Seahorse XF Cell Mito Stress Test

Mitochondrial Function Analysis for XFe96/XF Pro Analyzer

Assay Overview

Purpose

The Seahorse XF Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of live cells. This assay provides comprehensive information about:

- Basal respiration
- ATP production
- Proton leak
- Maximal respiration
- Spare respiratory capacity
- Non-mitochondrial respiration

Assay Principle

The assay uses sequential injection of mitochondrial inhibitors and uncouplers to reveal different aspects of mitochondrial function:

> **Injection 1 - Oligomycin:** Inhibits ATP synthase (Complex V), revealing ATP-linked respiration

> **Injection 2 - FCCP:** Uncoupler that collapses proton gradient, revealing maximal respiration

> **Injection 3 - Rotenone/Antimycin A:** Complex I & III inhibitors, revealing non-mitochondrial respiration

Timeline

1 **Day Prior (Evening)**
Seed cells (10,000-20,000/well), hydrate sensor cartridge

2 **Day of Assay (Morning)**
Prepare media, wash cells, prepare compounds, load ports

3 **Run Assay (~2-3 hours)**
Calibration (20-30 min) + Assay run (~90-120 min)

Important Notes:

- Optimal cell density and FCCP concentration must be determined empirically for each cell type
- Never use vacuum aspirator when washing cells
- Do NOT seed cells in corner wells (A1, A12, H1, H12) - these are background wells
- Use compounds on the same day they are reconstituted

Day Prior to Assay

☐ **Turn on XF Analyzer**

Turn on the Agilent Seahorse XFe96/XF Pro Analyzer and let it warm up overnight (minimum 5 hours).

☐ **Prepare Cells for Seeding**

Harvest and resuspend cells to desired density (10,000-20,000 cells per well).

Cell Seeding Calculation:

For **2.0×10^4 cells/well** in 80 µL:
• Required concentration: 2.5×10^5 cells/mL
• Volume needed: ~8 mL for one plate

Cells/Well	Stock (1×10^6/mL)	Media	Total
0.5×10^4	0.5 mL	7.5 mL	8.0 mL
1.0×10^4	1.0 mL	7.0 mL	8.0 mL
2.0×10^4	**2.0 mL**	**6.0 mL**	**8.0 mL**
3.0×10^4	3.0 mL	5.0 mL	8.0 mL

Seeding Tips:

- Ensure single-cell suspension (no aggregates)
- Mix thoroughly before and during seeding
- Use multichannel pipette (8- or 12-channel)

- [ ] **Seed Cells in XF96 Cell Culture Microplate**

  Dispense 80 µL of cell suspension per well.

  > **CRITICAL: Do NOT seed cells in corner wells!**
  > Wells A1, A12, H1, and H12 are background correction wells. Add 80 µL of medium ONLY (no cells).

  Pipetting Technique:

  1. Hold pipette at ~45° angle, touch tip halfway down well wall
  2. Dispense slowly - tip should be slightly submerged after dispensing
  3. Remix cells in reservoir every 8-12 wells to prevent settling

- [ ] **Rest Plate at Room Temperature**

  IMPORTANT: Let plate sit at room temperature for 30 minutes before transferring to incubator.

  This prevents edge effects caused by rapid temperature changes.

  > 💡 **Tip:** Place plate toward the back of the biosafety cabinet during the rest period.

- [ ] **Transfer to Incubator**

  After 30-minute rest, transfer plate to properly humidified 37°C CO_2 incubator.

  Incubate overnight (18-24 hours). Place plate toward back of incubator to minimize temperature fluctuations.

- [ ] **Hydrate Sensor Cartridge**

  Add 200 µL of Seahorse XF Calibrant to each well of the sensor cartridge utility plate.

  Incubate overnight at 37°C in a non-CO_2 incubator.

  > **Note:** Ensure sensor cartridge is properly seated on utility plate before adding calibrant.

- [ ] **Design Experiment in Wave Software**

  Create assay template with proper plate layout and injection strategy.

  Recommended protocol: 3 basal measurements, inject oligomycin, 3 measurements, inject FCCP, 3 measurements, inject Rot/AA, 3 measurements.

Day of Assay

Part A: Assay Medium Preparation

- [ ] **Prepare XF Assay Medium (50 mL)**

Supplement Seahorse XF DMEM or RPMI medium (pH 7.4) with:

Supplement	Stock Conc.	Final Conc.	Volume to Add
Glucose	1.0 M	5 mM	**250 µL**
L-Glutamine	200 mM	1 mM	**250 µL**
Sodium Pyruvate	100 mM	1 mM	**500 µL**
XF Base Medium			**49 mL**

📝 **User's Custom Media Recipe (for 50 mL):**

Component	Volume (µL)
Seahorse Base Medium	48,900
Pyruvate	500
Glucose	100
Glutamine	500
Total Volume	50,000

💡 **Note:** Medium composition can be adjusted based on cell type and experimental goals. pH adjustment is not necessary when using recommended concentrations.

