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Glycerol Cell-Based Assay Kit

9/16

(Catalog # K977-100; 100 assays; Store at -20°C)

I. Introduction:

Metabolic diseases such as type II diabetes and fatty liver are becoming more prevalent among an aging population, and are a burden to the healthcare system. Such diseases revolve around the body's ability to control triglyceride (lipid) metabolism. Events such as fasting can lead to cell autophagy, corresponding with removal of lipid droplets. A number of pathologies can lead to steatosis, bringing about triacylglyceride buildup and release of free fatty acids and free glycerol. Glycerol molecules can then be reabsorbed by the liver or kidney and can be metabolized to re-enter the energy store by pathways such as glycolysis and gluconeogenesis. The glycerol concentration can thus be used as a measure of lipid metabolism and fat mobilization. BioVision's Glycerol Cell-Based Assay Kit provides a simple, sensitive, straight-forward assay for determination of glycerol concentrations in various cell and tissue culture samples. The assay uses a glycerol-specific enzymatic reaction to convert the probe into a fluorescent product in the presence of glycerol. Glycerol levels can be detected as low as 50 pmol per well, or 1.0 µM in culture medium. Chloroquine (included in the kit as a control) induces steatosis and blocks autophagy, thus leading to a buildup of lipid droplets and an increase in the free glycerol concentration.

II. Applications:

- Determination of glycerol concentration in cell culture media

III. Sample Type:

- Cultured Adipocytes
- Liver/Hepatocytes/Liver cell lines

IV. Kit Contents:

Components	K977-100	Cap Code	Part Number
Glycerol Assay Buffer	25 ml	WM	K977-100-1
Glycerol Probe (in DMSO)	1 vial	Red	K977-100-2
Glycerol Enzyme Mix	1 vial	Green	K977-100-3
Free Glycerol Standard (100 mM)	0.2 ml	Yellow	K977-100-4
Chloroquine (5 mM)	120 µl	Purple	K977-100-5

V. User Supplied Reagents & Equipment:

- Cell culture medium (without Phenol Red)
- PBS, DMSO
- Fluorescence Plate Reader
- 96-well tissue culture clear plate
- 96-well black flat bottom plate

VI. Storage and Reagents Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. *Assay should be performed under sterile conditions*. Read entire protocol before performing the assay.

- **Glycerol Assay Buffer, Glycerol Probe, Free Glycerol Standard, and Chloroquine (5 mM):** Store at -20°C. Warm to RT before use. Stable for six months.
- **Glycerol Enzyme Mix:** Add 220 µl of Glycerol Assay Buffer to the vial. Pipet up and down to dissolve. Aliquot and store at -20°C. Stable for two months.

VII. Glycerol Assay Protocol:

1. Cell Culture:

a. Day 0: Dilute cells to $5x10^4$ cells/ml in desired media. Seed $1x10^4$ cells (200 µl)/well in a clear 96-well tissue culture plate. Grow cells overnight in a 37°C/5% CO_2 incubator. For positive control: plate $1x10^4$ HepG2 cells in 200 µl EMEM.

Note: Media containing Phenol Red may interfere with the measurement and reduce sensitivity. For this reason, we advocate use of Phenol Red-free media when seeding cells.

b. Day 1: After 24 hr, treat cells with compounds of interest diluted in 50 µl media. Leave one well as Media Control well to which you add 50 µl of media. For compounds with unknown effects, a titration will be necessary. Prepare a Solvent Control well with the same final concentration of solvent in test wells used to solubilize the compounds of interest. For positive control: Dilute 5 mM Chloroquine stock 10-fold by adding 90 µl EMEM to 10 µl Chloroquine (5 mM), generating 0.5 mM Chloroquine (Working Stock). Add 50 µl Working Stock to 10^4 HepG2 cells in 200 µl media, e.g. EMEM.

c. Day 2-4: Examine cells periodically for changes in morphology. After desired incubation period, proceed to section 2: Sample Preparation.

Note: For a positive control, HepG2 liver cells treated with 100 µM chloroquine will show measurable increases in glycerol concentration after 48 hours.

2. Sample Preparation: Upon completion of the experiment, cell culture media from the culture plate can be tested directly. Pipet 50 µl from the culture plate into a corresponding well on a black 96-well flat bottom plate for fluorescent glycerol determination.

Note: For HepG2 cells treated with chloroquine, 48 hours is an appropriate length of time for the treatment. Media may be taken and glycerol concentration tested after this time frame. Cells may begin to die after 72 hours.

3. **Standard Curve Preparation:** Add 10 µl of the Glycerol Standard (100 mM) to 990 µl Glycerol Assay Buffer to generate 1 mM Glycerol Standard. Mix well. Further dilute 20 µl 1 mM Glycerol Standard with 180 µl Glycerol Assay Buffer to obtain a 100 µM Working Glycerol Standard. Add 0, 2, 4, 6, 8, and 10 µl of the Working Glycerol Standard to each well individually to generate standards of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 nmol/well. Adjust the volume of each well to 50 µl with Assay Buffer.
4. **Reaction Mix**: Mix enough reagent for the number of samples and standards to be performed: For each well (samples and standards), prepare 50 µl Reaction Mix. For sample background wells, prepare 50 µl Background Control Mix:

	Reaction Mix (each well)	Background Control Mix (each well)
Assay Buffer	46 µl	48 µl
Glycerol Probe	2 µl	2 µl
Glycerol Enzyme Mix	2 µl	-

Add 50 µl Reaction Mix and 50 µl Background Control Mix to their respective sample wells. Before reading samples, mix for 5 sec.

