Cultured Human Adipocyte Lipolysis Assay Kit
Non-Esterified Fatty Acids Detection
500 Point Assay Kit

Cat# LIP-2RB

INSTRUCTION MANUAL ZBM0031.03

STORAGE CONDITIONS

- **Reagents & Buffers:** 4°C
- **Vehicle & Controls:** -20°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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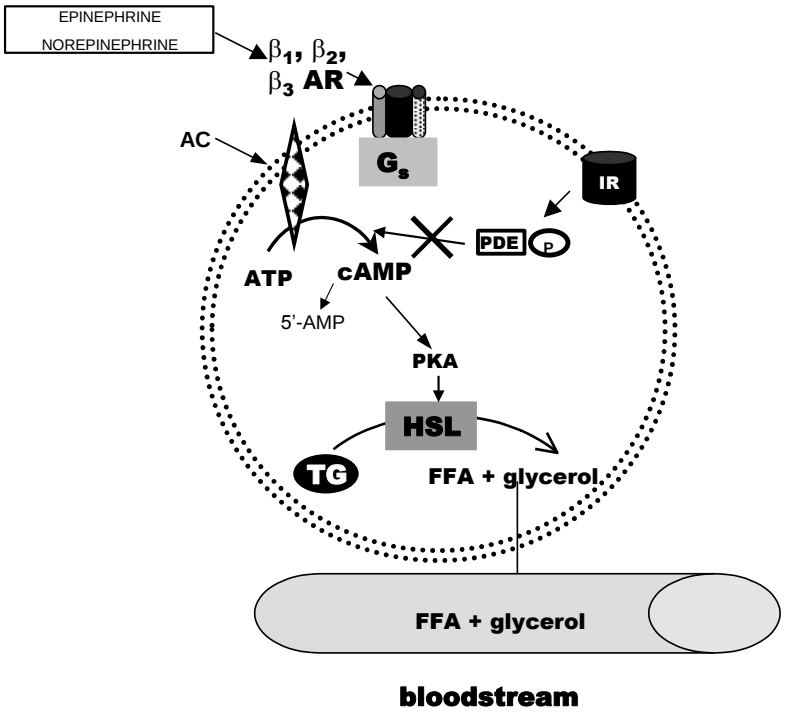
INTRODUCTION

Lipolysis plays a central role in the regulation of energy balance. 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. Elevated adipocyte lipolysis has been observed in obese and diabetic individuals (Arner 1996). Alterations in lipolytic capacity have also been implicated in the susceptibility to obesity of African-American individuals versus their Caucasian cohorts (Danadian *et al.* 2001).

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 glycerol and FFA (increased lipolysis). Phosphodiesterases (PDE) are enzymes that hydrolyze 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), is used as the positive control if your test compounds are suspected PDE inhibitors. Isoproterenol, a non-specific β-adrenergic agonist is used as the positive control if your test compounds affect lipolysis via β-adrenergic receptors.

This lipolysis assay kit provides the tool to study chemical compounds that may influence lipolysis in cultured human adipocytes.

Figure 1. Overview of adipocyte lipolysis

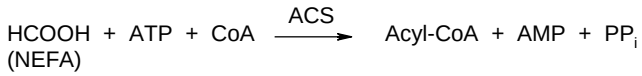


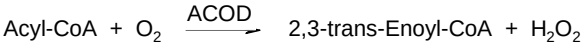
ABBREVIATIONS:

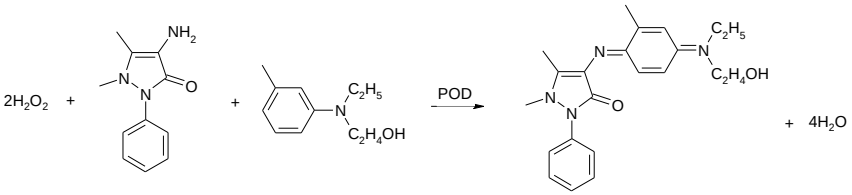
AC	adenylate cyclase
AR	adrenergic receptors
G_s	G protein coupled receptor
FFA	free fatty acids
PKA	protein kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
IR	insulin receptor
PDE	phosphodiesterase
TG	triglyceride

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
LIP-2/3 Assay Buffer	500 ml	---	BOTTLE	1	4°C
Wash Buffer	250 ml	---	BOTTLE	1	4°C
Vehicle	0.1% DMSO in LIP-2 Assay Buffer	PURPLE	1 ml / VIAL	5	-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	5	-20°C
FFA Standard	1mM Stock. See page 5 for standard curve preparation	AMBER	100 μl / VIAL	5	4°C
FFA Diluent A		YELLOW	50ML	1	4°C
FFA Diluent B*  Warning		PINK	25ML	1	4°C
FFA Reagent A**  Warning	Reconstitute using 50 ml FFA Diluent A. Discard remainder after 10 days	YELLOW	BOTTLE	1	4°C
FFA Reagent B	Reconstitute using 25 ml FFA Diluent B per bottle. Discard remainder after 10 days	PINK	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 37ºC
- Large gauge needle
- Blank 96 well plates
- Cultured human adipocytes
- Tubes for diluting standards

** Warning. May cause an allergic skin reaction. See SDS for more details*
*** Warning. H302 - Harmful if swallowed; H402 - Harmful to aquatic life See SDS for more details*

ASSAY PROCEDURE

1. Please observe your cells under a microscope prior to performing the assay.

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. 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.

