Title

Quantification of cell energetics in human subcutaneous adipose progenitor cells after target gene knockdown



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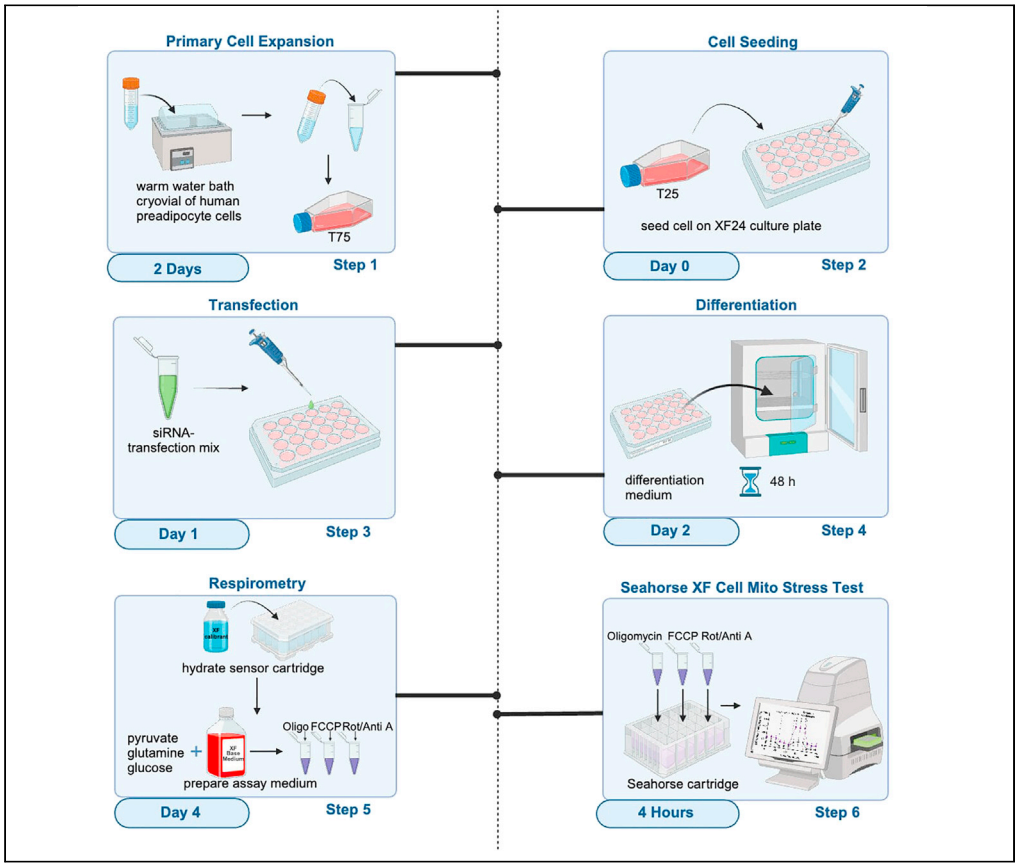


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Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Culture and differentiation of human subcutaneous preadipocytes

Effective knockdown of a specific gene in human subcutaneous preadipocytes

Analysis of the effect of a specific gene on cellular metabolism during adipogenesis

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# Quantification of cell energetics in human subcutaneous adipose progenitor cells after target gene knockdown

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## SUMMARY

**Pro-preadipocytes are adipocyte progenitor cells within subcutaneous adipose tissue that are conserved in human adipose tissue with distinct cellular energetics. Here, we detail a protocol to quantify cellular oxygen consumption rates of primary human cells harvested from adipose tissue. We describe steps for primary cell expansion, cell seeding, transfection, differentiation, and respirometry followed by Agilent Seahorse Analytics. The measurement of bioenergetic profiles and resulting data further expand our knowledge of the functional properties of primary cells isolated from adipose tissue.**

**For complete details on the use and execution of this protocol, please refer to Chen et al. (2023).[1]**

## BEFORE YOU BEGIN

### Thawing of cells and initiation of cell culture

*Note:* Human subcutaneous preadipocyte cells should be stored in liquid nitrogen until use.

