Cellulite Treatment Screening
Human Adipocyte Lipolysis Assay Kit
Non-Esterified Fatty Acids Detection

Cat# LIP-11

INSTRUCTION MANUAL ZBM0018.02

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

For *in vitro* Use Only

LIMITED PRODUCT WARRANTY

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?

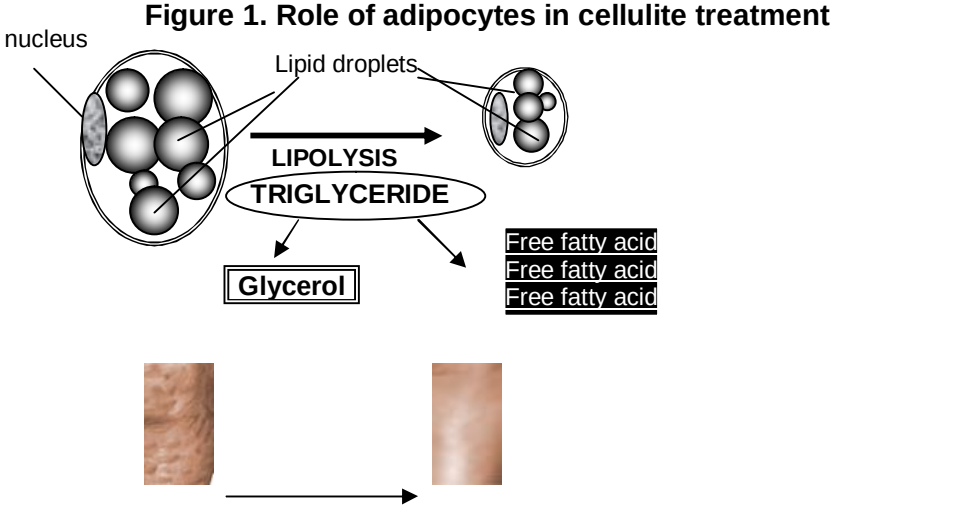
Cellulite is a term applied to a skin condition associated with the localized fat deposits that present as lumps and dimples appearing on the thighs of many women. Although cellulite primarily afflicts the thighs, hips and buttocks, it may also be present on the stomach and upper arms. Cellulite is simply 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

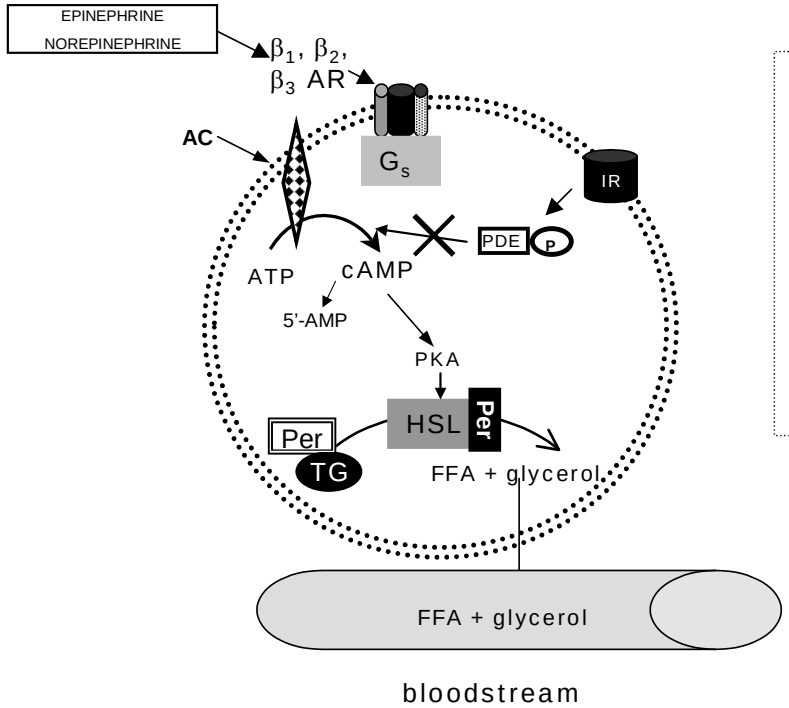


WHAT IS THE SCIENCE BEHIND THIS KIT?

