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ab133130 – Glycerol Assay Kit (Cell-Based)

Instructions for Use

For studying triglyceride/ fatty acid cycling and its regulation in adipocytes or hepatocytes.

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

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1. Overview

ab133130 provides a convenient tool for studying triglyceride/ fatty acid cycling and its regulation in adipocytes or hepatocytes. This kit will allow investigators to screen compounds involved in lipid storage and metabolism. Chloroquine is included in the kit as a positive control for screening compounds that induce lipid droplet accumulation and free glycerol release from hepatocytes.

2. Background

In mammals, triglycerides are constantly synthesized from fatty acids and segregated into cytosolic lipid droplets, mainly in adipocytes, as the major energy storage depot. During fasting, triglycerides stored in adipose tissue and liver are hydrolyzed by hormone-sensitive lipase and adipose triglyceride lipase to produce free fatty acids and glycerol. Triglyceride/ fatty acid cycling is important in metabolic regulation and heat production, and is highly regulated by enzymes such as phosphenolpyruvate carboxykinase (PEPCK) and lipases. Quantitative changes in the triglyceride/fatty acid cycle have been related to the increased metabolic rate of cachectic patients with esophageal cancer and to metabolic syndrome. Abnormal triglyceride accumulation in the form of lipid droplets can occur in adipocytes and/ or hepatocytes of obese mammals. *In vitro*, dramatic lipid accumulation can be observed in well-differentiated 3T3-L1 cells, or HepG2 cells treated with steatosis-inducing compounds such as chloroquine. Triglycerides stored in these lipid droplets can be hydrolyzed into free fatty acids and glycerol which are subsequently released into the surrounding environment. The amount of glycerol released into the medium is proportional to the triglyceride/fatty acid cycling rate.

3. Components and Storage

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item	Quantity	Storage
Glycerol Standard Solution	1 vial	-20°C
Free Glycerol Assay Reagent (10X)	2 vials	-20°C
Chloroquine Positive Control (25 mM)	1 vial	-20°C

Materials Needed But Not Supplied

- HepG2 cell line and associated cell culture media (other cell lines such as 3T3-L1 pre-adipocytes can also be used).
- Adjustable pipettes and a repeat pipettor.
- A 12-, 24-, or 96-well plate for culturing cells.
- A spectrophotometer or 96-well plate reader capable of measuring absorbance at 540 nm.

4. Pre-Assay Preparation

A. Treatment of Cells

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

1. Seed a 96-well plate with 10^4 cells/well. Grow cells overnight.
2. The following day, treat cells with experimental compounds or vehicle for 72 hours, or for the period of time used in your typical experimental protocol. Chloroquine, a compound known to induce lipid droplet accumulation, is included in the kit as a positive control. The recommended concentration is 25 µM. A measurable amount of free glycerol is released into the culture medium after 24-48 hours of treatment.
3. Terminate the experiment and examine the effect of experimental compounds on free glycerol release using the assay procedure described.

B. Reagent Preparation

Free Glycerol Assay Reagent (10X)

Reconstitute each vial of the Free Glycerol Assay Reagent with 5 ml of distilled water (each vial is sufficient for half of a 96-well plate). The prepared Free Glycerol Assay Reagent should be stable for approximately two weeks 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 eight clean test tubes and label them #1 through #8. Aliquot 100 µl of PBS or water to tubes #2 - #8. 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 for tubes #4-7. Do not add any standard to tube #8. This tube will be your blank for the standard curve.

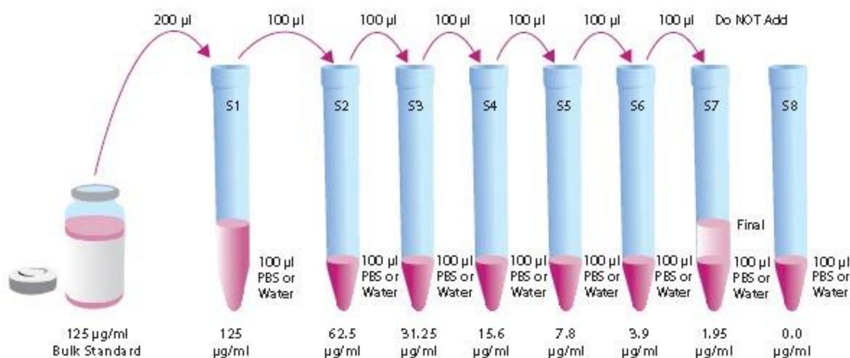

Figure 1. Preparation of the Glycerol standards

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 test compounds or vehicle. We recommend that standards be run in duplicate and that each treatment be performed in triplicate.

Pipetting Hints:

- *Use different tips to pipette each reagent.*
- *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

NOTE: Perform all steps at room temperature.

1. Collect cell culture supernatants from each well and place in glycerol-free containers. Samples may be assayed immediately or stored at 4°C for up to two weeks.
2. To perform the assay, transfer 25 µl of the standards prepared above into a new 96-well plate. We recommend that the standards be run in duplicate.

3. Transfer 25 µl of each supernatant collected in step #1 to triplicate wells on the new plate.

4. Add 100 µl of reconstituted Free Glycerol Assay Reagent per well to the standards and to two of each set of triplicate wells from step #3. Add 100 µl of distilled water to the third well of each sample from step #3. These will be the blanks for your samples.

5. Incubate for 15 minutes at room temperature.

6. Read the absorbance at 540 nm.

6. Data Analysis

A. Calculations

1. Calculate the average absorbance of each standard and sample.

2. Subtract the absorbance value of the standard 8 (0 µg/ml) from all other values (both standards and samples). This is the corrected absorbance.

