

Performing Bioenergetic Analysis: Seahorse XFe96 Analyzer

Introduction

Skeletal muscle is among the most metabolically active tissues that rely on oxidative phosphorylation, as demonstrated by the high density of spatially organized mitochondria present in myotubes. Dysregulation of mitochondrial activity in skeletal muscle has been implicated in several insulin resistance pathologies, such as diabetes mellitus and obesity.

iCell® Skeletal Myoblasts, derived from human induced pluripotent stem cells, recapitulate biochemical and physiological characteristics of native human myoblasts with the ability to fuse into myotubes. Due to their human origin, high purity, and functional relevance, iCell Skeletal Myoblasts represent an in vitro test system for skeletal muscle biology interrogations in basic research and drug discovery.

The Seahorse XFe96 Analyzer (Agilent Technologies) is a non-invasive, label-free, high-throughput instrument that measures the metabolic activity of living cells by simultaneously monitoring mitochondrial respiration and glycolysis. iCell Skeletal Myoblasts can be cultured directly on an XF96 Cell Culture Microplate where energy metabolism can be modulated and analyzed. Together, iCell Skeletal Myoblasts and the Seahorse XFe96 Analyzer offer an in vitro platform for analyzing mitochondrial function, understanding pathophysiology, and evaluating therapeutic interventions in human skeletal muscle.

This Application Protocol describes how to handle iCell Skeletal Myoblasts for use on the Seahorse XFe96 Analyzer and provides basic instructions for bioenergetic data acquisition and analysis.

Required Equipment, Consumables, and Software

The following equipment, consumables, and software are required in addition to the materials specified in the iCell Skeletal Myoblasts User's Guide.

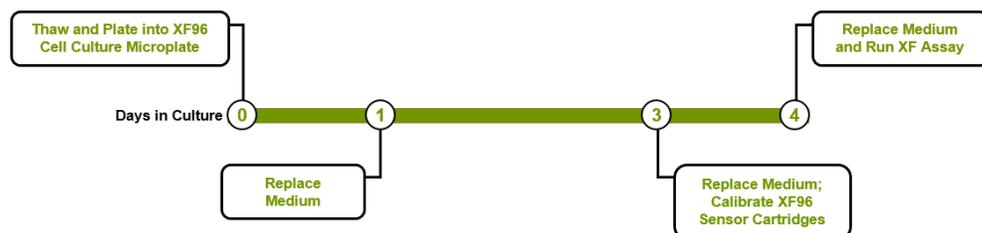
Item	Vendor	Catalog Number
Equipment		
8- or 12-well Multichannel Pipettor	Multiple Vendors	
Seahorse XFe96 Analyzer	Agilent Technologies	
Consumables		
iCell Skeletal Myoblasts	Cellular Dynamics International (CDI)	SKM-301-020-001-PT
Seahorse XFe96 FluxPak	Agilent Technologies	102601-100 02416-100
Seahorse XF Base Medium	Agilent Technologies	102353-100
Seahorse XF Cell Mito Stress Test Kit	Agilent Technologies	103015-100

Item	Vendor	Catalog Number
Software		
Wave Controller	Agilent Technologies	

Notes

Workflow

iCell Skeletal Myoblasts are thawed and plated into an XF96 Cell Culture Microplate previously coated with fibronectin. On day 1 and 3 post-plating, spent medium is replaced with Maintenance Medium. On day 3 post-plating, XF96 Sensor Cartridges are hydrated. On day 4 post-plating, spent medium is replaced with assay medium, and the XF assay is performed.



Methods

Thawing iCell Skeletal Myoblasts

1. Dilute 1 mg/ml fibronectin solution in sterile D-PBS to a final concentration of 10 µg/ml immediately before use.

Note: Reconstitute fibronectin in sterile water at 1 mg/ml according to the manufacturer's instructions. Aliquot and store at -20°C.

2. Coat an XF96 Cell Culture Microplate with 50 µl/well of 10 µg/ml fibronectin for at least 1 hour according to the iCell Skeletal Myoblasts User's Guide.
3. Thaw iCell Skeletal Myoblasts according to the User's Guide.
4. Remove a sample of cells to perform a cell count using a hemocytometer (using trypan blue exclusion to identify viable cells).
5. Dilute the cell suspension in Maintenance Medium to 412,500 viable cells/ml.
6. Aspirate the fibronectin solution. Immediately add 80 µl/well of the cell suspension (33,000 cells/well).

Note: CDI recommends seeding the 60 inner wells of an XF96 Cell Culture Microplate to avoid edge effects. Add the Maintenance Medium to all outer wells and all wells not containing cells to minimize the occurrence of edge effects.

7. Place the XF96 Cell Culture Microplate in a biological safety cabinet at room temperature for 20 - 30 minutes to allow the myoblasts to settle and ensure an even distribution.
8. Culture iCell Skeletal Myoblasts in a cell culture incubator at 37°C, 5% CO₂.

Maintaining iCell Skeletal Myoblasts

1. Maintain iCell Skeletal Myoblasts according to the User's Guide, gently replacing spent medium with Maintenance Medium on day 1 post-plating.
2. Maintain the cells according to the User's Guide, replacing medium every other day.