1. Thaw the cryovial of human subcutaneous (abdominal) preadipocyte cells in a warm water bath for no more than 2 min.
   a. Decontaminate the external surface of the cryovial of cells.
2. Add thawed cell suspension to 10 mL of pre-warmed Preadipocyte Growth Medium-2 (PGM-2).
   a. Wash cells with PGM-2 and centrifuge at 300 × *g* for 10 min at 20°C–26°C (room temperature).

⚠ CRITICAL: When washing, leave at least 1 mL of wash at the bottom of the tube.

3. Resuspend cells in 5 mL of PGM-2.
   a. Plate the preadipocytes into a T75 tissue culture flask and add 10 mL more PGM-2 to the flask.
   b. Move on to subculturing step after cells have reached 80% confluency.

*Note:* Change the PGM-2 every 3–4 days while culturing. The culture will have a doubling time of 36–48 h.

⚠ CRITICAL: Do not allow the cells to become completely confluent.

### Subculturing of human preadipocyte cells in T25 flask

4. Remove the spent media and carefully add 2 mL–3 mL pre-warmed PBS to the side of the flask opposite the attached cell layer.
   a. Rock the flasks back and forth to rinse.
5. Discard the PBS wash solution and add 2 mL–3 mL of Trypsin-EDTA solution.
   a. Rock the flasks to equally distribute the solution over the cells, then place the flasks in the tissue culture incubator for 3–5 min.
   b. After 5 min, greater than 90% of the cells should be detached.
6. Stand each flask on its end and add 7 mL of room temperature PGM-2 to each flask.
   a. Equally distribute the solution over the cells.
7. Centrifuge the cells at 300 × *g* for 10 min at 20°C–26°C (room temperature) to remove the trypsin.
8. Resuspend the cells in 15 mL PGM-2.
   a. If necessary, remove an aliquot for cell counting.
9. Plate 5 mL of cell suspension into three respective T25 flasks.

*Note:* Primary human preadipocytes should not be passaged more than 5 times.

### Preparation of media

*Preadipocyte Growth Medium-2*

Timing: 30 min

Make the Preadipocyte Growth Medium-2 (PGM-2) by adding FBS, Glutamine, Gentamicin Sulfate, and Amphotericin to Preadipocyte Basal Medium-2. Volumes and concentrations of each reagent are indicated in the tables below. Reserve 100 mL of PGM-2 for preparation of Adipocyte Differentiation Medium.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Preadipocyte Growth Medium-2 BulletKit | Lonza | Cat# PT-8202 |
| 0.25% trypsin-EDTA (1×) | Gibco | Cat# 25200056 |
| Pierce BCA Protein Assay Kit | Thermo Fisher Scientific | Cat# 23227 |
| Protein extraction buffer | Thermo Fisher Scientific | Cat# 78510 |
| Complete protease inhibitor cocktail EDTA-free | Sigma-Aldrich | Cat# 11836170001 |
| FBS | Lonza | Cat# PT-9502 |
| L-glutamine | Lonza | Cat# PT-9502 |
| GA-1000 (gentamicin sulfate and amphotericin) | Lonza | Cat# PT-9502 |
| Glucose | Agilent | Cat# 103577-100 |
| Pyruvate | Agilent | Cat# 103578-100 |
| Glutamine | Agilent | Cat# 103579-100 |
| Seahorse XF DMEM medium | Agilent | Cat# 103575-100 |
| GlutaMAX | Thermo Fisher Scientific | Cat# 35050061 |
| Indomethacin | Lonza | Cat# PT-9502 |
| Insulin | Lonza | Cat# PT-9502 |
| Isobutylmethylxanthine | Lonza | Cat# PT-9502 |
| Dexamethasone | Lonza | Cat# PT-9502 |
| Lipofectamine RNAiMAX | Thermo Fisher Scientific | Cat# 13778100 |
| Agilent Seahorse XF Cell Mito Stress Test Kit | Agilent | Cat# 103015-100 |
| Penicillin-Streptomycin | Thermo Fisher Scientific | Cat# 15140122 |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Critical commercial assays | | |
| Seahorse XF24 FluxPak | Agilent | Cat# 102340-100 |
| Experimental models: Cell lines | | |
| Human subcutaneous preadipocytes | Lonza | Cat# PT-5020 |
| Oligonucleotides | | |
| sihNOCT | Thermo Fisher Scientific | Cat# 1299001 |
| Control siRNA-A | Santa Cruz Biotechnology | Cat# Sc-37007 |
| Software and algorithms | | |
| Seahorse Wave Desktop | Agilent | Online |
| Spreadsheet software | N/A | N/A |