- [ ] **Warm Assay Medium**

  Place assay medium in 37°C water bath until ready to use.

Part B: Wash Cells

⚠️ **CRITICAL: Never use vacuum aspirator!**

Always use multichannel pipette to remove media. Vacuum aspiration will damage the cell monolayer.

- [ ] **Check Cell Monolayer**

  View cells under microscope to confirm:

  - Healthy morphology
  - Even distribution
  - No contamination
  - Background wells are empty

- [ ] **Remove Growth Medium**

  Using multichannel pipette, remove all but 20 µL of growth medium from each well.

Proper Technique:

1. Place plate on stable, flat surface
2. Slide pipette tips gently down well wall to bottom
3. Remove slowly (~60 µL from 80 µL starting volume)
4. Leave 20 µL minimum to prevent cells from drying

- [ ] **First Wash**

  Add 200 µL of warmed assay medium to each well.

  Dispense gently along upper well wall (not directly onto cells).

- [ ] **Remove First Wash**

  Remove all but 20 µL of wash medium.

- [ ] **Second Wash**

  Add 200 µL of assay medium to each well.

- [ ] **Remove Second Wash and Add Final Volume**

  Remove all but 20 µL, then add 160 µL of assay medium for final volume of 180 µL/well.

- [ ] **Check Cells Post-Wash**

  View cells under microscope to ensure monolayer is intact.

- [ ] **Incubate Cells**

  Incubate at 37°C in non-CO_2 incubator for 60 minutes.

  This step is critical for CO_2 equilibration and accurate ECAR measurements!

Part C: Prepare Compounds

⚠️ **Use compounds same day only!**

Do not refreeze reconstituted compounds. Discard any unused material.

- [ ] **Prepare Working Solutions (User's Protocol)**

  Dilute stock solutions to working concentrations in assay medium:

  User's Specific Dilutions (for 3 mL each):

  Oligomycin (Port A): 30 µM working stock

  • From 12 mM stock → 30 µM working
  • 7.5 µL stock + 3,000 µL assay media
  • Load 20 µL per well in Port A

FCCP (Port B): 15 µM working stock

- From 40 mM stock → 15 µM working
- 1.125 µL stock + 3,000 µL assay media
- Load 22 µL per well in Port B

Rotenone/Antimycin A (Port C): 60 µM working stock

- From 10 mM stock → 60 µM working
- 18 µL stock + 3,000 µL assay media
- Load 25 µL per well in Port C

Note: These concentrations are user-specific. Standard protocol uses different concentrations (1.5 µM oligomycin, 0.5-1.0 µM FCCP, 0.5 µM Rot/AA).

- [ ] ### Load Sensor Cartridge Ports

Using multichannel pipette, load compounds into injection ports:

Port A	Port B	Port C
Oligo	**FCCP**	**Rot/AA**
20 µL	22 µL	25 µL

Loading Tips:

- Load from left to right (A → B → C)
- Pipette slowly to avoid bubbles
- Ensure each port is completely filled
- Check for bubbles - remove if present

Running the Assay

- [ ] ### Open Wave Software

Browse for and open your saved assay design file.

- [ ] ### Start Calibration

Click "Run" or "Start Run" and follow prompts.

Place loaded sensor cartridge with calibrant plate into analyzer.

⏱️ Calibration takes 15-30 minutes

- [ ] ### Check Cell Plate

During calibration, verify cells under microscope one final time.

Ensure no evaporation or contamination has occurred.

- [ ] **Load Cell Plate**

  When prompted after calibration, replace calibrant plate with cell culture microplate.

  Click "Start" or "I'm ready" to begin assay.

- [ ] **Monitor Assay Progress**

  Assay typically runs 90-120 minutes.

  Expected Assay Timeline:

  - Basal measurements: ~20 min
  - Oligomycin response: ~20 min
  - FCCP response: ~20 min
  - Rotenone/Antimycin A response: ~20 min

- [ ] **Collect Assay Data**

  When assay completes, export data file from Wave software.

  Save plate for normalization if needed (protein, DNA, or cell count).