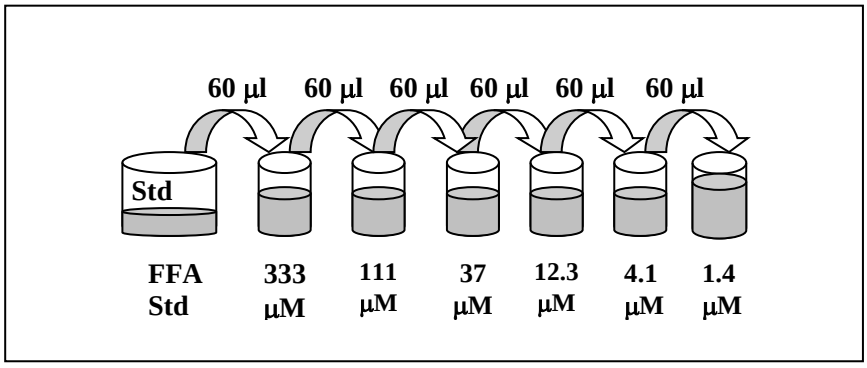
4. Remove all the media and Wash Buffer from the cells from triplicate wells. Treat the cells with 75 µl of the test compounds resuspended in Assay Buffer three (3) wells at a time. Treat with the diluted Isoproterenol as positive control. Use the Assay Buffer alone as one of the vehicle controls. Please 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°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 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 50ml FFA Diluent A to the 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 another empty 96 well plate. [This is most easily accomplished using a multi-channel pipet.] Add 50 μl of each standard to empty wells.

9. Add the reconstituted FFA Reagent A to a disposable tray. 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. Prepare the FFA Reagent B by adding 25ml FFA Diluent B to the Reagent B 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).

11. Add the reconstituted FFA Reagent B to another disposable tray (not provided). 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. Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).

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.

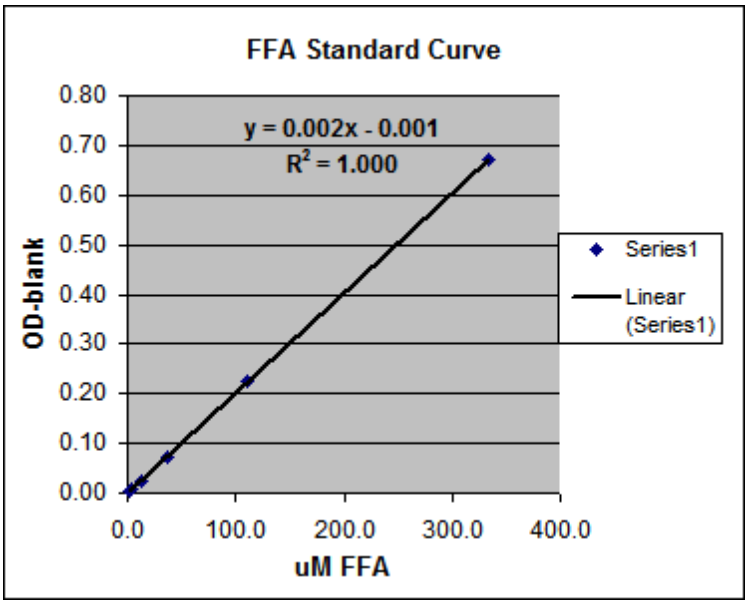
13. The optical density of each well is then measured at 540 nm.

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? How long can I store the samples before I complete the assay? Yes. The conditioned media can be immediately stored at -80°C for a maximum of 7 days. Bring the conditioned media 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: LIP-2RB PROCEDURE FLOWCHART

ON DAY OF ASSAY

Make all test compound dilutions in Assay Buffer.

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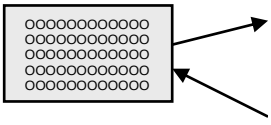
Remove 120 µl media from all wells. Add 200 µl Wash Buffer to all wells.



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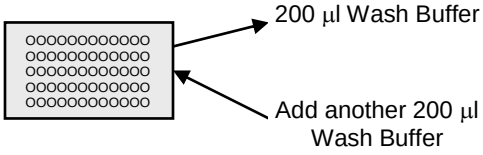
Remove 200 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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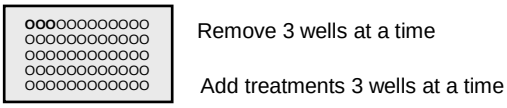
Remove all media & Wash Buffer. Add 75 µl treatments/controls to 3 wells at a time.



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Incubate 3-5 hours at 37ºC.

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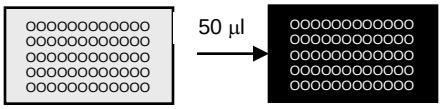
Transfer 50 µl/well conditioned media to a blank assay plate. Add 50 µl/well standards to empty wells.



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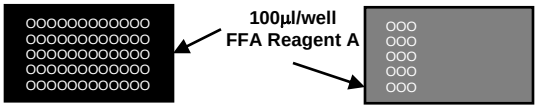
Reconstitute FFA Reagent A using Diluent A. Add 100µl/well. Incubate 10 minutes @ 37°C.



An additional plate may be necessary for the assay of standards.

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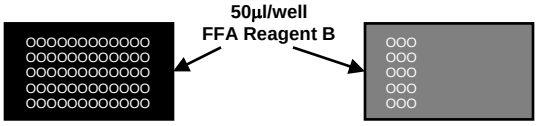
Reconstitute FFA Reagent B using Diluent B. Add 50µl/well. Incubate 10 minutes @ 37°C.



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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.

* blank 96 well plates not provided in –RB kits