## MATERIALS AND EQUIPMENT

### Preparation of necessary solutions for cell growth and differentiation

| Preadipocyte Growth Medium-2 (PGM-2) | | | |
|---|---|---|---|
| Reagent | Stock concentration | Final concentration | Amount |
| Glutamine | 200 mM | 2 mM | 500 µL |
| FBS | N/A | 10% (v/v) | 5 mL |
| Gentamicin Sulfate | 30 mg/mL | 30 µg/mL | 50 µL |
| Amphotericin | 15 µg/mL | 15 ng/mL | |
| Preadipocyte Basal Medium-2 | | | 44.5 mL |
| **Total** | | **N/A** | **50 mL** |
| Store at 4°C for no more than 5 days. | | | |

| Adipocyte differentiation medium | | | |
|---|---|---|---|
| Reagent | Stock concentration | Final concentration | Amount |
| Preadipocyte Growth Medium-2 | | | 10 mL |
| IBMX | 500 mM | 500 µM | 10 µL |
| Dexamethasone | 1 mM | 1 µM | 10 µL |
| Insulin | 85 µM | 850 nM | 100 µL |
| Indomethacin | 50 mM | 100 µM | 20 µL |
| **Total** | | **N/A** | **10 mL** |
| Prepare fresh for each experiment. | | | |

### Preparation of respiration assay medium and injection stock solutions for oxygen consumption assay analysis

*Alternatives:* There are a variety of media and injection solutions available for oxygen consumption analysis assays that can be used depending on specific experimental objectives.

| Injection stock solutions | | |
|---|---|---|
| Reagent | Stock concentration | Volume of assay medium |
| Oligomycin | 100 µM | 630 µL |
| FCCP | 100 µM | 720 µL |
| Rot/AA | 50 µM | 540 µL |
| Store at -20°C, avoid repeated freeze and thawing of the injection stock solutions. | | |

| Reagent | Final concentration in well (μM) | Stock solution volume (μL) | Respiration assay medium volume (μL) | 10× concentration in port (μM) | Volume added to port (μL) |
|---|---|---|---|---|---|
| Injection volumes and concentrations | | | | | |
| Port A Oligomycin | 1 | 140 | 1260 | 10 | 56 |
| Port B FCCP | 4 | 600 | 900 | 40 | 62 |
| Port C Rot/AA | 1 | 340 | 1360 | 10 | 69 |

The volumes in the tables above are for a Seahorse XF24-well cell culture microplate (V7-PS TC-related).

Prepare fresh injection working solutions in Respiration Assay Medium. Do not store injection working solutions. The volume added to each port depends on the initial volume of Respiration Assay Medium per well. We calculated the added volume of injection working solution based on a starting volume of 500 μL Respiration Assay Medium per well.

Respiration assay medium

| Reagent | Stock concentration | Final concentration | Volume/Weight |
|---|---|---|---|
| Seahorse XF DMEM Medium | | | 38.92 mL |
| Pyruvate | 100 mM | 1 mM | 0.4 mL |
| Glutamine | 200 mM | 2 mM | 0.4 mL |
| Glucose | 2.5 M | 7 mM | 0.28 mL |
| **Total** | **N/A** | **N/A** | **40 mL** |

Prepare fresh for each experiment.