🔍 **What to Expect:**

- **Basal OCR:** Steady oxygen consumption at baseline
- **After Oligomycin:** OCR decreases (ATP-linked respiration blocked)
- **After FCCP:** OCR increases to maximum (uncoupled respiration)
- **After Rot/AA:** OCR drops to near zero (mitochondrial respiration shut down)

Data Analysis

Seahorse XF Mito Stress Test Report Generator

The Seahorse XF Mito Stress Test Report Generator automatically calculates mitochondrial parameters from exported Wave data.

Steps:

1. Export assay data from Wave to Excel format
2. Open Seahorse XF Mito Stress Test Report Generator
3. Load exported Excel file
4. Review automatically calculated parameters
5. Export report (PDF or Excel)

Key Parameters Calculated

Basal Respiration
Baseline oxygen consumption before any compound injection. Reflects cellular ATP demand under baseline conditions.

ATP Production
Decrease in OCR after oligomycin injection. Represents respiration dedicated to ATP synthesis.

Proton Leak
OCR remaining after oligomycin that is not coupled to ATP production. Can indicate mitochondrial damage.

Maximal Respiration
Maximum OCR achieved after FCCP injection. Shows maximum capacity of electron transport chain.

Spare Respiratory Capacity
Difference between maximal and basal respiration. Indicates ability to respond to increased energy demand.

Non-mitochondrial Respiration
OCR remaining after rotenone/antimycin A. Represents oxygen consumption by non-mitochondrial enzymes.

Data Quality Checks

Review these quality metrics:

- **Background wells:** Should show minimal, consistent OCR
- **Coefficient of variation (CV):** Should be <15% for replicates
- **FCCP response:** Should show clear increase in OCR
- **Rotenone/AA response:** Should reduce OCR to near baseline
- **Edge effects:** Check if edge wells differ significantly from center wells

Normalization

To account for differences in cell number between wells, normalize OCR data to:

- Total protein content (BCA or Bradford assay)
- DNA content (PicoGreen or similar)
- Cell count (automated counting or manual)
- Imaging-based cell quantification (e.g., BioTek Cytation)

📚 **Resources:**

- Agilent Cell Analysis Learning Center: www.agilent.com/en/products/cell-analysis
- Reference: Divakaruni et al. (2014) Methods in Enzymology, Vol 547, Chapter 16

Troubleshooting

Problem: High variability between replicates (CV >20%)

Possible Causes & Solutions:

- **Uneven cell seeding:** Ensure cells are thoroughly mixed before and during seeding. Remix cell suspension every 8-12 wells.
- **Cell damage during washing:** Use gentle technique, never vacuum aspirator. Leave 20 µL minimum between washes.
- **Edge effects:** Ensure 30-min room temp rest after seeding. Use properly humidified incubator.

Problem: No response to FCCP

Possible Causes & Solutions:

- **FCCP concentration too low:** Perform FCCP titration (0.125-2.0 µM) to find optimal concentration for your cell type.
- **FCCP concentration too high:** Excessive FCCP can inhibit respiration. Try lower concentration.
- **Degraded FCCP:** Prepare fresh working solution. Do not reuse reconstituted compounds.

Problem: High background OCR in empty wells

Possible Causes & Solutions:

- **Cells in background wells:** Verify background wells (A1, A12, H1, H12) contain no cells under microscope.
- **Contaminated medium:** Use fresh, sterile assay medium. Filter if necessary.

Problem: Low or no basal respiration

Possible Causes & Solutions:

- **Too few cells:** Increase seeding density. Optimal range is typically 10,000-20,000 cells/well.

- **Unhealthy cells:** Check cell viability and morphology before seeding. Use cells at optimal passage number.
- **Insufficient substrate:** Verify assay medium contains glucose, glutamine, and pyruvate at recommended concentrations.

Problem: Incomplete inhibition by rotenone/antimycin A

Possible Causes & Solutions:

- **High non-mitochondrial respiration:** This is normal for some cell types. Remaining OCR represents non-mitochondrial oxygen consumption.
- **Insufficient compound delivery:** Verify ports loaded correctly and compounds diluted properly.

Problem: Cells detached during washing

Possible Causes & Solutions:

- **Aggressive pipetting:** Dispense media gently along well wall, never directly onto cells.
- **Weakly adherent cells:** Use "gentle washing" method with smaller volumes (see protocol for details).
- **Cells not fully adhered:** Allow longer incubation time after seeding (up to 48 hours for some cell types).

💡 Additional Resources:

- Agilent Cell Analysis Technical Support: Contact for specific troubleshooting
- Cell Line Reference Database: Check optimal conditions for your cell type
- Agilent Learning Center: Basic procedures and best practices guides