

ab133115 – Adipolysis Assay Kit

Instructions for Use

For studying the adipolysis of triglycerides in differentiated 3T3-L1 cells

This product is for research use only and is not intended for diagnostic use.

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1. Overview

ab133115 Adipolysis Assay Kit provides a convenient tool for studying the adipolysis of triglycerides in differentiated 3T3-L1 cells, using a common adipogenesis induction procedure. This kit will allow investigators to screen compounds involved in lipid storage and metabolism. Isoproterenol is included in the kit as a positive control for screening pharmaceuticals that regulate FFA release from adipocytes.

2. Background

Obesity is a growing concern worldwide and has reached epidemic proportions in the United States. It is a risk factor in many of the major chronic diseases that afflict our society, including cardiovascular disease, diabetes mellitus, and cancer. In recent years, numerous studies have focused on identifying the mechanism of development of obesity, which is a process of increasing the number of fat cells (fat cell hyperplasia), enlargement of fat cells with each cell carrying greater amounts of fat (fat cell hypertrophy), or both. The ability to regulate the number of fat cells and/or the size of fat cells is a key in the development and physiology of obesity and also in the origin of chronic disease.

Adipose tissue plays an important role in energy homeostasis. Mammals have both brown adipose tissue (BAT), which utilizes lipids to generate heat in a process known as thermogenesis, and white adipose tissue (WAT) which stores excess energy as triglycerides in lipid droplets. Triglycerides can be broken down into free fatty acids (FFA) and glycerol, through a process of adipolysis (also referred as lipolysis). Adipolysis is a highly regulated process which allows appropriate delivery of FFA to meet energy needs. Appropriate FFA levels are important for optimal human health but excess FFAs are generally associated with development of insulin resistance, hypertriglyceridemia, reduced hepatic insulin clearance, and impaired β -cell insulin secretion. It is well documented that adipolysis is under tight hormonal control. For example, insulin inhibits adipolysis in adipose tissue and tumor necrosis factor- α (TNF- α) stimulates it. However, other unknown autocrine/paracrine factors involved in adipolysis regulation remain to be elucidated.

3T3-L1 cells are a well-characterized model for studying lipid metabolism, including the uptake and release of fatty acids from fat cells. This model shares similar morphology, gene expression, and metabolism with adipocytes *in vivo*. During terminal differentiation, the fibroblast-like pre-adipocytes undergo a series of morphological and biochemical changes to eventually accumulate lipid droplets. Triglycerides stored in these lipid droplets can be stimulated by known factors or compounds to release FFA and glycerol. The latter can be measured by incubation with glycerol kinase, glycerol phosphate oxidase, and horseradish peroxidase in the presence of a

colorimetric substrate to generate a chromophore detectable at 540 nm. The amount of glycerol released into the medium is proportional to the level of triglyceride storage and/or degree of adipolysis.

3. Components and Storage

Item	Quantity	Storage
Adipolysis Assay IBMX Solution (1,000X)	1 vial	-20°C
Adipolysis Assay Insulin Solution (1,000X)	1 vial	-20°C
Adipolysis Assay Dexamethasone Solution (1,000X)	1 vial	-20°C
Adipolysis Assay Isoproterenol Solution	1 vial	-20°C
Glycerol Standard Solution	1 vial	-20°C
Free Glycerol Assay Reagent (10X)	2 vials	-20°C

Materials Needed But Not Supplied

- 3T3-L1 preadipocyte cell line and associated tissue culture media
- Dulbecco's Modified Eagles Medium
- Calf Serum
- Fetal Bovine Serum
- Adjustable pipettes and a repeat pipettor
- A 12-, 24-, or 96-well plate for culturing cells and induction of adipogenesis
- A 96-well plate reader capable of absorbance measurements at 540 nm.

4. Pre-Assay Preparation

A. Media Preparation

Induction Medium

For a 96-well plate, prepare Induction Medium by adding 10 μ l of the following to 10 ml of DMEM containing 10% FBS:

Adipolysis Assay IBMX Solution (1000X)

Adipolysis Assay Insulin Solution (1000X)

Adipolysis Assay Dexamethasone Solution (1000X)

Insulin Medium

For a 96-well plate, prepare Insulin Medium by adding 10 μ l of Adipolysis Assay Insulin Solution (1,000X) to 10 ml of DMEM containing 10% FBS.

B. Induction Procedure

The following protocol is designed for a 96-well plate. For other sizes of plates the volume of medium/solution to add to each well should be adjusted accordingly.