3. Subtract the absorbance of each sample blank from the absorbance of the corresponding samples. This is the corrected absorbance of each sample.

4. Graph the corrected absorbance values of each standard as a function of the final glycerol concentration. See Figure 2, on page 10, for a typical standard curve.

5. Calculate the amount of glycerol in each sample using the equation obtained from the linear regression of the standard curve by substituting the corrected absorbance values for each sample into the equation.

$$\text{Free glycerol } (\mu g/ml) = \frac{A_{540} - (\text{y-intercept})}{\text{Slope}}$$

B. Performance Characteristics

Tube No.	Glycerol Concentration (µg/ml)	Average A_{540}	Corrected Absorbance
1	125	1.023	0.983
2	62.5	0.548	0.508
3	31.25	0.294	0.254
4	15.6	0.166	0.126
5	7.8	0.105	0.065
6	3.9	0.073	0.033
7	1.95	0.060	0.020
8	0	0.040	0.000

Table 1. Typical results

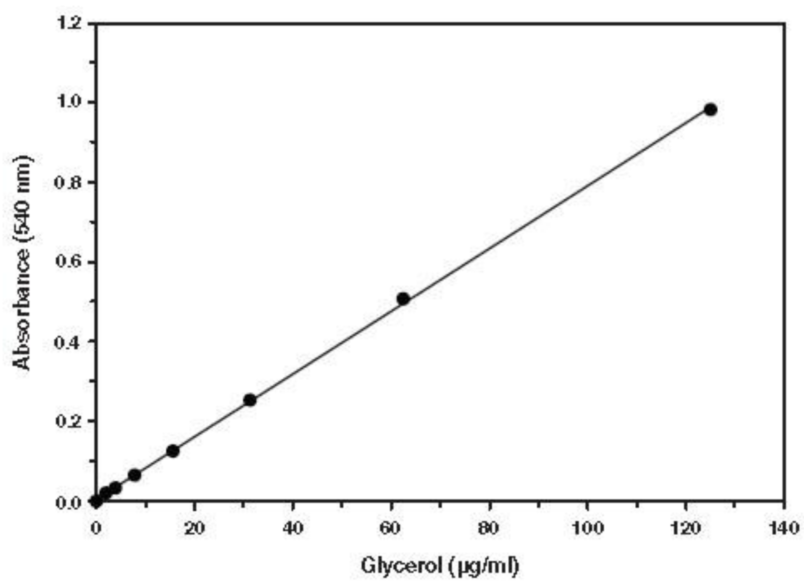

Figure 2. Typical glycerol standard curve.

Chloroquine Concentration (μM)	A_{540} with Free Glycerol Detection Reagent	A_{540} with distilled water (Blank)	Corrected Absorbance	Free Glycerol released (μg/ml)
200	0.123	0.080	0.043	8.3
100	0.111	0.078	0.033	7.06
50	0.090	0.077	0.0134	4.71
25	0.083	0.078	0.005	3.65
12.5	0.080	0.077	0.003	3.45
6.25	0.078	0.076	0.002	3.33
0	0.074	0.074	0	3.09

Table 2. Glycerol release from cells treated with different doses of chloroquine.

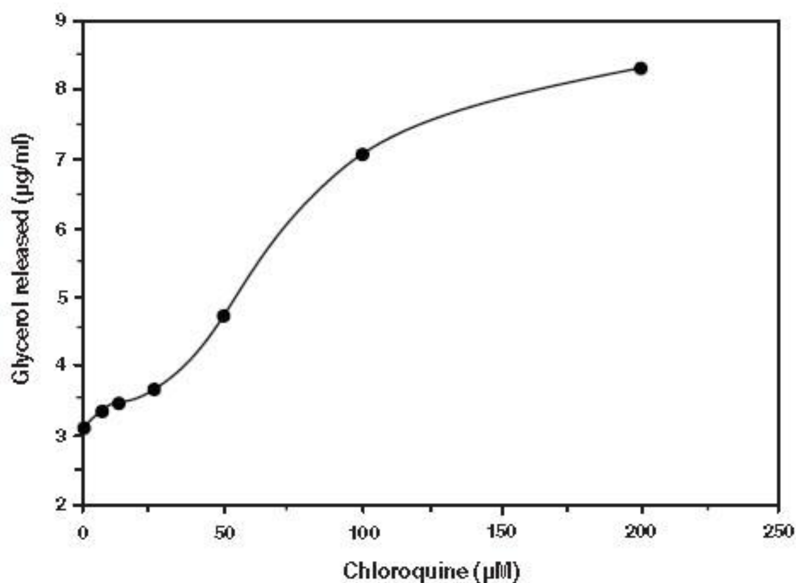

Figure 3. Glycerol release from HepG2 cells treated with chloroquine. HepG2 cells were seeded at a density of 10^4 cells/well in a 96-well plate and grown overnight in a 37°C incubator. The next day, cells were treated with vehicle or different doses of chloroquine for 24 hours. At the end of this incubation, supernatants were collected and analyzed for free glycerol according to the procedure described above.

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7. Troubleshooting

Problem	Possible Causes	Recommended Solutions
No difference among different treatments including the positive control	Cell density too high or cells overgrown	Plate cells at a lower density
Cells treated with experimental compound do not release measurable free glycerol	Experimental compounds may have a cytotoxic effect on the cells which results in cell death or the compounds may not cause the release of glycerol from this particular cell line	Lower the concentration of the experimental compounds or test the compounds in a different cell line

Technical Support

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