Cultured Human Adipocyte Lipolysis Assay Kit-500 point assay kit 96-well Format

Cat# LIP-1-RB

INSTRUCTION MANUAL ZBM0027.04

STORAGE CONDITIONS

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

Glycerol Reagent A & Buffers:

Store at 4°C.

Glycerol Standards & Controls:

Store at -20°C

ALL ZEN-BIO INC PRODUCTS ARE FOR RESEARCH USE ONLY. NOT APPROVED FOR HUMAN OR VETERINARY USE OR FOR USE IN DIAGNOSTIC OR CLINICAL PROCEDURES.

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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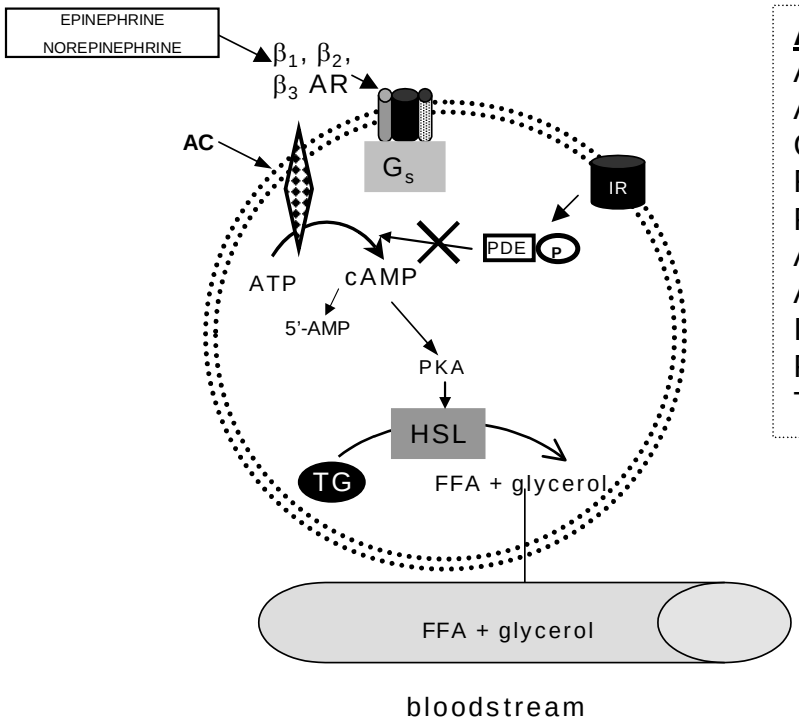
INTRODUCTION

Lipolysis plays a central role in the regulation of energy balance. Lipolysis is the process in which triglycerides (TG) 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). Excessive free fatty acid production is believed to contribute to insulin resistance in skeletal muscle that is observed in obesity. Hormone sensitive lipase is the rate-limiting enzyme catalyzing triglyceride breakdown. Perilipins, one of the PAT (perilipins, adipophilin, TIP47 proteins) family of lipid-associated proteins, are implicated in adipocyte lipolysis by mediating the interaction of HSL with the triacylglycerol molecule (Brasaemle *et al.* 2004; reviewed in, Tansey *et al. 2004.)* The presence of these proteins corresponds to lipolytic stimulation in cultured adipocytes (Braemle *et al.* 2004).

The sympathetic nervous system also 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 (Robidoux *et al*. 2004).

This lipolysis assay kit provides the tool to study chemical compounds that may influence lipolysis in cultured human adipocytes. This kit detects lipolysis as the amount of glycerol released from mammalian cells in a 96-well format. This kit contains sufficient volume of reagents to assay 500 data points (~5 96 well plates).

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

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-ethyl-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.

GLYCEROL + ATP $\longrightarrow$ G-1-P + ADP

G-1-P + O_2 $\longrightarrow$ DAP + H_2O_2

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

ITEMS INCLUDED IN THE KIT _____

ITEM	DESCRIPTION	UNIT	QTY	STORAGE
LIP-1 Assay Buffer	500 ml	BOTTLE	1	4°C
Wash Buffer	250 ml	BOTTLE	1	4°C
Glycerol Reagent A (cat# RGTA-40)	40-ml- Reconstitute with 40 ml deionized water prior to use.	40ML BOTTLE	1	4°C
Glycerol Reagent A (cat# RGTA-10)	11-ml- Reconstitute with 11 ml deionized water prior to use.	11ML BOTTLE	1	4°C
Glycerol standard (cat# LIP-GLYSTAN) Orange cap	Glycerol @ 1mM [Reconstitute with 400 µl Wash Buffer to make the 200 µM glycerol standard; see page 6 for recommended dilution scheme]	100 µl / VIAL	2	-20°C
Vehicle Control Green cap	0.1% DMSO in Assay Buffer	1 ml / VIAL	2	-20°C
Positive control Blue cap	Isoproterenol, 10 mM in DMSO. Dilute to 1 µM in Assay Buffer before use! (i.e.1 µl in 10 ml Assay Buffer)	10 µl / VIAL	2	-20°C

