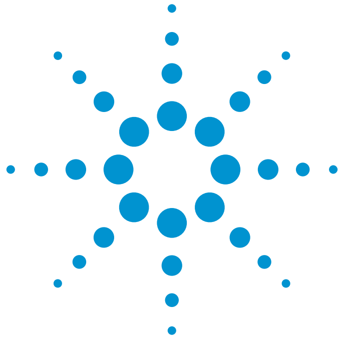


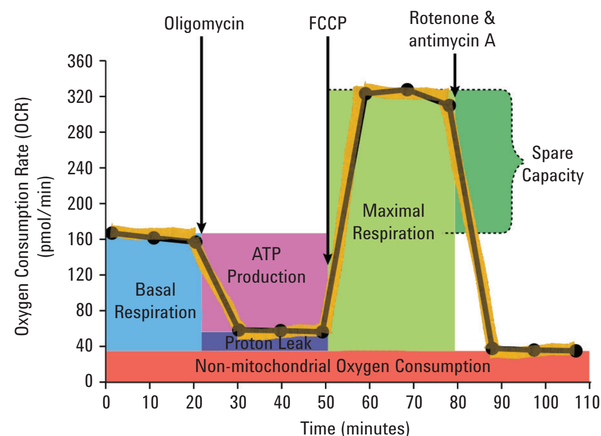
3T3-L1 Pre-Adipocyte Differentiation

Technical Overview

Introduction

The pre-adipocyte 3T3-L1 cell culture line, derived from mouse embryos, is a widely used model for the study of adipocyte differentiation. Robust and well-characterized, 3T3-L1 pre-adipocytes differentiate and form fat pads indistinguishable from normal adipose tissue when injected into mice.

In this protocol, differentiation is achieved *in vitro* through the sequential application of adipogenic factors to 3TC-L1 cells over a period of several days in culture. Following differentiation, metabolic functionality of adipocytes is characterized using the Agilent Seahorse XF Analyzer and the Agilent Seahorse XF Cell Mito Stress Test Kit. The Seahorse XF Cell Mito Stress Test Kit measures the four key parameters of mitochondrial function: basal respiration, ATP turnover, proton leak, and maximal respiration, revealing critical information not evident in basal metabolism measurements alone.



Agilent Seahorse XF Cell Mito Stress Test Profile. The fundamental parameters of mitochondrial function: basal respiration, ATP turnover, proton leak, and maximal respiration, or spare capacity.

Reagents and Materials

- Agilent Seahorse XF/XFe96 FluxPak (p/n 102416-100)
- Agilent Seahorse XF Cell Mito Stress Test Kit (p/n 103015-100)
- Gelatin (Sigma G9391)
- 3T3-L1 cells (ATCC CL-173)

Media

(Except where otherwise indicated, media and chemical additives were obtained from Sigma-Aldrich):

- Growth Medium (DMEM + 10 % FCS)
- Induction Medium (DMEM + 10 % FCS supplemented with IBMX (3-isobutyl-1-methylxanthine), DEXA (dexamethasone), TZD (thiazolidine) and insulin
- Agilent Seahorse XF Assay Medium (p/n 102365-100) supplemented with 25 mM glucose, 1 mM pyruvate

Cell Line Differentiation

The following cell seeding and differentiation protocol was developed by researchers at the Hotamisligil laboratory in conjunction with Agilent Seahorse Bioscience field application scientists, and is published with their permission.

Plate coating procedure

Prepare gelatin-coated 96-well Agilent Seahorse XF Cell Culture Microplates according to manufacturers' instructions with the following adaptations:

1. Prepare a 0.2 % w/v solution of gelatin in sterile tissue-culture grade H_2O at 37 °C.
2. Gently rock the plate to coat with 10 µL/cm^2 gelatin solution (XF microplates surface area = 0.13 cm^2), and pour off any excess.
3. Prep the coated plates for cell seeding by exposure to two 60 minute doses of UV radiation generated by the ultraviolet lamps in the cell culture hood (class II laminar flow biological safety cabinet).
4. Allow the coated plates to dry for 2 hours at room temperature (18–22 °C) prior to cell seeding.