Transfection mix

| Reagent | Stock concentration | Final concentration | Volume for 1 well | Volume for 28 wells | With 10% extra |
|---|---|---|---|---|---|
| OptiMEM | N/A | N/A | 37.5 μL + 37.5 μL | 1050 μL + 1050 μL | 1155 μL + 1155 μL |
| Lipofectamine RNAiMAX | N/A | N/A | 2.25 μL | 63 μL | 69.3 μL |
| siRNA | 10 μM | 37.5 nM (in well) | 0.75 μL | 21 μL | 23.1 μL |
| **Total** | **N/A** | **N/A** | **75 μL** | **2184 μL** | **2402.4 μL** |

Prepare fresh for each experiment.

## STEP-BY-STEP METHOD DETAILS

### Knockdown of a specific gene in human subcutaneous progenitor cells followed by oxygen consumption analysis

Timing: 2 h + transfection

While this protocol uses a 24-well cell culture microplate, the methodology and rationale of this protocol can be extended to a 96-well cell culture microplate-based assay.

*Day 0*

1. After cells grow to confluence in the T25 flask, remove the spent media and carefully add 1 mL of pre-warmed PBS to the side of the flask opposite the attached cell layer.
   a. Rock the flasks back and forth to rinse.

*Note:* The more passages the cells undergo, the longer the cells will take to become confluent.

2. Discard the PBS wash solution and add 800 μL-1 mL of Trypsin-EDTA solution.
   a. Rock the flasks to equally distribute the solution over the cells, then place the flasks in the tissue culture incubator for 3–5 min.

b. After 5 min, greater than 90% of the cells should be detached.
3. Stand each flask on its end and add 4 mL of room temperature PGM-2 to each flask.
   a. Equally distribute the solution over the cells.
4. Centrifuge the cells at 300 × *g* for 10 min at 20°C–26°C (room temperature) to remove the trypsin.
5. Resuspend the cells in 5 mL PGM-2 and remove an aliquot (20 μL) for cell counting.
6. Plate approximately 15,000 human subcutaneous preadipocyte cells (125 μL) per well into the Seahorse XF24 cell culture microplate.

   ***Note:*** We suggest leaving four wells as control wells for this protocol, specifically wells A1, B3, C4, and D6.

7. Plate approximately 15,000 human subcutaneous preadipocyte cells (125 μL) per well into 8 wells in a standard 48-well tissue culture plate.

   ***Note:*** The volume of cell suspension added into each well of the Seahorse XF24 cell culture microplate will vary based on the final cell yield calculated after conducting a cell count.

8. Gently shake the Seahorse XF24 cell culture microplate in all directions by hand before allowing the plate to rest at 20°C–26°C (room temperature) in the cell culture hood for 10 min.
   a. This enables equal distribution of the cells within the well.
   b. Repeat this step with the standard 48-well tissue culture plate.
9. Place both the Seahorse XF24 cell culture microplate and the standard 48-well tissue culture plate in the tissue culture incubator (with 5% $CO_2$) overnight.

*Transfection (Day 1)*

10. Retrieve the Seahorse XF24 cell culture microplate and the standard 48-well tissue culture plate from the incubator.
    a. View the cells under the microscope and confirm that the cells are 80% confluent.
11. Make a transfection mix using OptiMEM, Lipofectamine RNAiMAX, and siRNA stock solutions.
    a. Dilute the Lipofectamine RNAiMAX (2.25 μL per well) in OptiMEM (37.5 μL per well), and siRNA (0.75 pM per well) in OptiMEM (37.5 μL per well) respectively.
    b. Add at least 10% extra volume when making the transfection mix to ensure that all wells will be filled equally.

| Reagent | Volume OptiMEM | Volume RNAiMax (to be diluted in 37.5 μL OptiMEM) | Volume siRNA (10 μM stock, to be diluted in 37.5 μL OptiMEM) | Volume cell suspension |
|---|---|---|---|---|
| Per well | 75 μL | 2.25 μL | 0.75 μL | 125 μL |

12. Add the diluted siRNA to the diluted Lipofectamine RNAiMAX reagent in a 1:1 ratio and incubate at 20°C–26°C (room temperature) for 5 min.
13. After 5 min, add 75 μL of transfection mix into each well in both the Seahorse XF24 cell culture microplate and the standard 48-well tissue culture plate.
14. Place both the Seahorse XF24 cell culture microplate and standard 48-well standard tissue culture plate at the back of the tissue culture incubator to limit environmental changes.
    a. Allow the plates to rest overnight in the cell culture incubator.