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

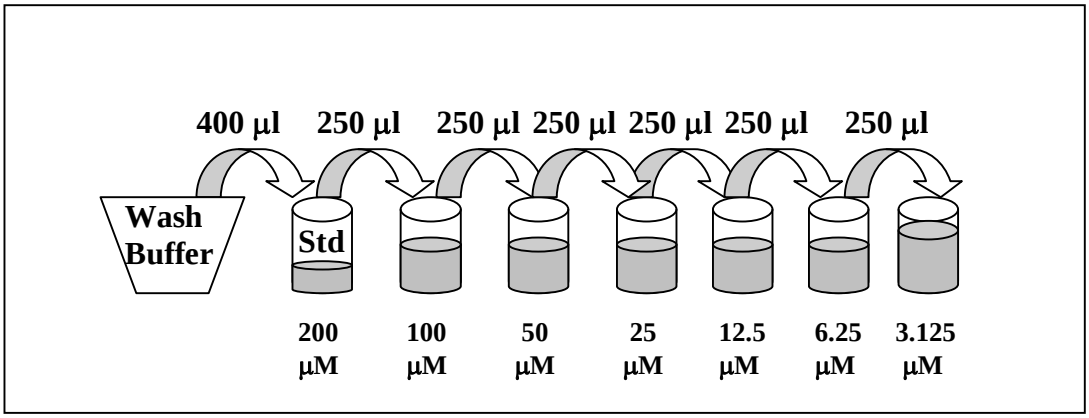
- Clean/disposable pipettor trays
- Blank 96 well plates
- Multi-channel Pipet , single channel pipet and pipet tips
- Plate reader with a filter of 540 nm
- Incubator at 37^{o}C
- Large gauge needle
- Cultured human adipocytes
- Tubes for diluting glycerol standards

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 Assay Buffer (500 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. 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. You can treat with isoproterenol in assay buffer as the positive control (not provided in this kit). Use the Assay Buffer alone as one of the vehicle controls. Please be sure to include your vehicle (if your test compounds are not dissolved in DMSO). The assay should be performed in triplicate.
5. 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^{o}C with the treated cells. When performing the assay, add 100 µl of Glycerol Reagent A following the instructions in Steps 10 and 11.
6. Incubate the plates at 37^{o}C-humidified incubator for 3 hours (for time course experiments the longest time point is usually 24 hours).
7. **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 eight fewer data points can be assayed with this kit.

8. At this time prepare the Glycerol Reagent A by adding 40 ml or 11 ml room temperature deionized water per bottle following the instructions on the bottle. Gently invert bottle to mix contents. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. 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). You may order additional glycerol standards for the Lipolysis Kit (cat# LIP-GLYSTAN).

9. At the end of the incubation, 100 μl of the conditioned media is removed and transferred to the corresponding well of another blank plate. [This is most easily accomplished using a multi-channel pipet.] Add 100 μl of each glycerol standard to any remaining empty wells in one of the blank assay plates.

10. Add the reconstituted Glycerol Reagent A solution to a disposable tray (not provided). Add 100 μl of Reagent A to each well of the assay plates containing samples. 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^{o}C (room temperature) for 15 minutes.

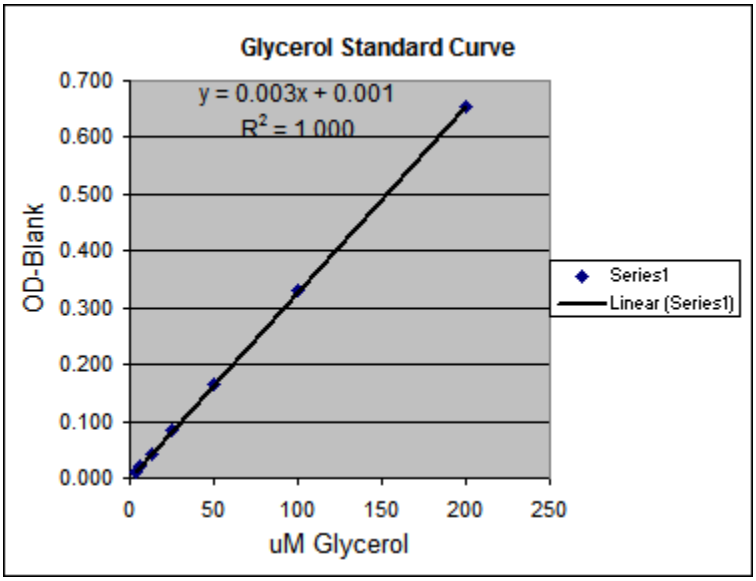
11. The optical density of each well is then measured at 540 nm.

