Adipolysis Assay Kit

Cat. No. OB100

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Not for use in diagnostic procedures

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Introduction

Obesity is a significant clinical problem that contributes to life-threatening diseases such as diabetes and atherosclerosis. The identification of pathways leading to increased adipose tissue formation, and reversal of lipid stores in adipose tissue, raises the prospect of preventing or reversing obesity through pharmacological means. The process of adipogenesis, the formation of adipose tissue, has become better understood by the study of several cell types that can be induced to undergo differentiation into adipocytes. The first, and best characterized, model of adipogenesis *in vitro* is the 3T3-L1 cell line, a substrain of Swiss 3T3 mouse cell line (Kehinde and Green, 1974). 3T3-L1 cells propagated under normal conditions have a fibroblastic phenotype. However, when treated with a combination of dexamethasone, isobutylmethylxanthine (IBMX or MIX) and insulin, 3T3-L1 cells adopt a rounded phenotype and within 5 days begin to accumulate lipids intracellularly in the form of lipid droplets (Rubin *et al*., 1978).

Adipolysis refers to lipolysis, the degradation of triglyderide stores, in differentiated adipocytes. Several compounds, including isoproterenol and tumor necrosis factor-α (TNF-α, have been shown to stimulate adipolysis in differentiated 3T3-L1 and primary human adipocytes. Isoproterenol is a nonselective agonist of the beta-adrenergic class of GPCRs, which stimulate cAMP levels in adipocytes (Robidoux *et al*., 2004). Subsequent activation of PKA by elevated cAMP results in phosphorylation of perilipin, which is located at the surface of the lipid droplet (Sztalryd *et al.*, 2003). Although perilipin inhibits basal lipolysis by non-hormone sensitive lipases, phosphorylated perilipin recruits the hormone-sensitive lipase (HSL) to the surface of the lipid droplet (Zhang *et al*., 2003). HSL cleaves triglycerides into their constituent fatty acids and free glycerol, which can be assayed as a marker of adipolysis. Although the mechanism by which TNF-α induces adipocyte lipolysis has yet to be completely elucidated, activation of the MAPK family, downregulation of $G\alpha_i$, and/or downregulation of perilipin appear to play a role (Rydén *et al*., 2002; Rydén *et al*., 2004). In addition, extracellular glucose is required for the TNF-α-mediated adipocyte lipolysis (Green, *et al*., 2004). Glycerol generated by triglyceride breakdown is released into the extracellular space through aquaporin adipose (Kishida *et al.*, 2000). Extracellular glycerol is easily assayed by incubation with glycerol kinase (to produce glycerol phosphate), glycerol phosphate oxidase (to produce H_2O_2), and horseradish peroxidase in the presence of a colorimetric substrate.

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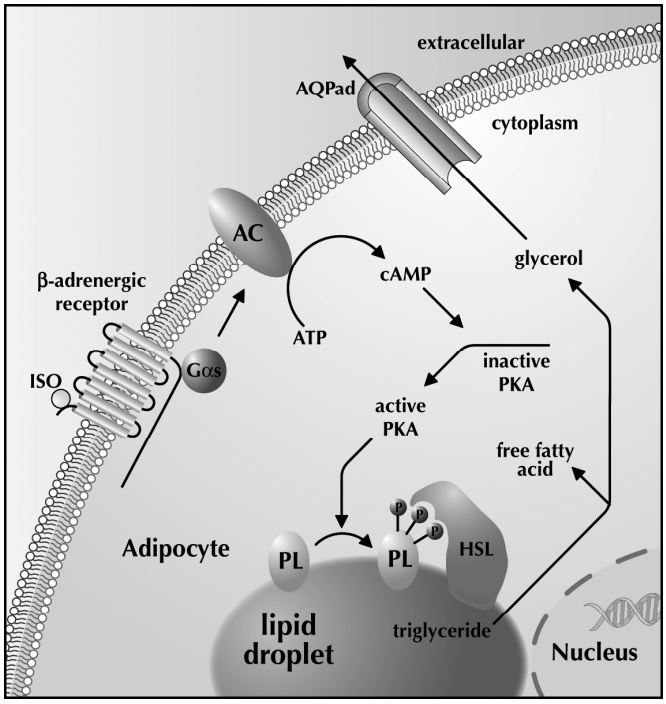

