Modulating Glucose Uptake in Skeletal Myotubes:

Insulin Induction with Bioluminescent Glucose Uptake Analysis

Introduction

The skeletal muscle is one of the primary targets of insulin-stimulated glucose uptake. Insulin induces an increase in glucose transporter activity (primarily GLUT4) by triggering a translocation of the intracellular glucose transporters to the plasma membrane. Abnormalities in molecular mechanisms of insulin-mediated glucose uptake are associated to metabolic disorders, such as type 2 diabetes.

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.

Insulin-induced glucose uptake can be measured using the glucose analog 2-deoxyglucose (2-DG). The 2-DG is taken up by cells and phosphorylated to 2-DG6P that accumulates in the cells. The 2-DG uptake is then determined using a coupled bioluminescent assay, in which the 2-DG6P is oxidized in the presence of G6PDH resulting in the generation of NADPH that can be detected and quantified.

This Application Protocol describes how to analyze insulin-induced glucose uptake in iCell Skeletal Myoblasts fused into myotubes as a human-based in vitro model to study modulation of glucose metabolism in myotubes.

Required Equipment and Consumables

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

Item	Vendor	Catalog Number
Equipment		
Plate Reader	Multiple Vendors	
Consumables		
iCell Skeletal Myoblasts	Cellular Dynamics International (CDI)	SKM-301-020-001-PT
8-bromo-cyclic AMP	Axxora	BLG-B007
96-well White Cell Culture Plates	Corning	3610
Bioluminescent Glucose Uptake Assay Kit	Promega	Contact Promega's Custom Assay Services
$CaCl_2$	Multiple Vendors	
CHIR99021	Stemgent	04-0004

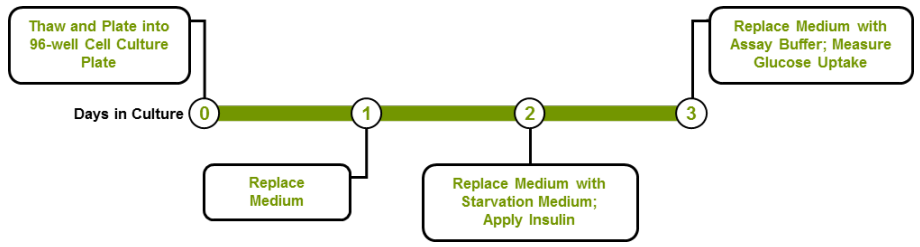
Item	Vendor	Catalog Number
Cytochalasin B	Sigma	C6762-5MG
Dorsomorphin	Sigma	P5499
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Life Technologies	14190
Fatty Acid Free BSA	Sigma	A8806-5G
Fibronectin	Roche Applied Science	11051407001 11080938001
HEPES, 1 M Solution	Thermo Fisher	15630-106
Insulin*	Life Technologies	12585-014
KCl	Multiple Vendors	
KnockOut Serum Replacement	Life Technologies	10828-010
MEM Alpha, No Nucleosides	Life Technologies	12561-056
$MgSO_4$	Multiple Vendors	
NaCl	Multiple Vendors	
Sterile Distilled Water	Multiple Vendors	

* Insulin from other vendors may be acceptable. CDI recommends to perform a titration to identify the optimal concentration to be applied on iCell Skeletal Myoblasts to induce glucose uptake. Contact CDI's Technical Support (support@cellulardynamics.com; +1 (877) 320-6688 (US toll-free) or (608) 310-5100) for more information.

Notes

Workflow

iCell Skeletal Myoblasts are thawed and plated into a fibronectin-coated 96-well cell culture plate in MEM alpha-based maintenance medium. On day 1 post-plating, spent medium is replaced with fresh MEM alpha-based maintenance medium. On day 2 post-plating, spent medium is replaced with starvation medium, and cells are treated with insulin. On day 3 post-plating, glucose uptake is measured using a bioluminescent assay.



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. Add 50 µl/well of the 10 µg/ml fibronectin solution to a 96-well cell culture plate to evenly coat the bottom of the wells.

Notes

3. Incubate at 37°C for at least 1 hour.
4. Prepare and store MEM alpha-based maintenance medium.
   a. Combine the following components:

Component	Amount	Final Concentration
MEM Alpha, No Nucleosides	94 ml	N/A
8-bromo-cyclic AMP (100 mM)	1 ml	1 mM
CHIR99021 (20 mM)	10 µl	2 µM
Dorsomorphin (5 mM)	20 µl	1 µM
KnockOut Serum Replacement	5 ml	5%

   b. Filter the medium using a 0.2 µm PES filter unit.
   c. Prepare working aliquots of the medium.
   d. Store the medium at 4°C, protected from light, for up to 1 week.
5. Equilibrate an aliquot of MEM alpha-based maintenance medium to room temperature.
6. Thaw iCell Skeletal Myoblasts according to the iCell Skeletal Myoblasts User's Guide using MEM alpha-based maintenance medium to dilute the cell suspension.
7. Remove a sample of cells to perform a cell count using a hemocytometer (using trypan blue exclusion to identify viable cells) or an automated cell counter.
8. Dilute the cell suspension in MEM alpha-based maintenance medium to 800,000 viable cells/ml.
9. Aspirate the fibronectin solution. Immediately add 100 µl/well of the cell suspension (80,000 cells/well) into the 96-well cell culture plate.
10. Culture iCell Skeletal Myoblasts in a cell culture incubator at 37°C, 5% CO_2.

Maintaining iCell Skeletal Myoblasts

1. Maintain iCell Skeletal Myoblasts according to the User's Guide, gently replacing spent medium with MEM alpha-based maintenance medium on day 1 post-plating.
2. Culture iCell Skeletal Myoblasts in a cell culture incubator at 37°C, 5% CO_2.