*Day 2 (after transfection)*

15. Retrieve both the Seahorse XF24 cell culture microplate and standard 48-well tissue culture plate from the incubator.

16. Remove the PGM-2 and add 200 μL of fresh Adipocyte Differentiation Medium to induce white adipocyte differentiation from human subcutaneous preadipocyte cells into each well in the Seahorse XF24 cell culture microplate.
    a. Repeat this step with the standard 48-well tissue culture plate.
17. Return both the Seahorse XF24 cell culture microplate and standard 48-well tissue culture plate to the back of the incubator for 48 h.
18. After the 48 h have elapsed, view the cells under the microscope to assess cell health and morphology.
    a. The cells should now be ready for oxygen consumption analysis.
    b. Cells can be harvested from the standard 48-well tissue culture plate to assess knockdown efficiency at this time.

*Note:* Before undergoing differentiation, human subcutaneous preadipocytes will show fibroblast-like physical features. After undergoing successful differentiation into white adipocytes, cells will accumulate fat droplets and develop a rounder shape.

### Sensor cartridge hydration and creating the seahorse assay (Day 4)

Timing: 30 min + overnight hydration

*Note:* The Seahorse XF24 sensor cartridge needs to be hydrated the day prior to conducting the respiration assay.

19. Invert the Seahorse XF24 sensor cartridge such that it is upside down.

⚠ CRITICAL: Do not touch the sensors on the Seahorse XF24 sensor cartridge. Should the Seahorse XF24 sensor cartridge sensors encounter any surface or object, the final data will be skewed, potentially leading to misinterpretation of results.

20. Fill each well of the utility plate with 1 mL of XF Calibrant, carefully lower the Seahorse XF24 sensor cartridge back onto the utility plate and ensure that each sensor is properly submerged.
21. Place the Seahorse XF24 sensor cartridge and utility plate overnight in a humidified, non-$CO_2$ incubator at 37°C.

Create a Seahorse assay template including the following protocol using Wave Desktop:

| Steps | Time | Cycles |
|---|---|---|
| Calibration | | |
| Equilibration | | 3 |
| Mix | 3 min | |
| Wait | 2 min | |
| Measure | 2 min | |
| Injection, Port A (Oligomycin) | | 3 |
| Mix | 3 min | |
| Wait | 2 min | |
| Measure | 2 min | |
| Injection, Port B (FCCP) | | 3 |
| Mix | 3 min | |
| Wait | 2 min | |
| Measure | 2 min | |
| Injection, Port C (Antimycin A) | | 3 |
| Mix | 3 min | |
| Wait | 2 min | |
| Measure | 2 min | |

### Respiration media preparation (Day 4)

Timing: 45 min

On the same day the sensor cartridge hydration is completed, the Respiration Assay Medium is prepared.

22. Aliquot 40 mL of Seahorse XF DMEM Medium into a 50 mL conical tube to be used for washing steps.
    a. Incubate the Seahorse XF DMEM Medium, Seahorse XF 1.0 M Glucose Solution, Seahorse XF 100 mM Pyruvate Solution, and Seahorse XF 200 mM Glutamine Solution (aliquot) at 37°C until ready for use.
23. Begin preparing the Respiration Assay Medium.
    a. Add appropriate volumes of the XF supplements (XF Glucose, XF Pyruvate, and/or XF Glutamine solution) indicated in the tables above.
    b. It is recommended to incubate the Respiration Assay Medium at 37°C for no more than 4 h as substrates, such as glutamine, can degrade.