Figure 1. Induction of adipolysis by β-adrenergic receptor agonists. Binding of isoproterenol (ISO) to the β-adrenergic receptor activates the G-protein, G_s, which stimulates adenylate cyclase (AC) to produce cyclic AMP (cAMP). Protein kinase A (PKA) is activated by cAMP to phosphorylate the lipid droplet surface protein, perilipin (PL). Hormone-sensitive lipase (HSL) docks onto phosphorylated PL and breaks down triglyceride into glycerol and free fatty acid. Glycerol is released into the extracellular space through aquaporin adipose (AQPad).

Application

The Millipore Adipolysis Assay Kit provides the necessary reagents for differentiating adipocytes and analyzing triglyceride mobilization by glycerol release. The kit includes IBMX Solution, Dexamethasone Solution and Insulin Solution for addition to basal media (supplied by the user) to stimulate conversion of preadipocytes to lipid droplet-containing adipocytes. In addition, the kit supplies media optimized for application of the test substance to the differentiated adipocytes. A positive control Isoproterenol Solution is provided to stimulate high levels of adipolysis. Also included is a reagent cocktail for the coupled enzymatic analysis of free glycerol released into the medium, and a glycerol standard.

The Millipore Adipolysis Assay Kit is a convenient and sensitive tool for analysis of small molecules and proteins to stimulate or inhibit triglyceride breakdown in cultured adipocytes.

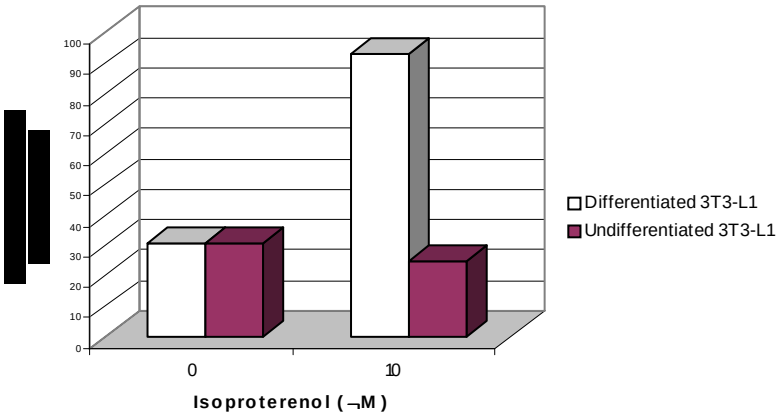

Figure 2. Adipolysis assay. 3T3-L1 cells were plated in 24-well plates at 60,000 cells/well. The cells were incubated in DMEM containing calf serum alone (undifferentiated), or with fetal calf serum (FCS), IBMX and dexamethasone (differentiated) for 2 days. Cells were then incubated with the same media, except with insulin substituted for IBMX and dexamethasone for 2 days. Cells were then incubated with DMEM + CS (undifferentiated) or DMEM + FCS (differentiated) for 3 days. Cells were washed and incubated with Incubation Solution with 2% BSA alone or containing 10 µM Isoproterenol (Isoproterenol Positive Control Working Solution) for 1 h. The culture supernatant was assayed for glycerol with Free Glycerol Assay Reagent, calibrated using Glycerol Standard Solution.

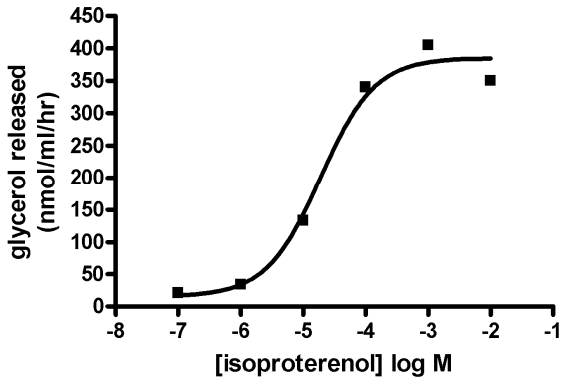

Figure 3. Dose response of isoproterenol in adipolysis assay. 3T3-L1 cells were differentiated and washed as in Figure 2. Cells were treated with increasing concentrations of isoproterenol, and assayed for glycerol release.

