Cellulite Treatment Screening Kit
Human Adipocyte Lipolysis Assay
For Detection of Both Free Glycerol And Non-Esterified Fatty Acids

Cat# LIP-12

INSTRUCTION MANUAL ZBM0019.03

STORAGE CONDITIONS

- **Human Adipocytes**

  All orders are delivered via Federal Express Priority courier at room temperature.
  All orders must be processed immediately upon arrival.

  NOTE:
  Domestic customers: Assay must be performed 5-7 days AFTER receipt.
  International customers: Assay must be performed 3-5 days AFTER receipt

- **Reagents & Buffers:** 4°C
- **Vehicle & Controls:** -20°C
- **Assay plate A (96-well) cultured human adipocytes:** 37°C

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This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

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INTRODUCTION

WHAT IS CELLULITE?

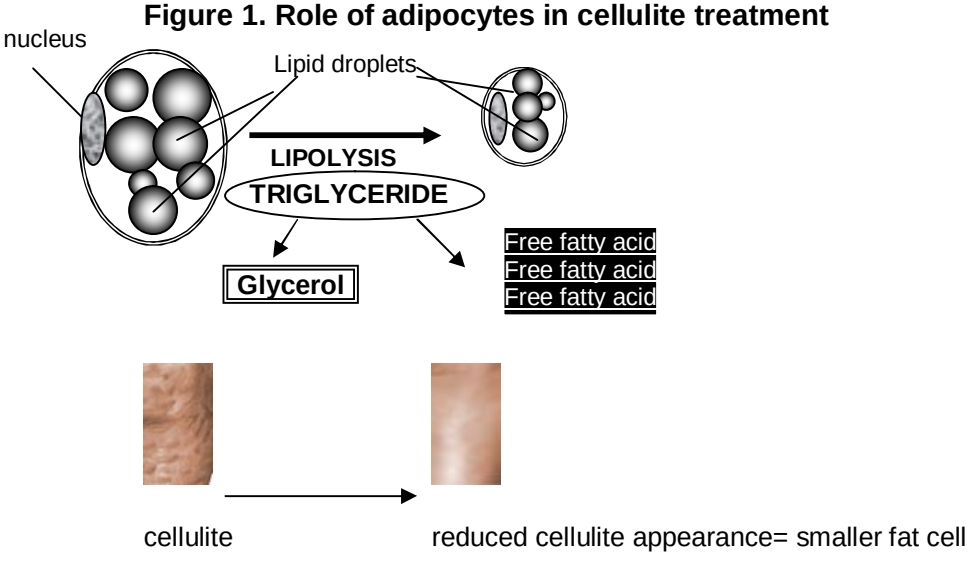
Cellulite is a term applied to a skin condition associated with the localized fat deposits that present as lumps and dimples appearing on the thighs of many women. Although cellulite primarily afflicts the thighs, hips and buttocks, it may also be present on the stomach and upper arms. Cellulite is simply made up of ordinary fatty tissue. Fibrous strands called connective tissue which separate the skin from the underlying fatty tissue form separate compartments under the skin that contain fat cells. The appearance is frequently described as "orange peel skin" or said to have a “cottage cheese appearance”. Cellulite afflictions are a stubborn problem causing emotional and psychological distress to many women. Although the etiology of cellulite is poorly understood, the main factor appears to be local accumulation of fat in a regional compartment.

HOW CAN THIS KIT HELP MY RESEARCH?

Adipocytes (fat cells) are the principle cells implicated in fat storage by adipose tissue. It has been proposed that the anatomical structure of subcutaneous adipose tissue is a major contributor to the appearance of cellulite. The histological studies of subcutaneous tissues from men and women suggest that the fat lobules are larger and more vertical in women than men. As a result, these larger, less restricted lobules can express outward against the dermis causing the bumps and dimples characteristic of cellulite. The femoral subcutaneous fat deposits in women also tend to be more lipogenic and less lipolytic than abdominal subcutaneous or visceral fat due to the difference in the distribution of α and β adrenergic receptors on adipocytes in these different regions. When these fat cells increase in size, the skin compartment bulges, which forms the noticeable “dimpling” or “cottage cheese” look. These fat cells contain triglycerides which must be broken down before fat cells can be reduced in size. The more triglyceride in fat is broken down, the smaller the fat cells under the skin, leaving the skin appearing smoother (less cellulite). Increased lipolysis or fat reduction of the subcutaneous adipose (fat under the skin) means more triglyceride is broken down to lead to smaller fat cells and a reduction of the cellulite appearance.