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:

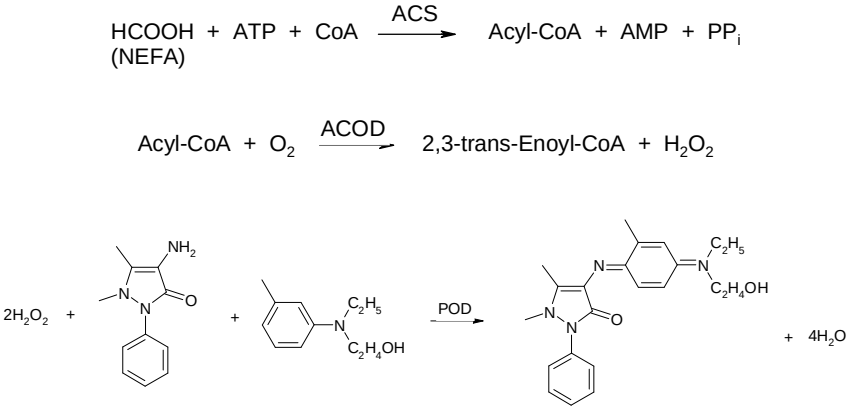
AC	adenylate cyclase
AR	adrenergic receptors
G_s	G protein coupled receptor
FFA	free fatty acids
PKA	protein kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
IR	insulin receptor
PDE	phosphodiesterase
Per	Perilipin
TG	triglyceride

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 the non-esterified fatty acids (NEFA) 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

Assessment of lipolytic activity is 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.



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, Plate B	96-well assay plate, blank	---	PLATE	1	-----
Assay Plate, Plate C	96-well assay plate, blank (for standards)	---	PLATE	1	-----
LIP-2/3 Assay Buffer	100 ml	---	BOTTLE	1	4°C
Wash Buffer	50 ml	---	BOTTLE	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
FFA Standard	1mM Stock. See page 5 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
Tray	For multi-channel pipetters, clear polyvinyl	CLEAR	EACH	2	-----

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 FFA 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.

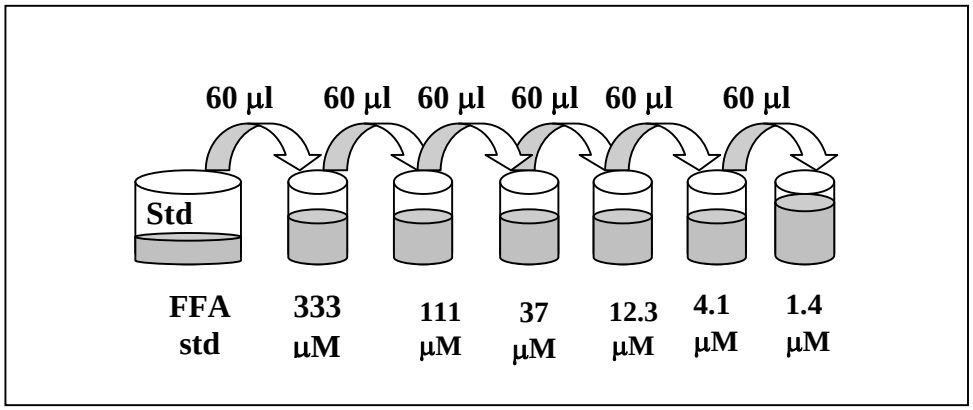
4. Remove all the media and Wash Buffer from the cells from triplicate wells. Treat the cells with 75 μl of the test compounds resuspended in LIP-2/3 Assay Buffer three (3) wells at a time. Treat with the diluted Isoproterenol as positive control. Use the Assay Buffer alone as one of the vehicle controls. 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.

6. Prepare the standard curve using the STANDARD SOLUTION as follows:

Briefly spin down the contents of the free fatty acid 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.

7. 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).

8. At the end of the incubation, 50 μl of the conditioned media is removed and transferred to the corresponding well of Plate B. [This is most easily accomplished using a multi-channel pipet.] Add 50 μl of each standard to empty wells (use PLATE C if necessary).

9. 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.

10. 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).

11. 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.

12. 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.