Kit Components

1. IBMX Solution - (Catalog No. 90355) - One vial containing 250 µL of 0.5 M 3-isobutyl-1-methylxanthine (IBMX) in DMSO.

2. Insulin Solution – (Catalog No. 90356) - One vial containing 250 µL of 10 mg/mL recombinant human insulin.

3. Dexamethasone Solution – (Catalog No. 90357) - One vial containing 100 µL of 10 mM dexamethasone in ethanol.

4. Isoproterenol Stock Solution – (Catalog No. 90539) – One vial containing 250 µL 10mM isoproterenol in water.

5. 30% BSA Solution – (Catalog No. 90540) – One bottle containing 8 mL of filtered 30% Bovine Serum Albumin in PBS, aseptically filled.

6. Glycerol Standard Solution – (Catalog No. 90541) – One vial containing 0.25 mL of a 0.26 mg/mL glycerol standard.

7. Free Glycerol Assay Reagent – (Catalog No. 90542) – two bottles containing lyophilized Free Glycerol Assay Reagent.

   After reconstitution in 10 mL distilled water per bottle, Free Glycerol Assay Reagent contains:
   0.75 mM ATP
   3.75 mM Magnesium salt
   0.188 mM 4-aminoantipyrine
   2.11 mM sodium-N-ethyl-N(3-sulfopropyl) m-anisidine
   1.25 units/mL microbial glycerol kinase
   2.5 units/mL microbial glycerol phosphate oxidase
   2.5 units/mL horseradish peroxidase
   Buffer pH 7.0
   0.05% sodium azide

8. Wash Solution – (Catalog No. 90544) - One bottle containing 200 mL of filtered Hanks' Balanced Salt Solution, aseptically filled

9. Incubation Solution – (Catalogue No. 90543) – One bottle containing 100 mL of filtered Hanks' Balanced Salt Solution, aseptically filled.

Materials Not Supplied

- 3T3-L1 Preadipocyte cell line (can be obtained from ATCC).
- Media for propagation of 3T3-L1 cells – DMEM with 10% calf serum (not fetal calf serum) and antibiotics.
- Trypsin/EDTA solution for passaging cells
- Adipogenesis basal media – DMEM with 10% fetal calf serum and antibiotics.
- 24-well or other size tissue culture plates for induction of adipogenesis.
- Spectrophotometer or 96-well plate reader capable of detecting 540 nm, for the most sensitive quantitation of the Free Glycerol Assay Reagent chromophore. A spectrophotometric plate reader set at 562 nm, typically used for Bicinchoninic Acid-based protein assays, is also effective.

Precautions

- Please refer to the Material Safety Data Sheet at www.millipore.com for further precautions.

Storage

Note: *Kit components require two different storage temperatures.*

Dexamethasone Solution, IBMX Solution, Insulin Solution and Isoproterenol Stock Solution should be stored at –20ºC protected from light. Glycerol Standard Solution, Free Glycerol Assay Reagent, Sterile 30% BSA Solution, Wash Solution and Incubation Solution should be stored at 2 - 8°C.

Preparation of Reagents

Note*: Reagents 1-6 should be prepared and opened only in a sterile laminar flow hood with proper sterile technique.*

1. Adipogenesis Initiation Media: DMEM/10% fetal calf serum/0.5 mM IBMX/1 µM dexamethasone – Dilute IBMX Solution 1:1000 and Dexamethasone Solution 1:10,000 in DMEM/10% fetal calf serum containing antibiotics of choice. Store at 4ºC for up to 6 weeks.

2. Adipogenesis Progression Media: DMEM/10% fetal calf serum/10 µg/mL insulin – Dilute Insulin Solution 1:1000 in DMEM/10% fetal calf serum. Store at 4ºC for up to 6 weeks.