Seeding cells

1. Dispense media only in columns 1 and 12.
2. Seed 5,000 3T3-L1 cells/well (DMEM + 10 % FCS; 37 °C at 10 % CO_2). In this experiment, columns 1 and 12 are used as the background correction wells for the XF assay– do not seed cells in these two columns, include medium only.
3. Allow the cells to grow for two days until they reach confluence.
4. Once the cells have reached confluence, maintain cells for two days in growth medium, then begin differentiation protocol.

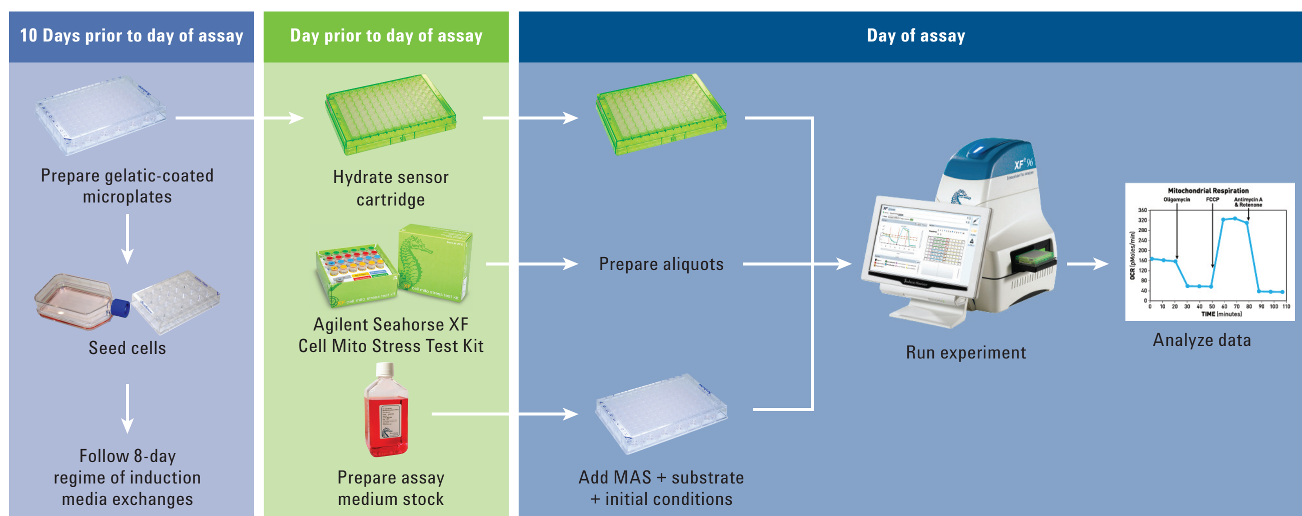


Figure 1. Assay workflow.

Differentiation protocol

Two days after the cells become confluent, exchange growth medium for induction medium and culture according to Table 1.

On day 8, the cells will be differentiated adipocytes.

Table 1. Differentiation protocol.

Timeline	Induction medium
Day 0–4	DMEM Supplemented with 10 % FCS + 500 µM IBMX + 1 µm DEXA + 1 µm TZD + 5 µg/mL insulin (change medium after day 2)
Day 4–6	DMEM Supplemented with 10 % FCS + 1 µm TZD + 5 µg/mL insulin
Day 6–9	DMEM Supplemented with 10 % FCS + 0.5 µg/mL insulin

Agilent Seahorse XF Cell Mito Stress Test

Refer to the Agilent Seahorse XF Cell Mito Stress Test User Manual for detailed instructions.

Note: Seahorse XF Cell Mito Stress Test compounds (Oligomycin and FCCP) must be optimized prior to running the stress test. Follow the instructions in the Seahorse XF Cell Mito Stress Test Kit User Manual to optimize these compounds. The concentrations cited below are provided as an example. These concentrations correspond to the injections used to generate the example data included in the Results section of this protocol.