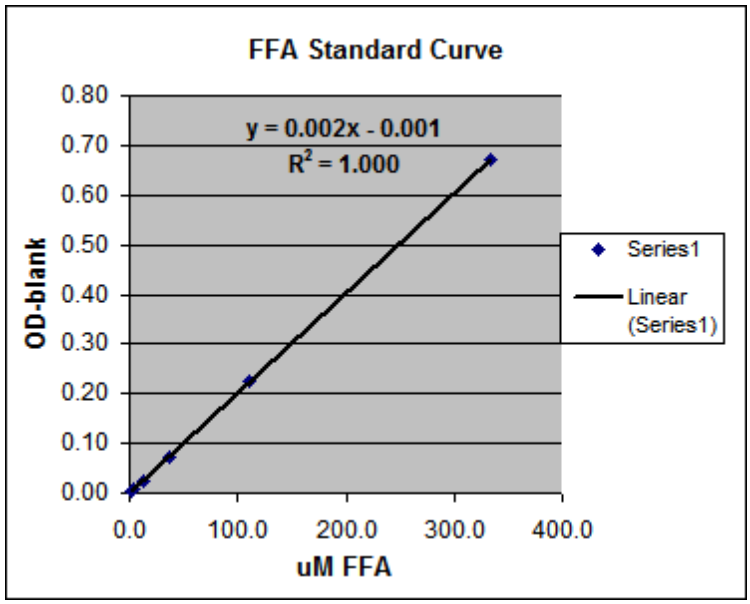
13. The optical density of each well is then measured at 540 nm.

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.

FREQUENTLY ASKED QUESTIONS

1. **I do not have time to perform the assay. Can I freeze the conditioned media in PLATE B? How long can I store the samples before I complete the assay?** Yes. The conditioned media in PLATE B can be immediately stored at -80°C for a maximum of 7 days. Bring the conditioned media in PLATE B to room temperature BEFORE adding the FFA Reagents A and B and completing the assay

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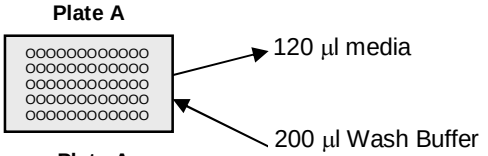
Remove 120 µl media from all wells. Add 200 µl Wash Buffer to all wells.



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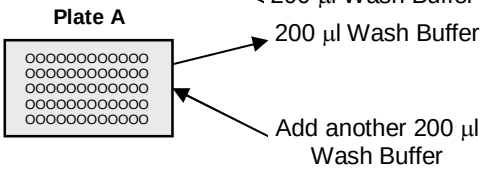
Remove 200 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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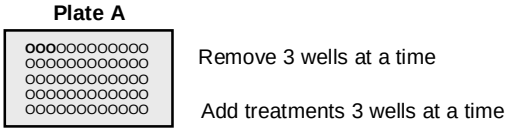
Remove all media & Wash Buffer. Add 75 µl treatments/controls to 3 wells at a time.



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Incubate 3-5 hours at 37°C.

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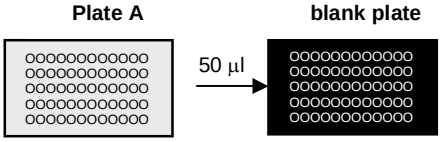
Remove 50 µl/well conditioned media from Plate A to one of the blank assay plates provided. Add 50 µl FFA standards to empty wells.



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

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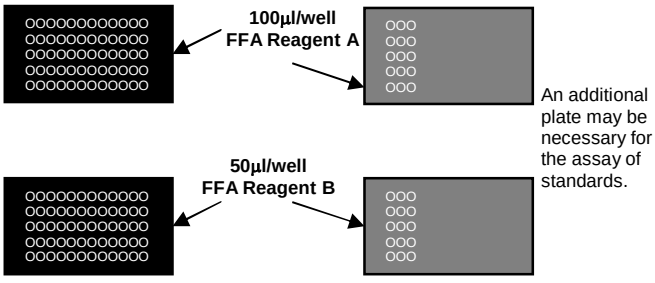
Reconstitute FFA Reagent B using Diluent B. Add 50µl/well. Incubate 10 minutes @ 37°C.



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

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

REFERENCES

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