Topically applied lipolytic agents can distribute or reduce local fat accumulation and improve the aesthetic appearance of the skin (Mas-Chamberlin *et al.* 2006, Hexsel *et al*. 2005, Huber *et al.* 2004). Testing lipolytic activity of potential treatments for cellulite requires screening many compounds and plant extracts. Prior to beginning a clinical trial of the product, one would need to establish validity of the lipolytic activity in human adipocytes.

Figure 1. Role of adipocytes in cellulite treatment

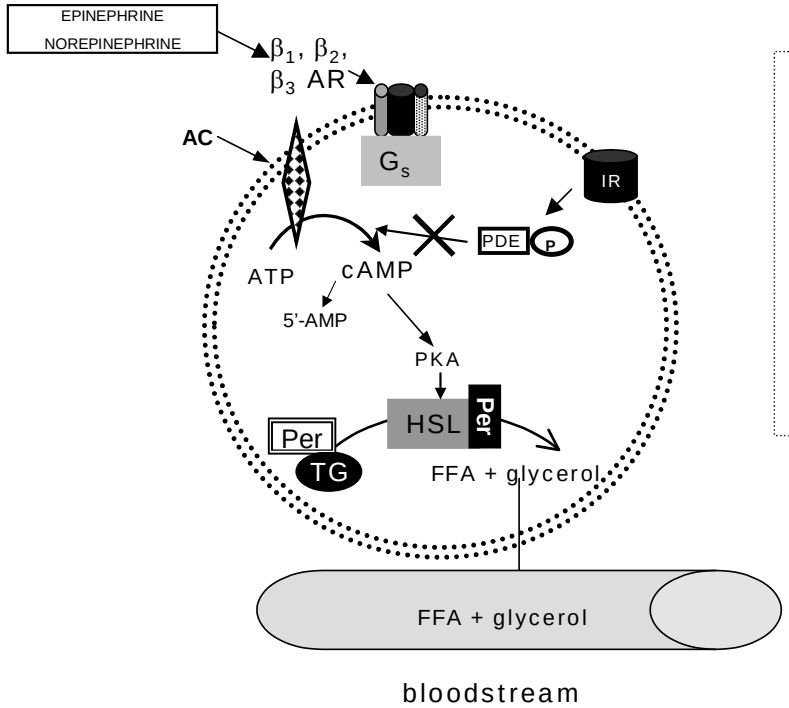


WHAT IS THE SCIENCE BEHIND THIS KIT?

Lipolysis is the process in which triglycerides are hydrolyzed into glycerol and free fatty acids. This process releases free fatty acids (FFA) into the bloodstream where they may be either re-esterified by the adipocyte or travel to other tissues and exert other effects throughout the body. The sympathetic nervous system plays a key role in the regulation of lipid mobilization. The main lipolytic pathway involves beta-agonists (β-agonists), which activate β-adrenergic receptors via the intracellular G_s proteins in adipocytes. This leads to the activation of adenylate cyclase (AC), which then increases cyclic AMP (cAMP) levels. Elevated cAMP acts as a second messenger to activate hormone sensitive lipase (HSL). HSL, the rate-limiting enzyme regulating adipocyte lipolysis, then catalyzes the hydrolysis of triglycerides and results in the release of one molecule of glycerol and 3 molecules of free fatty acids (FFA; increased lipolysis). Phosphodiesterases (PDE) are enzymes that transform cAMP to 5'-AMP (5 prime adenosine monophosphate). This action results in a decrease in lipolysis. PDE inhibitors increase intracellular cAMP levels. 3-isobutyl-1-methylxanthine (IBMX), a non-specific inhibitor of cAMP phosphodiesterases (PDE), can be used as a positive control if your test compounds are suspected PDE inhibitors. PDE inhibitors can be found as an ingredient in mesotherapy solution for the treatment of cellulite (Snyder *et al.* 2005) Isoproterenol; a non-specific β-adrenergic agonist is used as the positive control if your test compounds affect lipolysis via β-adrenergic receptors.