1. Seed a 96-well plate with 3×10^4 cells/well.
2. Grow preadipocytes to confluence in DMEM containing 10% calf serum.
3. Two days post confluence (Day 0), change the medium to either Induction Medium (treatment group) or fresh DMEM containing 10% FBS (control group).
4. Three days after the induction (Day 3) change the medium to Insulin Medium (treatment group) or fresh DMEM containing 10% FBS (control group).
5. Five days after the induction (Day 5) change the medium to fresh Insulin Medium (treatment group) or DMEM containing 10% FBS (control group).
6. Monitor visible accumulation of lipid droplets under a microscope two days later (Day 7). If more differentiation is desired, change medium to fresh Insulin Medium (treatment group) or DMEM containing 10% FBS (control group) and every three days monitor the degree of differentiation under a microscope for several more

days. More than 80% of the cells are usually differentiated by Day 7. Cells may be maintained for up to two weeks after this point for the Adipolysis Assay.

C. Reagent Preparation

Free Glycerol Assay Reagent (10X)

Free Glycerol Assay Reagent is unstable. Store the un-reconstituted Free Glycerol Assay Reagent (10X) at -20°C. We recommend that you prepare only the amount you need for your assay. Each vial is to be reconstituted with 5 ml of distilled water and is enough for half of a 96-well plate. The prepared Free Glycerol Assay Reagent should be stable for approximately two weeks stored at 4°C.

Glycerol Standard Solution

The Glycerol Standard Solution is provided at a concentration of 125 µg/ml. To prepare the glycerol standard curve for this assay: obtain six clean test tubes and label them #1 through #6. Aliquot 100 µl of culture medium into tubes #2-#6. Transfer 200 µl of Glycerol Standard Solution into tube #1. Serially dilute the standard by removing 100 µl from tube #1 and placing it into tube #2; mix thoroughly. Next remove 100 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat it for tubes #4 and #5. Do not add any standard to tube #6. This tube will be your blank.

5. Assay Protocol

A. Plate Setup

Each plate should contain a glycerol standard curve, wells containing medium only, and wells containing supernatants from samples treated with and without test compounds. It is recommended that standards be run in duplicate and that each treatment be performed in triplicate. A suggested plate format is shown in Figure 1, below. The user may vary the location and type of wells present as necessary for each particular experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44
E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37	NSB	NSB
F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38	PC	PC
G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39	Blk	Blk
H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40	C1	C1

S1-S6 - Standards 1-6 1-28 – Samples

Figure 1. Sample plate format

Pipetting Hints

- *It is recommended that an adjustable pipette be used to deliver reagents to the wells.*
- *Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times)*
- *Do not expose the pipette tip to the reagent(s) already in the well.*

B. Performing the Assay

1. Carefully aspirate the culture medium from each well.
2. Replace with fresh medium containing test compounds at desired concentrations. The Isoproterenol Solution may be used as a positive control by diluting the solution as provided 1:1,000 into culture medium to yield a 10 μM solution. Negative controls with medium alone should also be included.
3. Incubate cells at 37°C for the desired time period. Isoproterenol begins to induce adipolysis within one hour and the amount of detectable free glycerol increases linearly afterwards. Users of the kit must determine the optimal incubation time period for each experimental compound.

4. Collect cell culture supernatants from each well into glycerol-free containers. Samples may be assayed immediately or stored at -20°C .
5. To perform the assay, transfer 25 μl of the standards (tubes 1-6) prepared above into a new 96-well plate. We recommend that the standards be run in duplicate.
6. Transfer 25 μl of each supernatant collected in step 4 to the corresponding wells on the new plate. Depending on the experimental compounds being tested, samples may need to be diluted with culture medium prior to addition to the plate in order to fall within the range of the standard curve.
7. Add 100 μl of diluted Free Glycerol Assay Reagent per well and incubate for 15 minutes at room temperature.
8. Read absorbance at 540 nm.

6. Data Analysis

A. Data Analysis

Preparation of the Data

Subtract the absorbance reading obtained for the S6 wells from all absorbance readings including the rest of the standard curve. This is the corrected absorbance value.

Plotting the Standard Curve

Make a plot of absorbance at 540 nm as a function of glycerol concentration and determine the equation of the line. See Figure 3. for a typical standard curve.