Inducing Glucose Uptake in iCell Skeletal Myoblasts

On day 2 post-plating, iCell Skeletal Myoblasts require a medium replacement to starvation medium. Glucose uptake can be induced with insulin on day 2 post-plating for a 24-hour induction.

1. Prepare starvation medium.
   a. Combine the following components:

Component	Amount	Final Concentration
MEM Alpha, No Nucleosides	47.5 ml	N/A
Fatty Acid Free BSA, 10% in D-PBS	2.5 ml	0.5%

b. Filter the medium using a 0.2 µm PES filter unit.

c. Do not store starvation medium. Use only on the day of preparation.

2. Equilibrate starvation medium at room temperature.

3. Remove the 96-well cell culture plate containing iCell Skeletal Myoblasts from the cell culture incubator and gently wash cells twice with 100 µl/well of starvation medium to remove the spent medium.

4. Add insulin at ≥100 nM final concentration in 100 µl/well of starvation medium to induce glucose uptake.

5. Culture iCell Skeletal Myoblasts in a cell culture incubator at 37°C, 5% CO_2.

Measuring Glucose Uptake in iCell Skeletal Myoblasts

On day 3 post-plating, iCell Skeletal Myoblasts appear fused into myotubes. Optimal glucose uptake can be measured after treatment with insulin for 24 hours.

1. Prepare assay buffer.

   a. Combine the following components:

Component	Amount	Final Concentration
Sterile Distilled Water	44.7 ml	N/A
$CaCl_2$, 1 M	50 µl	1 mM
Fatty Acid Free BSA, 10% in D-PBS	2.5 ml	0.5%
HEPES, 1 M	1 ml	20 mM
KCl, 1 M	250 µl	5 mM
$MgSO_4$, 1 M	125 µl	2.5 mM
NaCl, 5 M	1.4 ml	140 mM

   b. Adjust the pH to 7.4

   c. Adjust the volume to 50 ml.

   d. Do not store assay buffer. Use only on the day of preparation.

2. Remove the 96-well cell culture plate containing iCell Skeletal Myoblasts from the cell culture incubator and gently wash cells twice with 100 µl/well of assay buffer to remove the spent medium.

3. Add the non-selective GLUT inhibitor cytochalasin B to the cells at a final concentration of 10 µM in 45 µl/well of assay buffer and incubate at 37°C for 20 minutes before measuring glucose uptake.

4. Add 5 µl/well of 1 mM 2-DG to reach a final concentration of 100 µM. Gently mix by pipetting up and down 3 - 5 times.

   Note: *2-DG is provided in the Bioluminescent Glucose Uptake Assay Kit.*

5. Incubate the plate at room temperature for 10 minutes.

6. Perform the glucose uptake measurement according to the Bioluminescence Glucose Uptake Assay Kit manufacturer's instructions.

Notes

Notes

Data Analysis

iCell Skeletal Myoblasts begin to fuse into myotubes on day 1 post-plating, and on day 3, they are ready for measuring insulin-induced glucose uptake. A titration of insulin is recommended to identify the optimal concentration to induce the glucose uptake. CDI recommends to perform the assay using 4 replicates per data point in each experiment. The insulin-dependent response can be quantified using the GLUT inhibitor cytochalasin B. The signal generated in presence of cytochalasin B is subtracted from all samples to generate normalized fold-change data relative to untreated conditions (control).

Example Data

Figure 1 illustrates representative assay data for evaluating insulin-induced glucose uptake.

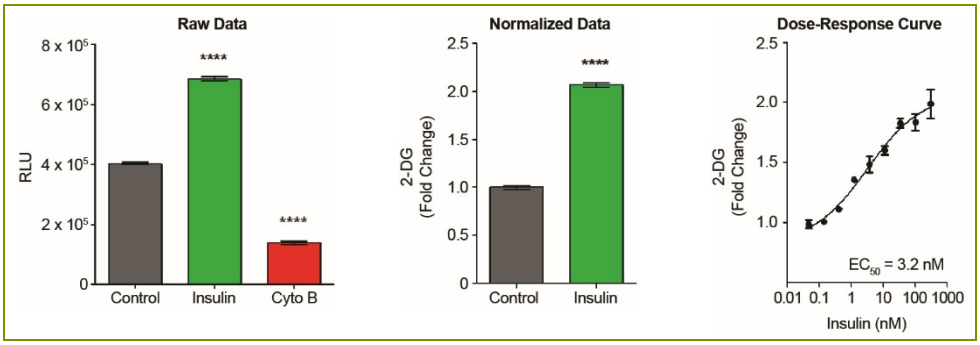


Figure 1: iCell Skeletal Myoblasts Provide a Human System That Is Robust and Easy to Use for Measuring Insulin-dependent Glucose Uptake

On day 3 post-plating, insulin-dependent glucose uptake is quantified using the Bioluminescent Glucose Uptake Assay Kit. Stimulation with insulin results in a twofold increase in 2-DG uptake while treatment with the GLUT inhibitor cytochalasin B (cyto B) in the presence of insulin reduces the assay signal (mean ± SEM; n = 3).

Summary

iCell Skeletal Myoblasts, derived from human iPS cells, form myotubes in culture within 3 days post-plating and provide a relevant and consistent in vitro cellular system for studying glucose uptake. The methods and data presented here highlight an HTS-compatible 96-well assay format for modulating glucose metabolism in skeletal myotubes using a bioluminescent assay.

Notes

Version: November 2015
AP-SKMGLU151102