Among the methods for stimulating lipolysis, the most commonly known and used is that which consists of inhibiting the phosphodiesterase in order to prevent or at least limit the rate of degradation of cyclic AMP. In effect, the phosphodiesterase destroys cyclic AMP by transforming it into 5'AMP so that it cannot function as a lipolysis activator. Among the common agents for treatment of cellulite as slimming agents are xanthine analogs such as caffeine or theophylline. These agents block the antilipolytic action of adenosine, a potent endogenous inhibitor of lipolysis. Other known methods in lipolysis stimulation are achieved by inhibiting phosphodiesterase in order to prevent or at least limit the degradation of cAMP. Other existing methods for the treatment of cellulite have been the stimulation of adenylate cyclase to increase cAMP levels or to block the antilipolytic inactivation of adenylate cyclase (α-2-adrenergic antagonists). Greenway *et al*. (1995) disclose that isoproterenol, a known β agonist adrenergic stimulator, is effective for the treatment of cellulite by stimulating lipolysis; furthermore, creams based on yohimbine, a known α2-blocker, applied to women's skin showed a decrease in thigh circumference.

Figure 2. Overview of adipocyte lipolysis



ABBREVIATIONS:

AC	adenylate cyclase
AR	adrenergic receptors
G_s	G protein coupled receptor
FFA	free fatty acids
PKA	protein kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
IR	insulin receptor
PDE	phosphodiesterase
Per	Perilipin
TG	triglyceride

WHAT DOES THIS KIT MEASURE?

This kit provides the tools to study chemical compounds that may influence lipolysis in cultured human adipocytes. This kit specifically measures both the non-esterified fatty acids (NEFA) and the glycerol released by the breakdown of triglyceride. **NOTE: 3 fatty acid molecules are released per triglyceride molecule resulting in a 3:1 fatty acid to glycerol concentration.**

PRINCIPLE OF THE ASSAY

Detection of Free Glycerol

Lipolytic activity is assessed by the measurement of glycerol released into the medium. Glycerol released to the medium is phosphorylated by adenosine triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5'-diphosphate (ADP) in the reaction catalyzed by glycerol kinase. G-1-P is then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethytl-N-(3-sulfopropyl)m-anisidine (ESPA) with H_2O_2, which shows an absorbance maximum at 540nm. The increase in absorbance at 540nm is directly proportional to glycerol concentration of the sample.

$$\text{GLYCEROL} + \text{ATP} \longrightarrow \text{G-1-P} + \text{ADP}$$

$$\text{G-1-P} + O_2 \longrightarrow \text{DAP} + H_2O_2$$

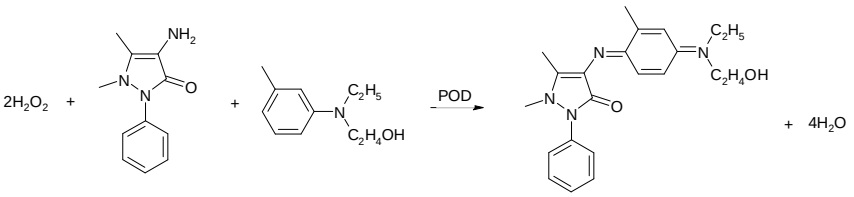
$$H_2O_2 + \text{4-AAP} + \text{ESPA} \longrightarrow \text{Quinoneimine dye} + H_2O$$

Detection of Non-Esterified Fatty Acids (Free Fatty Acids;FFA)