Determination of Glycerol Concentration

$$\text{Glycerol concentration } (\mu\text{g/ml}) = \left[\frac{A_{540} - (\text{y-intercept})}{\text{Slope}} \right] \times \text{Sample Dilution}$$

B. Performance Characteristics

Sample Data

The standard curve presented below is an example of the data typically produced with 3T3- L1 culture medium. However, your results will not be identical to these. You must run a new standard curve with each experiment.

Tube #	Glycerol Concentration ($\mu\text{g/ml}$)	A_{540}	Corrected Absorbance
1	125	1.262	1.178
2	62.5	0.68	0.596
3	31.25	0.376	0.292
4	15.625	0.227	0.143
5	7.813	0.152	0.068
6	0	0.084	0.000

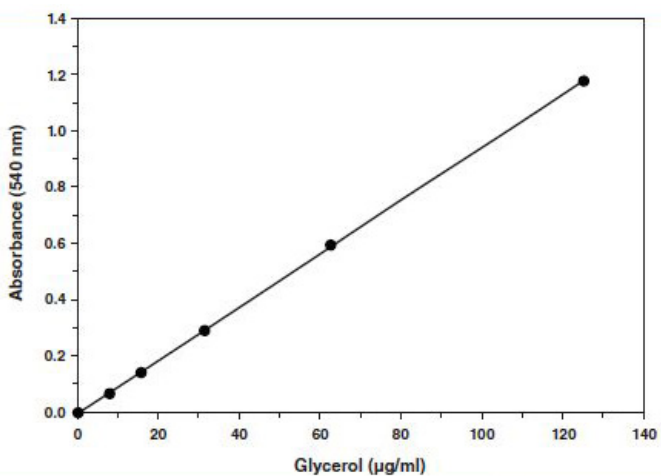


Figure 2. Glycerol standard curve. Example of standard curve of typical data produced with 3T3-L1 culture medium.

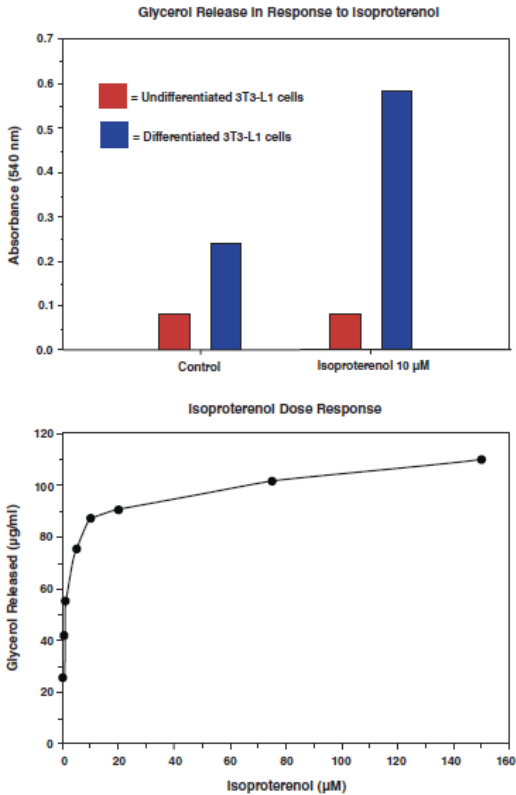


Figure 3. Adipolysis assay. Differentiated 3T3-L1 cells were treated with various concentrations of isoproterenol for 48 hours and the culture supernatant was assayed for free glycerol. *Top panel:* glycerol release from differentiated and undifferentiated 3T3-L1 cells as determined using Free Glycerol Assay Reagent. *Bottom panel:* isoproterenol dose response curve generated by plotting the isoproterenol concentration versus the glycerol released after 24 hours.

7. Troubleshooting

Problem	Possible Causes	Recommended Solutions
Cells treated with induction medium do not form lipid droplets	Cells are from a late passage and may have lost the capacity to differentiate	Use cells at a low passage number
The absorbance reading of the samples is higher than the range of the standard curve	The free glycerol concentrations of the samples are too high and therefore out of the detection range of the assay	Dilute the samples in culture medium so the concentrations fall within the range of the standard curve

UK, EU and ROW

Email: technical@abcam.com
Tel: +44 (0)1223 696000
www.abcam.com

US, Canada and Latin America

Email: us.technical@abcam.com
Tel: 888-77-ABCAM (22226)
www.abcam.com

China and Asia Pacific

Email: hk.technical@abcam.com
Tel: 400 921 0189 / +86 21 2070 0500
www.abcam.cn

Japan

Email: technical@abcam.co.jp
Tel: +81-(0)3-6231-0940
www.abcam.co.jp