### Washing the cells and adding fresh respiration assay medium (Day 4)

Timing: 30 min

Once fresh Respiration Assay Medium is prepared, the cells are washed prior to adding the medium into each well in the microplate.

⚠ CRITICAL: Only process one-half or one-third of the microplate at a given time to prevent the cells from drying out. Practice changing the medium prior to conducting the assay.

24. Retrieve the Seahorse XF24 cell culture microplate from the incubator.
25. View cells under a microscope to assess cell health and document for reference or publication.
26. Carefully remove all but 50 μL of the Assay Differentiation Medium.
    a. Add 200 μL of fresh Respiration Assay Medium.
    b. Repeat this step one more time.

*Note:* Using a multichannel pipette will eliminate variability between results.

27. Add 500 μL of Respiration Assay Medium to each well.
28. View the cells under the microscope and check for any cell damage.
29. Incubate the Seahorse XF24 cell culture microplate at 37°C in a non-$CO_2$ incubator for 60 min prior to assay commencement.

### Preparing and loading the injection ports and starting the assay (Day 4)

Timing: 30 min + respiration assay run time

⚠ CRITICAL: It is imperative that all working solutions that are to be loaded into the sensor cartridge's injection ports are prepared freshly on the day of the assay.

30. Retrieve the Agilent Seahorse XF Cell Mito Stress Test Kit from -20°C storage and allow the compounds to warm to room temperature for approximately 15 min.
31. Resuspend the Oligomycin, FCCP, and Antimycin A with the freshly prepared Respiration Assay Medium using the volumes described in the table(s) above.
32. Gently pipette the medium up and down roughly 10 times to solubilize the compounds.

a. These are the compound stock solutions.

33. Use Oligomycin, FCCP, and Antimycin A stock solutions to make working solutions for loading into the injection ports of the sensor cartridge.
    a. Load the sensor cartridge ports with the working solutions (multichannel pipettes and reagent reservoirs are recommended to maintain consistency).[2]

⚠ CRITICAL: Before loading the sensor cartridge, utility plate, or cell culture plate into the instrument tray, ensure that all external packaging has been removed. Make sure there is a secure fit between the utility plate, the cell culture plate, and the instrument tray, respectively. Conduct a final check to make sure all pieces are orientated correctly.

34. Place the utility plate with the loaded sensor cartridge onto the instrument tray.
    a. Start the respiration assay 40 min after the Seahorse XF24 cell culture microplate was placed in a non-$CO_2$ incubator; calibration takes 20 min.
35. When prompted, replace the utility plate with the Seahorse XF24 cell culture microplate.
36. Click "Start." The assay will take approximately 2 h.
37. Once all measurements are completed, an "Unload Sensor Cartridge" message will be displayed.
    a. Once this message appears, click "Eject" to eject both the sensor cartridge and the Seahorse XF24 cell culture microplate.
38. Separate the sensor cartridge from the Seahorse XF24 cell culture microplate.

⚠ CRITICAL: Check the injection ports of the sensor cartridge for any injection medium that has remained stuck in the port(s). These failed injections can be easily visualized and identified. Take note of any and all ports that were not completely emptied during the assay and plan for subsequent data analysis.

*Optional:* Should you decide to conduct further analysis on the DNA, RNA, or protein content of the cells, the Seahorse XF24 cell culture microplate can be stored at -80°C at this time.

### Testing the protein content of each well

Timing: 2 h

39. Wash each well twice in the Seahorse XF24 cell culture microplate with 500 µL PBS.
40. Lyse the cell with 50 µL Protein Extraction Buffer containing Complete Protease Inhibitor Cocktail EDTA-free.
    a. Gently pipette up and down 10 times to homogenize.
41. Centrifuge the homogenate at 4°C at 15,000 × *g* for 20 min.
    a. The supernatant contains the protein fraction.
42. Analyze the protein concentration using a protein quantification assay using bicinchoninic acid.

## EXPECTED OUTCOMES

### Knockdown of a specific gene in human subcutaneous preadipocytes

Expect 75%–95% knockdown efficiency of mRNA of the targeted gene 72 h after treatment when compared to a negative control that shows neither cell damage nor cytotoxicity.