Assessment of lipolytic activity can also be detected through a coupled reaction to measure non-Esterified fatty acids (NEFA) released by adipocytes. The initial step, carried out by acyl-CoA synthetase (ACS), produces fatty acyl-CoA thiol esters from the NEFA, ATP, Mg, and CoA in the reaction. The acyl-CoA derivatives react with oxygen in the presence of acyl-CoA oxidase (ACOD) to produce hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine which forms a purple product that absorbs light at 550nm. This allows the concentration of NEFA to be determined from the optical density measured at 540 - 550nm.

$$\text{HCOOH (NEFA)} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACS}} \text{Acyl-CoA} + \text{AMP} + PP_i$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\text{ACOD}} \text{2,3-trans-Enoyl-CoA} + H_2O_2$$



NOTE: 3 fatty acid molecules are released per triglyceride molecule resulting in a 3:1 fatty acid to glycerol concentration.

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Adipocytes, Plate A	Cultured human subcutaneous adipocytes	---	PLATE	1	37°C
Assay Plate	96-well assay plate, blank	---	PLATE	3	-----
Wash Buffer		---	50ML	1	4°C
Vehicle	0.1% DMSO in LIP-2/3 Assay Buffer	PURPLE	1 ml / VIAL	1	-20°C
Positive control	Isoproterenol, 10 mM in DMSO. Dilute to 1 μM in Assay Buffer before use! (i.e.1 μl in 10 ml Assay Buffer)	BLUE	10 μl / VIAL	1	-20°C
Glycerol Reagent A	Reconstitute with 11.0 ml deionized water prior to use.		BOTTLE	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl		EACH	4	-----
Glycerol standard	Glycerol @ 1mM [Dilute with 400 μl Wash Buffer to make the 200 μM glycerol standard; see page 7 for recommended dilution scheme]	ORANGE	100 μl / VIAL	1	-20°C
LIP-2/3 Assay Buffer	100 ml	---	BOTTLE	1	4°C
FFA Standard	1mM Stock. See page 6 for standard curve preparation	AMBER	100 μl / VIAL	1	4°C
FFA Diluent A		YELLOW LABEL	10.5 ML	1	4°C
FFA Diluent B		PINK LABEL	5.5 ML	1	4°C
FFA Reagent A	Reconstitute using 10.5 ml FFA Diluent A. Discard remainder after 10 days	YELLOW LABEL	BOTTLE	1	4°C
FFA Reagent B	Reconstitute using 5.5 ml FFA Diluent B. Discard remainder after 10 days	PINK LABEL	BOTTLE	1	4°C

Other equipment/reagents required but not provided with the kit:

- Multi-channel Pipet , single channel pipet and pipet tips
- Plate reader with a filter of 540 nm
- Incubator at 37^{o}C
- Large gauge needle
- Tubes to dilute standards

ASSAY PROCEDURE

1. Preadipocytes are plated in 96 well plates and allowed to differentiate under standard Zen-Bio differentiation conditions for 1 week. Upon arrival, remove 150µl of the shipping medium from each well and discard. Place the plate (Plate A) in your incubator for 5-7 days (3-5 days for international customers) to allow the cells to recover from the stress of shipping. To ensure optimal performance, **DO NOT** feed the cells fresh medium during this time. Please observe the cells under a microscope prior to performing the assay [see the photograph in the Certificate of Analysis for the lot # of Plate A].

2. Make your stock solution using whatever vehicle is appropriate for your test compounds. Dilute your stock solutions to their final concentration in LIP-2/3 Assay Buffer (100 ml is available). NOTE: if desired, maintain a constant concentration of solvent by preparing all compound dilutions in the highest concentration of that solvent. Dilute your controls in assay buffer. Prepare all vehicles as appropriate for your compounds, 0.1% DMSO has been included as the vehicle for the positive controls. Include the Assay Buffer alone as a vehicle control. PLEASE NOTE: ZEN-BIO DOES NOT RECOMMEND THE USE OF SOLVENTS AT CONCENTRATIONS ABOVE 1%.

