oligomycin, FCCP, and rotenone/antimycin A are already standardized and validated for the specific cell type, an absence of OCR response to the added compound may originate from injection-related issues during step 24 (Figure 6D). The presence of air bubbles in a port or an improper pipetting technique can compromise the accurate delivery of compounds into the wells. To mitigate this, change pipette tips whenever air bubbles are observed during the loading of the XFe96/XF Pro sensor cartridge with the ETC drugs. Additionally, refer to Methods video S1 for a validated loading technique to ensure precise and consistent preparation of the cartridge with the ETC drugs.

Problem 4

During step 61, the generated curves appear flat, indicating that the cells exhibit consistently low or undetectable OCR values and do not respond to the compounds added during the assay. This result might be expected in some conditions, such as when cytopathic effects are induced at late time points of infection (*i.e.*, 3 dpi, Figure 6B). If cells were expected to consume oxygen, this might be due to technical difficulties (Figure 6E).

Potential solutions

- **Low cell abundance:** Standardize the optimal number of cells to be seeded during step 4. Insufficient cell confluency can lead to low OCR values and flat curves. It is essential to optimize cell density for reliable results. Since the optimal number of cells varies between cell lines, standardization by testing different cell concentrations is recommended. For Huh7.5 cells and other cell lines that we have tested, seeding approximately 50,000 cells per well in the XFe96/XF Pro Cell

Culture Microplate one day before the experiment ensures optimal performance. However, for (antiviral) drug testing, where cells are seeded four days in advance, a lower density of around 18,000 cells per well is suggested to maintain appropriate growth conditions. We recommend regularly monitoring the confluency, morphology, and viability of the cells using a light microscope to determine the optimal time point and cell concentration for the day of the assay.

- **Cell overconfluency:** Establish the optimal cell seeding concentration during step 4. Excessive cell confluency can lead to exacerbated cellular stress or cytostatic effects, which may negatively impact OCR and thus result in flat curves and a quiescent phenotype. Maintaining an optimal cell density is crucial to ensure accurate and reproducible results.
- **Treatment-induced cell stress/death:** Excessive cellular stress or cell death resulting from pretreatments, such as infection (step 2), transduction, or high drug concentrations, can induce apoptosis and adversely affect OCR measurements. This may lead to flat curves. Optimizing pretreatment parameters, such as drug concentration, is essential to minimize cellular stress and ensure accurate and reproducible results. This can also be a sign of the virus-induced cytopathic effects expected at the late time points of the infections. Low protein concentration is generally indicative of decreased cell viability.

Problem 5

If no change in OCR is observed following infection with ZIKV H/PF/2013 or DENV serotype 2 16681 in Huh7.5 cells, it may be due to low infection efficiency during step 3 of the flavivirus infection section of this protocol.

Potential solutions

- **Incorrect viral stock titration:** Confirm the viral stock titers used for the experiment by performing a plaque assay. Accurate titer determination is critical for calculating the MOI, which directly determines the number of cells that will be infected.
- **MOI optimization:** Optimize the MOI for each virus and cell line. When working with different DENV or ZIKV serotypes/strains, or when using a different cell line, test a range of MOIs and select the one that results in 90%–100% infection on the day of OCR measurement, as assessed by confocal microscopy using a viral marker.
- **Isolated low-efficiency infection event:** If infection efficiency appears inconsistent between experiments despite using an established MOI, consider the possibility of an isolated experimental issue. In such cases, we recommend collecting supernatant before seeding cells into the XFe96/XF Pro cell culture microplate (during step 1 of the "step-by-step method details" section). Then, perform a plaque assay using the collected supernatant to verify the infection rate for each experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Laurent Chatel-Chaix (laurent.chatel-chaix@inrs.ca).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Viviana Andrea Barragan Torres (viviana.barragan@inrs.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets and code.

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AUTHOR CONTRIBUTIONS

V.A.B.T.: study design, execution of the experiments, data analysis, and manuscript writing and editing. L.C.-C.: study design, funding, and manuscript editing. Both authors have read, reviewed, and agreed to this version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.104051.

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