Data Acquisition and Analysis

On day 3 post-plating into the XF96 Cell Culture Microplate, iCell Skeletal Myoblasts appear fused into myotubes. At day 4 post-plating, the preparation is suited for data acquisition. Refer to the manufacturer's instructions for the Seahorse XFe96 Analyzer to perform data acquisition and analysis.

Compound Application

1. Hydrate the XF96 Sensor Cartridge with 200 μ l/well of XF Calibrant Solution at approximately 24 hours before performing metabolic assessment of iCell Skeletal Myoblasts using the XF assay.
2. Add the desired additives to the XF Base Medium, if applicable, to create a complete assay medium on the day of the XF assay.
3. Adjust the pH of the assay medium to 7.4 and equilibrate it to approximately 37°C.
4. Remove 50 μ l/well of the spent medium from the XF96 Cell Culture Microplate, leaving approximately 30 μ l/well to avoid cell detachment.
5. Wash twice with 170 μ l/well of the assay medium, leaving approximately 30 μ l/well to avoid cell detachment, at approximately 45 - 60 minutes before starting the XF assay.
6. Add 150 μ l/well of the assay medium to reach a final volume of 180 μ l/well. Incubate the XF96 Cell Culture Microplate in a cell culture incubator at 37°C with atmospheric CO₂ levels (i.e. no additional CO₂) for 45 - 60 minutes.

Note: It is critical to incubate the XF96 Cell Culture Microplate in atmospheric levels of CO₂ before the assay because CO₂ outgassing from the XF96 Cell Culture Microplate can affect the ECAR (Extracellular Acidification Rate) readout.

Note: The total volume of 180 μ l/well is the recommended starting volume for the Constant Concentration protocol described in the manufacturer's instructions for the Seahorse XFe96 Analyzer.

7. Load injection ports A, B, and C of the XF96 Sensor Cartridge with the recommended volumes of the components of the XF Cell Mito Stress Test Kit (or a compound of interest, if applicable) according to the Constant Concentration protocol described in the manufacturer's instructions for the Seahorse XFe96 Analyzer.

Example Data

Representative respiratory profiles of iCell Skeletal Myoblasts are shown in Figure 1 to exemplify the effects of modulating energy metabolism through the addition of compounds having a direct effect on mitochondrial integrity and function. Data were acquired using the Wave Controller software set for a 3 minute mixing time and 3 minute measuring time. The basal mitochondrial respiration decreases after treatment with the ATP-synthase-inhibitor oligomycin. The oligomycin-insensitive respiration is due to proton leak. The subsequent addition of a proton ionophore, carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP), uncouples oxidative phosphorylation from the electron transport system and shows the maximal respiration rate in iCell Skeletal Myoblasts. The mitochondrial electron transport chain is inhibited completely by the addition of the complex I-specific inhibitor rotenone and the cytochrome c reductase-inhibitor antimycin A.

Notes

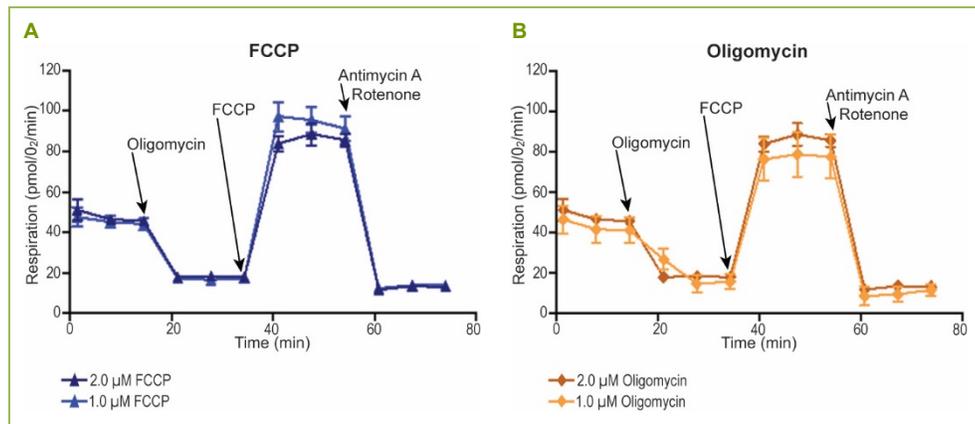


Figure 1: Representative Respiratory Profile of iCell Skeletal Myoblasts

iCell Skeletal Myoblasts were cultured on the XF96 Cell Culture Microplate for 4 days. The Maintenance Medium was replaced with XF Base Medium (pH 7.4) supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), and glucose (10 mM) at 45 - 60 minutes before performing the XF assay. Optimal concentration of oligomycin (1 μ M), FCCP (1 μ M), antimycin A (0.5 μ M), and rotenone (0.5 μ M) were added where indicated. Panels A and B show the concentration effect on the respiratory profile for FCCP and oligomycin, respectively.

Summary

iCell Skeletal Myoblasts form myotubes within 3 days post-plating, and provide relevant and consistent in vitro cellular system for studying mitochondrial activity in human myotubes. The Seahorse XFe96 Analyzer provides a label-free technology for non-invasive monitoring of basal oxygen consumption, glycolysis rate, ATP turnover, and spare respiratory capacity. The metabolic profile typical of skeletal myotubes can be monitored, and the treatment effects on energy metabolism can be detected and quantified. The methods and data presented here highlight how to obtain robust and relevant data with respect to the cellular bioenergetic function and responses to oxidative stress in living human myotubes.

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