3. Remove 120 µl medium from each well. Gently add 200 µl Wash Buffer to all wells. Remove 200 µl of the media and Wash Buffer from each well and replace with another 200 µl Wash Buffer.

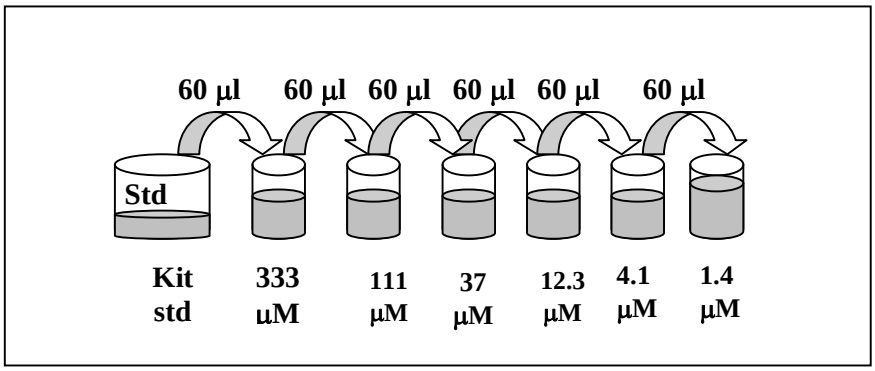
4. Remove all the media and Wash Buffer from the cells from triplicate wells. Treat the cells with 150 µl of the test compounds resuspended in Assay Buffer three (3) wells at a time. Treat with the diluted Isoproterenol as positive control. Use the Assay Buffer alone as one of the vehicle controls. Please be sure to include both the vehicle provided in the kit and your vehicle (if your test compounds are not dissolved in DMSO). The assay should be performed in triplicate.

5. Incubate the plates at 37^{o}C-humidified incubator for 3 hours (for time course experiments the longest time point recommended is 5 hours). Note: Treatment times longer than 3 hours will result in significant fatty acid reutilization by the adipocytes and may decrease signal relative to total lipolysis activity.

A. DETECTION OF NON-ESTERIFIED FATTY ACIDS

1. Prepare the standard curve using the FFA STANDARD SOLUTION as follows:

   Briefly spin down the contents of the FFA standard tube. Standards are: 0, 1.4, 4.1, 12.3, 37, 111, and 333 µM fatty acid. Prepare as follows:
   The kit standard solution is the 1.0 mM standard. Pipette 120 µl of LIP-2/3 Assay Buffer into 6 tubes (not provided). Pipette 60 µl of the FFA Standard Stock into a tube labeled 333 µM. Prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The Assay Buffer alone serves as the zero standard.



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that seven fewer data points can be assayed with this kit.

2. Also at this time prepare the FFA Reagent A by adding 10.5 ml FFA Diluent A per bottle and gently inverting. DO NOT VORTEX! Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).

3. At the end of the incubation, 30 µl of the conditioned media is removed and transferred to the corresponding well of a blank plate for assessment of non-esterified fatty acids. [This is most easily accomplished using a multi-channel pipet.] Add 30 µl of each standard to empty wells.

4. Add the reconstituted FFA Reagent A to one of the disposable trays provided in the kit. Add 100 µl of FFA Reagent A to each well. Gently shake the plate to ensure mixing. Place in a 37 °C incubator for 10 minutes.

5. Add 5.5 ml FFA Diluent B to the FFA Reagent bottle and gently invert. Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).

6. Add the reconstituted FFA Reagent B to the other disposable trays provided in the kit. Add 50 µl of FFA Reagent B to each well. Gently shake the plate to ensure mixing. Place in a 37 °C incubator for 10 minutes.