3. Adipogenesis Maintenance Media: DMEM/10% fetal calf serum

4. Negative Control Media: DMEM/10% normal calf serum (not fetal calf serum).

5. Incubation Solution + 2% BSA: Add 6.7 mL Sterile 30% BSA Solution to the 100 mL bottle of Incubation Solution. Store at 4℃ for up to 3 months.

6. Isoproterenol Positive Control Working Solution: Dilute Isoproterenol Stock Solution 1:1000 in Incubation Solution + 2% BSA to obtain a 10 µM isoproterenol solution. Other concentrations of isoproterenol may be used if desired.

7. Free Glycerol Assay Reagent: Reconstitute one with 10 mL distilled water and store at 4℃ for up to 60 days. For longer term storage, dispense into single use aliquots and freeze at –20℃.

8. Glycerol Standard Curve:
   Dilute stock glycerol standard solution 1:2.5 in water to make a 0.104 mg/mL glycerol solution (for a typical assay, add 24 µL to 36 µL water).

   Using seven glycerol – free tubes, perform 2-fold serial dilutions from 1:2.5 – 1:160, using Incubation Solution + 2% BSA as the diluent. For example, add 60 µL of 1:2.5 glycerol standard to 60 µL Incubation Solution + 2% BSA to make the 1:5 dilution. Mix well, add 60 µL of 1:5 dilution to 60 µL Incubation Solution + 2% BSA to make the 1:10 dilution, and continue with serial dilutions.

   In an eighth tube add just Incubation Solution + 2% BSA as a blank.

Dilution	Glycerol Concentration (µg/mL)	Glycerol Molarity (nmol/mL)
Undiluted Glycerol Standard Solution	260	2824
1:2.5	104	1129.6
1:5	52	564.8
1:10	26	282.4
1:20	13	141.2
1:40	6.5	70.6
1:80	3.25	35.3
1:160	1.625	17.65
Buffer control	0	0

Assay Instructions

Note: *The protocol below is for use with 24-well plates. For plates of different sizes, refer to the table in the section below titled "Suggested Volumes for Plate Sizes".*

1. Propagate cells as recommended by ATCC. Maintain the cells in DMEM/10% calf serum (not fetal calf serum). Plate cells at 4 x 10^5 per T75 flask, and passage every third day. Do not permit the cells to become confluent. Prepare stocks of frozen cells at the earliest passage possible, and also use thawed cells at the earliest passage possible.
2. Trypsinize cells. Neutralize by addition of 3 volumes of DMEM/10% calf serum. Count cells.
3. Resuspend cells at 30,000 cells/mL in DMEM/10% calf serum (not fetal calf serum).
4. Plate 2 mL cell suspension per well of 24-well plate.
5. Incubate for 1-2 days. Cells should be confluent at this point.
6. Remove approximately 1.8 mL media and add 2 mL Adipogenesis Initiation Media per well. Use care to replace media as gently as possible to avoid disturbing the monolayer.

   Optional*: For negative control wells employing undifferentiated 3T3-L1 cells, use Negative Control Media at this step and subsequent media changes.*
7. Incubate for 48 hours at 37ºC, 5% CO_2.
8. Remove 2 mL media, and add 2 mL Adipogenesis Progression Media per well. Use care to replace media as gently as possible to avoid disturbing the monolayer.
9. Incubate for 48 hours at 37ºC, 5% CO_2.
10. Remove 2 mL media and add 2 mL Adipogenesis Maintenance Media per well.