### Oxygen consumption analysis

Basal oxygen consumption in the first three measurements in the Seahorse is generally between 10–30 pmol $O_2$/min/µg protein (Figure 1). After the injection of Oligomycin, oxygen consumption should immediately decrease to under 20 pmol $O_2$/min/µg protein. This is explained by Oligomycin inhibition of the mitochondrial ATP synthase.[3] There can be variability in the magnitude of the decrease in basal oxygen consumption, as rates will depend on the type of cells and other

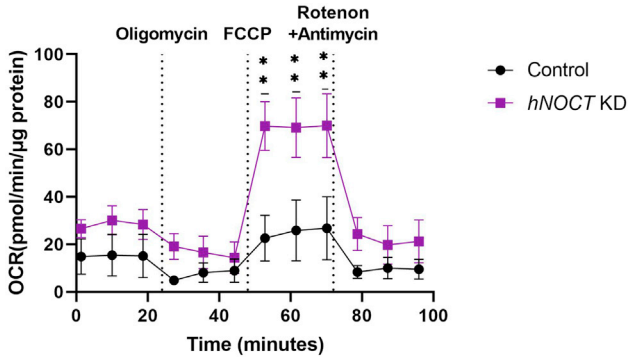


**Figure 1. Oxygen consumption rate data**

Quantification of adipocyte cell energetics after exposure to oligomycin, FCCP, and rotenone and Antimycin A following knockdown of *hNOCT*. All data presented are mean values ± SEM. Two-way ANOVA was used for statistical analysis. n = 3, **p < 0.01.

conditions. Despite these variations, a steep decrease in respiration rates and the oxygen consumption rates should be observed after the Oligomycin injection. Oxygen consumption should then increase after FCCP (carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone) is injected, generally expecting an increase to 20–70 pmol $O_2$/min/µg protein. This is because FCCP is a mitochondrial uncoupler: it is a protonophore that allows protons to cross membranes in the electron transport chain, which drives ATP-synthase and ATP production.[4] The last injection in the Seahorse includes Rotenone and Antimycin A. This addition inhibits cytochrome c reductase in complex III of the electron transport chain and, thus, fully blocks the electron transport chain, leaving only non-mitochondrial oxygen consumption remaining.[5]

## QUANTIFICATION AND STATISTICAL ANALYSIS

1. Login into Agilent Seahorse Analytics.
2. Upload the assay result file (*.asyr) into Agilent Seahorse Analytics.
3. Click the "Files" tab at the top of the screen and open the assay result file.
4. Expand the "Views" tab on the left-hand side of the screen to display the full list of analysis options.
5. Within the "Views" tab, add a "View" and click the "Assay Kit Comparison Views" tab. Select "XF Cell Mito Stress Test" and choose a widget for further analysis. To customize any data, double-click the widget to open the widget's editing portal.

   *Note:* The data represented below (Figure 1) was analyzed using the "Mitochondrial Respiration" widget. The widget necessary for analysis can vary depending on which Seahorse Kit is used.

6. Return to the "Analysis" view by clicking the back arrow.
7. Click the "Normalize" function in the upper right-hand corner of the screen.
8. Ensure the "Normalization Units" are in µg protein to assess the protein content of each well. Ensure the "Scale Factor" is 1. Input the protein concentrations of each well previously tested using the protein quantification assay into the "Normalization Values" table.
9. Return to the "Analysis" view by clicking the back arrow.

Click on the "Mitochondrial Respiration" widget and click on the "Normalization" toggle to turn "Normalization" on from its default state.

*Note:* Assessing the resulting data as a group can lead to misinterpretation of results. Make sure to assess the resulting data from each individual well, check for extreme outliers, and plan for subsequent data analysis.

*Note:* Wells in rows "A" and "H" and wells in columns "1" and "6" are frequently observed outliers.