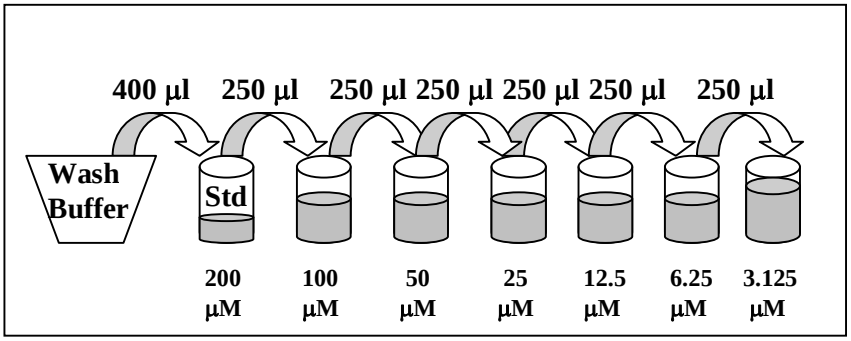
7. Allow the plate to equilibrate to room temperature for 5 minutes. During this time, ensure that there are no bubbles in the solution mixture. Use a large gauge needle or clean pipet tip to pop any bubbles as this will result in inaccurate absorbance readings.

8. The optical density of each well is then measured at 540 nm.

B. DETECTION OF FREE GLYCEROL

1. One hour prior to the assay, prepare the glycerol standards as follows:

   Briefly spin down the contents of the glycerol standard tube before reconstitution. Pipette 400 µl of Wash Buffer into the 1 mM glycerol standard tube provided and mix well by vortexing. This produces a diluted stock glycerol standard of 200 µM. Pipette 250 µl of wash buffer into 6 tubes (not provided). Using the newly diluted stock glycerol solution, prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The 200 µM stock dilution serves as the highest standard, and the wash buffer serves as the zero standard.



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that seven fewer data points can be assayed with this kit.

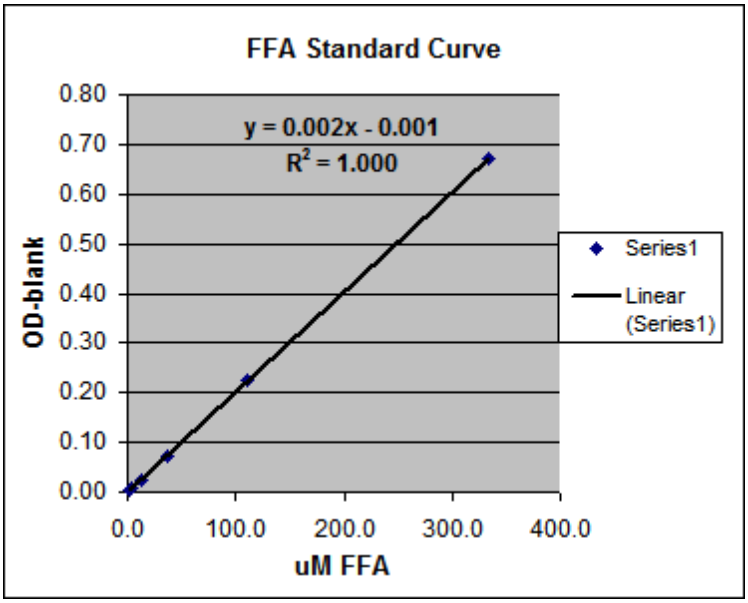
2. Also at this time prepare the Glycerol Reagent A by adding 11.0 ml room temperature deionized water per bottle and gently invert. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. Keep at room temperature. If using a Reagent A solution previously prepared and stored at 2-8°C, also bring to room temperature. Make sure there is enough Reagent A from one solution to treat all the points in the assay. It may be necessary to combine solutions. Store in a light protected bottle. Reconstituted Glycerol Reagent A is stable for 60 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).
3. At the end of the incubation, an additional 100 µl of the conditioned media is removed and transferred to the corresponding well of a blank plate for assessment of free glycerol. [This is most easily accomplished using a multi-channel pipet. Add 100 µl of each standard to empty wells.
4. OPTION: to determine if the compound alone reacts with the Glycerol Reagent A, prepare a fresh plate (not included in kit) containing 100 µl of the compound. This plate can be incubated at 37°C with the treated cells. When performing the assay, add 100 µl of Glycerol Reagent A following the instructions in Steps 5 and 6.
5. Add the reconstituted Glycerol Reagent A solution to one of the disposable trays provided in the kit. Add 100 µl of Reagent A to each well of the assay plates containing samples & standards. Gently, pipet up and down once to mix. Pop the bubbles using a large gauge needle or a clean pipet tip. The plate is then incubated at 25°C (room temperature) for 15 minutes.
6. The optical density of each well is then measured at 540 nm.