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

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

TROUBLESHOOTING

Problem	Suggestions
High background or the glycerol reagent A turns purple before the assay begins.	• Change pipet tips frequently • Use Glycerol Reagent A before the expiration date
No response to positive control	• Do not add the compounds and controls too fast. The cells can float if a solution is added too fast. • Make sure to starve the cells for 5-7 days BEFORE initiating treatment.
Edge effects	• Ensure a saturated humidity in the incubator to prevent evaporation from the outside wells
Inconsistent OD reading	• The Assay Buffer contains bovine serum albumin (BSA). Be careful when pipetting to avoid bubbles. If bubbles persist, burst the bubbles using a large gauge needle and read the plate again.

FREQUENTLY ASKED QUESTIONS

1. **I want to perform a lipolysis time course experiment. How many time points can I complete ?** We do not recommend performing more than 2 time points per assay. For time course experiments, add 250 µl assay medium with treatments per well. Remove 100 µl for each time point. Complete the assay using an equal volume Glycerol Reagent A.

2. **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 in regular polystyrene cell culture plates. Bring the conditioned media in the plate to room temperature BEFORE adding the Glycerol Reagent A and completing the assay.

3. **Can I buy the reagents separately?** The Glycerol Standard, cat# LIP-GLYSTAN and Glycerol Reagent A, cat# RGTA-10 (11 ml), RGTA-40 (40 ml) are sold separately. Assay Buffer is not sold separately.

4. **I need to know the concentration of the BSA in the Assay Buffer?** ZenBio, Inc does not provide the concentrations of the components of our media and buffers. If knowledge of the BSA concentration is critical to your experiment, you may order Assay Buffer WITHOUT BSA for no additional charge. Please note it on your order.

REFERENCES

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11. Tansey JT, Sztalryd C, Hlavin EM, Kimmel AR, Londos C. (2004) *IUBMB Life* 56(7): 379-85.

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

Make all test compound dilutions in Assay Buffer.

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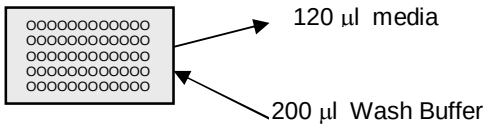
Remove 120 µl media from all wells. Add 200 µl Wash Buffer to all wells.



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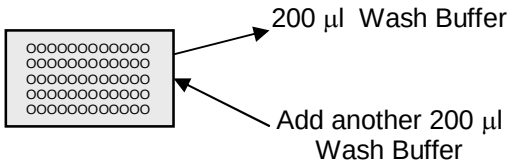
Remove 200 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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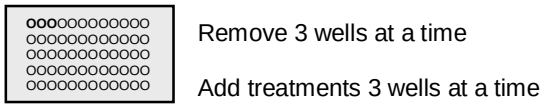
Remove all media & Wash Buffer. Add 150 µl treatments/controls to 3 wells at a time. OPTION: Add 100 µl/well compounds to a fresh plate without cells.



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Incubate 3-5 hours at 37°C.

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

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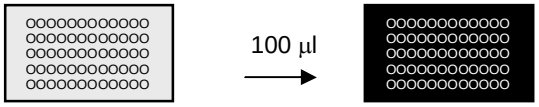
Remove 100 µl/well conditioned media to a blank assay plate (not provided). Add standard dilutions.



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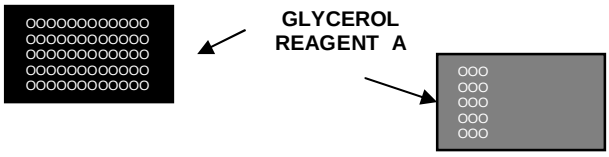
Add 100 µl/well reconstituted Glycerol Reagent A to the plate (including the glycerol standards at 100µl/well) and optional plate without cells.



An additional blank assay plate may be necessary for the assay of glycerol standards if al 96 wells are used. (not provided)

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

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