10. Click on individual wells within the "Plate Map" grid to remove outliers from the overall data analysis. Wells that are greyed out will not be included in the data analysis.
11. Export the resulting data from the assay to Microsoft Excel by clicking the "Export" icon. Extrapolate the data from the resulting Excel file and analyze it using GraphPad Prism.

The "Baseline" function on the Wave software can be used to scale oxygen consumption rate traces. This will express the data generated from the assay as a percentage of a selected rate measurement. This function can aid in visualizing the acute effect of administered injections. A general recommendation proposes selecting rate measurements prior to Oligomycin administration. However, using the "Baseline" function can lead to data misinterpretation. The function can potentially reduce information generated from the experiment. Scaled data should only be considered in conjunction with the original raw oxygen consumption data.

## LIMITATIONS

The most prominent limitation of this assay is the high price point of reagents. While the technology is advanced and precisely measures oxygen consumption rates and extracellular acidification rates, the price of the analyzer and its corresponding materials is high. These limit availability to laboratories and researchers.

A second limitation of this assay is that the scientist needs to be aware that the position of some wells within the Seahorse XF24 cell culture microplate in the Seahorse XFe24 Analyzer might cause technical variation in the results. For example, we observed that certain wells consistently had higher respiration rates compared to others, particularly those located around the periphery of the plate. There may be variability in this technical issue in different machines. If this is observed, it might be necessary to exclude the use of those wells when performing the assay.

A final limitation is that the commercial human subcutaneous cell line is not immortalized. To avoid error, the cells should not be passaged more than 5 times to maintain the cells' differentiation ability.

## TROUBLESHOOTING

### Problem 1

Low final human preadipocyte cell count (before you begin Step 2).

### Potential solution

It is likely that some of the cell pellet was removed while washing. To avoid a low final cell count, ensure to leave a minimum of 1 mL of the wash left at the bottom of the tube while washing.

### Problem 2

Cells do not reach 70%–80% confluency on Day 1 (Step 10).

### Potential solution

Time between seeding and human preadipocytes reaching 70% confluence (start of the transfection phase) is too long. Fresh preadipocyte cultures should have a doubling time of approximately 36–48 h. Over-passaged cells will negatively affect their proliferation potential.

### Problem 3

Basal oxygen consumption rate of cells is too low following transfection (Step 11).

### Potential solutions

Ensure cells get appropriate confluency per well of the Seahorse XF24 cell culture microplate before starting transfection procedure.

The transfection reagent is cytotoxic. Lower the concentration of Lipofectamine RNAiMAX.

A high amount of siRNA causes cytotoxic effects. Lower the concentration of the siRNA.

Treat the cells more gently, especially during the washing steps.

### Problem 4

The injection ports are not completely emptied during the assay (Step 32).

### Potential solutions

Take care to fill all injection ports regardless of use. Do not leave any ports empty.

Practice loading the injection ports prior to conducting the assay. Proper loading technique requires a specific pressure and speed to ensure the solution has filled each respective port. Applying too much pressure when loading can result in premature emptying or leaking. Similarly, loading the injection solution too quickly can result in premature emptying due to the injection solution only partially filling the injection port.

### Problem 5

There is low knockdown efficiency (Step 18).

### Potential solutions

Ensure you perform transfection only after the cells have reached 70%–80% confluency.

Take care to optimize the exposure time to the transfection agent. Keep the siRNA complexes with normal growth media 8–24 h after transfection has occurred.

Titrate the concentration of Lipofectamine RNAiMAX and the siRNA. Use high quality siRNA at the lowest effective concentration.

Reverse transfection has been reported to allow cells to be more effectively transfected.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Brian Feldman (brian.feldman@ucsf.edu).

### Materials availability

This study did not generate any unique materials or reagents.

### Data and code availability

This study did not generate unique datasets or code.

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## AUTHOR CONTRIBUTIONS

Conceptualization, B.J.F.; investigation, B.J.F. and L.L.; writing – original draft, A.M.G., L.L., T.N., and B.J.F.; writing – review and editing, B.J.F., A.M.G., and L.L.; funding acquisition, B.J.F.; supervision, B.J.F.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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