1. Perform a medium exchange on the cells in the plate by removing the differentiation medium from each well and replacing with Agilent Seahorse XF Assay Medium supplemented with 25 mM glucose + 1 mM pyruvate:

   a. Remove all but 10 µL of differentiation medium.

   b. Rinse cells twice with 215 µL Agilent Seahorse XF Assay Medium supplemented with 25 mM glucose + 1 mM pyruvate.

   c. Add 215 µL Agilent Seahorse XF Assay Medium supplemented with 25 mM glucose + 1 mM pyruvate. The final volume in each well is 225 µL.

2. Place the cells in a non-CO_2 incubator at 37 °C for 1 hour prior to the start of the assay.

3. Use the Seahorse XF Cell Mito Stress Test assay template provided in Wave software to run the assay, or customize the template to meet the needs of your experiment. Manually set up the assay for the XF Analyzer following steps 3a–g. Figure 2 shows an example plate map comparing two groups.

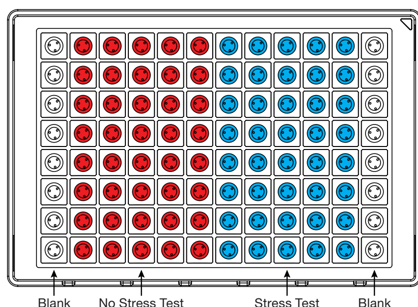


Figure 2. Example assay.

   a. Record three rate measurements to establish the basal rate.

   Note: In the example, the average baseline OCR is 150 pmol/min.

   b. Inject Port A: Group 1: Medium only (red); Group 2: Oligomycin (blue).

      i. Oligomycin injection concentration 10 µM (final concentration in the assay 1 µM)

      ii. Injection volume 25 µL

   Note: The concentration provided for example; must be optimized.

   c. Record six rate measurements following the oligomycin injection.

   d. Inject Port B: Group 1: Medium only; Group 2: FCCP.

      i. FCCP injection concentration 6 µM (final concentration in the assay 600 nM)

      ii. Injection volume 25 µL
      Note: The concentration provided for example; must be optimized.

   e. Record three rate measurements following the FCCP injection.

   f. Inject Port C: Group 1: Medium only; Group 2: Antimycin A and Rotenone.

      i. Antimycin A and Rotenone injection concentration 150 µM (final concentration in the assay 15 µM)

      ii. Injection volume 25 µL

   g. Record three rate measurements following the Antimycin A and Rotenone injection.

Results

The cells in Group 1 (red) received medium only. Cells in Group 2 (blue) demonstrated a robust response to the Agilent Seahorse XF Cell Mito Stress Test compounds.

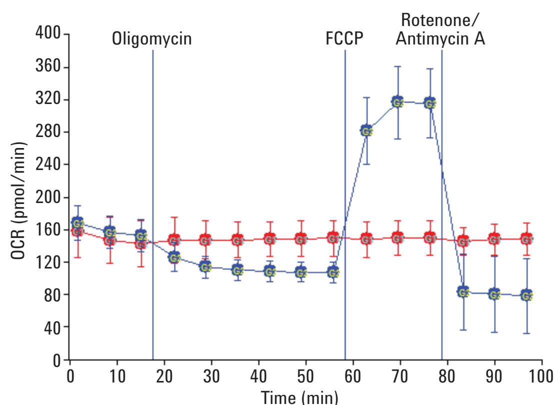


Figure 3. Differentiated adipocytes demonstrate a robust response in the Agilent Seahorse XF Cell Mito Stress Test.

Reference

1. Ntambi, J.a.Y.-C.K. Adipocyte Differentiation and Gene Expression. *Journal of Nutrition* **2000**, *130*, 3122S-3126S.

Further Information

1. Agilent Seahorse Bioscience Cellular Bioenergetics Webinar Series: *New dimensions in adipocyte metabolism: Using the XF to probe human and rodent adipocytes and adipose tissue* Presented by: Sheila Collins, PhD, Diabetes and Obesity Research Center, Sanford-Burnham Medical Research Institute and Einav Yehuda-Shnaidman PhD, RD, The Hebrew University of Jerusalem. Air date: February 9, **2011**.

2. Hotamisligil Laboratory website: *https://apps.sph.harvard.edu/publisher/upload/research/gsh-lab/research/protocols/index.html*

www.agilent.com

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