5. **Measurement:** Incubate plate at RT for 60 minutes and read the plate using a fluorescence microplate reader at Ex/Em = 535/587 nm.
6. **Calculations:** Subtract the 0 Glycerol standard reading from all standard readings, and plot the background-subtracted glycerol standards to generate the standard curve (from 0-1 nmol Glycerol). For sample readings, subtract the reading obtained from the parallel reaction containing Background Control Mix.

$$\text{Glycerol Concentration} = \left(\frac{\text{Glycerol amount from standard curve (nmol)}}{\text{vol.of sample(ml)}}\right) \quad \textbf{(nmol/ml or µM)}$$

Glycerol MW = 92.09 g/mol; 1 nmol Glycerol = 92.09 ng; 1 µg/ml Glycerol = 1.09 µM

Note:

a. To assess the effect of test compounds compare the glycerol amounts with the Media Control well value. In the case that Solvent Control well signal is significantly different from the Media Control well, use its values in assessing test compound effect.

b. Glycerol levels can vary substantially depending on the cell line, seeding density, etc. In some instances, dilution of the media may be required to obtain a measurable value within the range of the standard curve. In other instances, the assay can be run directly on the plate *in situ* if the user anticipates no more than approximately 1 nmole glycerol in the well.

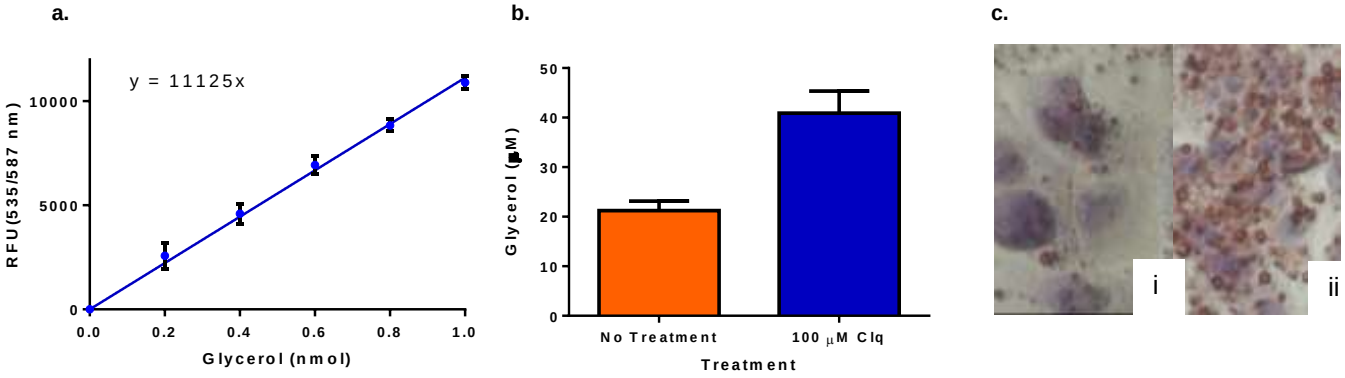

Figure 1: (a) Glycerol Standard Curve. (b) Chloroquine Treatment: HepG2 cells were plated overnight at 10^4 cells per well. Cells were then treated with chloroquine for 48 hours according to the protocol. **(c) Oil Red Staining:** HepG2 cells were untreated (i) or treated with 100 µM chloroquine (ii) and, after 48 hours, stained using Oil Red O lipid stain (Cat. #K580). Treatment with chloroquine induces steatosis, leading to a significant increase in lipid droplets and glycerol content of the cells.

VIII. RELATED PRODUCTS:

NAD/NADH Quantification Kit (K337)
Glucose Assay Kit (K686)
Ethanol Assay Kit (K620)
Pyruvate Assay Kit (K609)
Triglyceride Assay Kit (K622)
Fatty Acid Assay Kit (K612)
Free Glycerol Colorimetric/Fluorometric Assay Kit (K630)
Glycerol Assay Kit (K634)
HDL & LDL/VLDL Assay Kits (K586)
Cholesterol Assay Kit (K603)
3T3-L1 Differentiation Kit (K579)
Adipogenesis Colorimetric/Fluorometric Assay Kit (K610)
Glycerol-3-phosphate (G3P) Colorimetric Assay Kit (K641)
Hepatic Steatosis Assay Kit (K584)
Lipase Activity Colorimetric Assay Kit (K722)
Lipid (Oil Red O) Staining Kit (K580-24)
Lipolysis (Adipocyte) Colorimetric Assay Kit (K581)

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