FATTY ACID STANDARD CURVE

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 0μM standard from all OD values including the standard curve. . Note: 1mM standard is commonly omitted from analysis due to lack of linearity between 333 μM and 1mM. Optionally, a 4-parameter fit may be used to calculate standard curve.

uM FFA	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.05	0.048			0.049
1.4	0.051	0.053	0.002	0.004	0.003
4.1	0.056	0.058	0.007	0.009	0.008
12.3	0.070	0.075	0.021	0.026	0.024
37	0.119	0.122	0.070	0.073	0.072
111	0.274	0.277	0.225	0.228	0.227
333	0.689	0.750	0.640	0.701	0.671



Slope	0.002
Intercept	-0.001
R^2	1.000

y = observed O.D. minus the blank

x = concentration of FFA in μM

To calculate x for each y, (i.e. to change the observed O.D. into FFA concentration) use the following equation:

y=(slope) times (x) plus intercept

y=mx+b so x=(y-b)/m

x=(y – (-0.001))/0.002 where 0.002= slope of the line and -0.001= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number. Data are expressed as μM free fatty acids released.

OPTION: express data as Fold induction over appropriate vehicle

$$\text{Fold induction} = \frac{\mu\text{M free fatty acids SAMPLE}}{\mu\text{M free fatty acids VEHICLE}}$$

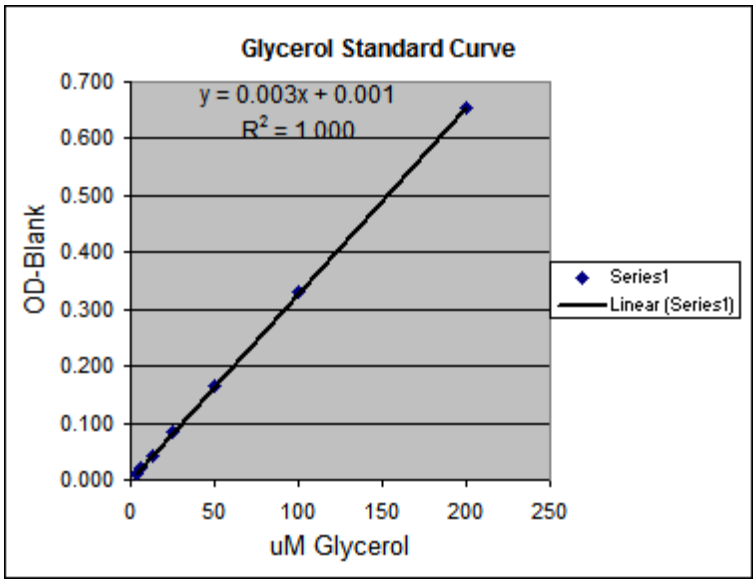
The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

GLYCEROL STANDARD CURVE

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 0μM standard from all OD values including the standard curve.

uM glycerol	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.044	0.041			0.043
3.125	0.054	0.053	0.012	0.011	0.011
6.25	0.062	0.063	0.020	0.021	0.020
12.5	0.083	0.084	0.041	0.042	0.041
25	0.126	0.125	0.084	0.083	0.083
50	0.205	0.208	0.163	0.166	0.164
100	0.372	0.374	0.330	0.332	0.331
200	0.698	0.697	0.656	0.655	0.655



Slope	0.003
Intercept	0.001
R^2	1.000

y = observed O.D. minus the blank
x = concentration of glycerol in μM
To calculate x for each y, (i.e. to change the observed O.D. into glycerol concentration) use the following equation:
y=(slope) times (x) plus intercept
y=mx+b so x=(y-b)/m
x=(y – (0.001))/0.003 where 0.003= slope of the line and 0.001= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

Any OD values greater than the highest standard (200 μM) □□□should be suspect. The compound should be re-assayed using a lower dose of the compound at treatment OR a dilute solution of the condition medium at the time of the assay.