11. Incubate for at least 48 hours at 37ºC, 5% CO_2. View cells under an inverted microscope; the cells should contain visible lipid droplets. Replace media every 48-72 hours. Cells can be used for adipolysis assay between 2 days and 14 days after addition of Adipogenesis Maintenance Media.
12. Remove media and wash twice with 1 mL aliquots of Wash Solution. To avoid disturbing the cell monolayer, use care to gently add and remove the solution each time.
13. Remove solution and add test compounds at desired dilutions in Incubation Solution + 2% BSA at 0.25 mL per well. For a positive control, add 0.25 mL/well Isoproterenol Positive Control Working Solution (10 μM). For a negative control, add 0.25 mL/well Incubation Solution + 2% BSA with no additions.
14. Incubate at 37℃ in a 5% CO_2 incubator, for a time period optimized for the test compound of interest. Isoproterenol-induced glycerol release is easily detectable within 1 h, and occurs in a linear fashion to at least 24 h. Other inducers of adipolysis, such as TNFα, may require the longer incubation period. See "Technical Hints" for time course considerations.
15. Collect culture supernatant in a glycerol-free container. Samples may be assayed immediately or stored at –20℃.
16. Dispense 25 μL each of blank (Incubation Solution + 2% BSA), Glycerol Standard Curve (0.4 μg glycerol/mL – 26 μg glycerol/mL, prepared as described in "Preparation of Reagents") and culture supernatant samples into separate wells of a 96-well microtiter plate.
17. Add 200 μL Free Glycerol Assay Reagent per well.
18. Incubate for 15 minutes at room temperature.
19. Read absorbance in spectrophotometric plate reader at 540 nm. A wavelength of 562 nm will also detect the chromophore.
20. Plot a standard curve based on the absorbances of the standards. From the standard curve, calculate glycerol content of samples in terms of nmol/mL, and divide by hours of incubation time to determine nmol/mL/hr.

Suggested Volumes for Plate Sizes

Plate size	Volume of cell suspension/media	Volume of Wash Solution per wash	Volume of Isoproterenol Positive Control or Sample
96-well	200 μL	100 μL	50 μL
48-well	800 μL	400 μL	100 μL
24-well	2 mL	1 mL	250 μL
12-well	4 mL	2 mL	500 μL

Technical Hints

- Monolayers of 3T3-L1 cells are fragile and are easily dislodged from their substratum. Disrupted 3T3-L1 monolayers tend to curl up and form bundles that are difficult to analyze for adipocyte differentiation. This property is increasingly evident with increasing passage number. Use cells at the earliest passage possible. In addition, remove and replace media gently to avoid disturbing the monolayer. Disruption of the monolayer is most evident in 96-well plates, and becomes less severe in plates with larger wells.

- Fetal calf serum contains low but significant amounts of IGF-1, which can stimulate differentiation of 3T3-L1 cells. To maintain 3T3-L1 cells in an undifferentiated state during propagation of stocks of 3T3-L1 cells, calf serum is recommended over fetal calf serum as a media supplement.
- After thawing 3T3-L1 cells, dispense the contents of the vial directly into the culture plate containing media. *Do not centrifuge the cells*, as this is more damaging to the cells than the presence of DMSO.
- If desired, glycerol assay results may be normalized to 1) cell number, as determined by trypsinization and counting, 2) DNA content, determined by fluorimetric methods, or 3) protein assay. For protein assay, the cell layer must be washed thoroughly with protein-free PBS or HBSS to remove BSA remaining from the incubation solution.
- Some compounds may induce adipolysis indirectly, by causing changes in gene expression that lead to triglyceride breakdown. Such compounds are likely to require a longer time course than the isoproterenol standard. For longer term experiments (greater than 24 hours), it is recommended to add the compound in Adipogenesis Maintenance Media (steps 11 and 12) to the experimental wells, and incubate for the desired time period. Media can then be removed and replaced with Incubation Solution + 2% BSA, alone or containing experimental compound (steps 13 and 14).
- In long term experiments, remaining triglyceride stores in the monolayer may be quantified by the Oil Red O staining method in Millipore's Adipogenesis Assay Kit (catalog # ECM950).

Troubleshooting

Problem	Cause	Remedy
Cells curl or ball up	Monolayer disturbed during media change	Do not completely remove media when changing, and add fresh media drop-wise to center of the well. Use larger wells
	Cells at late passage	Use cells in earliest passage possible
Cells treated with IBMX, dexamethasone and insulin do not form lipid droplets	Cells at late passage have lost capacity to differentiate	Use cells in earliest passage possible
Weak signal in isoproterenol-treated cells	Inadequate differentiation of cells	See above
	Volume of Incubation Solution + 2% BSA is too large, resulting in overly diluted released glycerol	Use smaller volume, just enough to cover cells
Background is high in Glycerol Assay	Contamination of tubes or solution with glycerol	Avoid glycerol-coated tubes

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