The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

Data are expressed as μM glycerol released.

OPTION: express data as Fold induction over appropriate vehicle

$$\text{Fold induction} = \frac{\mu\text{M glycerol SAMPLE}}{\mu\text{M glycerol VEHICLE}}$$

APPENDIX A: PLATE LAYOUT _____

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

APPENDIX B: PROCEDURE FLOWCHART

Remove 150µl of the shipping medium and place in your incubator for 5-7 days (3-5 days for international customers)

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ON DAY OF ASSAY

Make all test compound dilutions in Assay Buffer.

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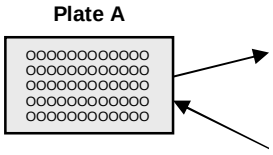
Remove 120 µl media from all wells. Add 200 µl Wash Buffer to all wells.



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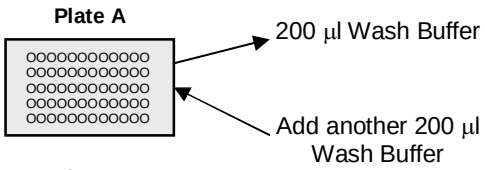
Remove 200 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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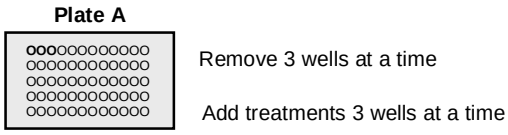
Remove all media & Wash Buffer. Add 150 µl treatments/controls to 3 wells at a time.



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Incubate 3-5 hours at 37°C.

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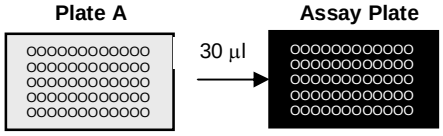
FREE FATTY ACID DETECTION

Remove 30 µl/well conditioned media from Plate A to Plate B. Add 30 µl FFA standards to empty wells.



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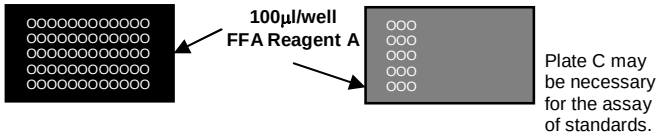
Reconstitute FFA Reagent A using Diluent A. Add 100µl/well. Incubate 10 minutes @ 37°C.



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Reconstitute FFA Reagent B using Diluent B. Add 50µl/well. Incubate 10 minutes @ 37°C.

↓

Place at room temp. for 5 minutes. Pop any bubbles in each well using a clean pipet tip or large gauge needle.

↓

Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

FREE GLYCEROL DETECTION

One hour prior to assay, reconstitute Glycerol Reagent A and prepare standards. Keep all at room temp.

↓

Remove 100 µl/well conditioned media from Plate A to a blank assay plate. Add 100 µl glycerol standards to empty wells.

↓

Add 100 µl/well reconstituted Glycerol Reagent A to a blank assay plate (including the glycerol standards at 100µl/well and optional plate without cells)

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Incubate at 25°C (room temperature) for 15 minutes. Pop the bubbles in each well.

↓

Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

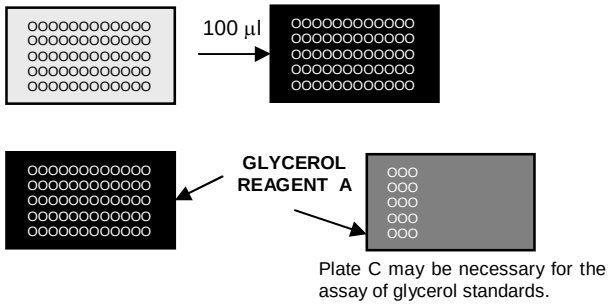

Plate C may be necessary for the assay of glycerol standards.

REFERENCES

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