Cultured Human Adipocyte Lipolysis Assay Kit

Cat# LIP-1; LIP-1-NC

INSTRUCTION MANUAL ZBM0002.04

STORAGE CONDITIONS

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

Assay Plate A: Cultured human adipocytes (LIP-1 kit ONLY)

37°C CO_2 incubator

Glycerol Reagent A & Buffers:

Store at 4°C.

Glycerol Standards & Controls:

Store at -20°C

Long-term storage:

LIP-1-NC (*Reagents Only*) kit: remove the glycerol reagent A from the box and place at 4°C, store the rest of the kit at -20°C. Reagents are good for 6 months if stored properly.

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.

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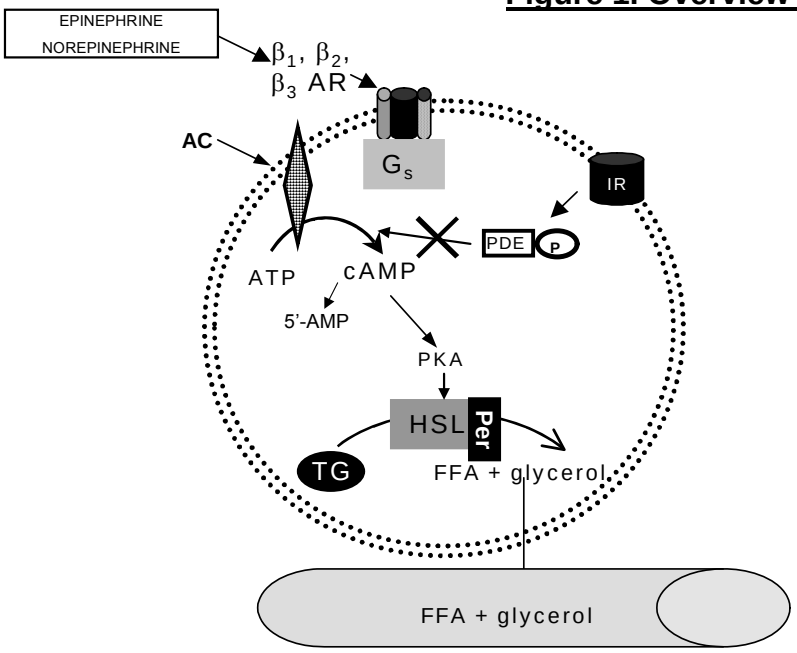
INTRODUCTION

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.

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 quinoeimine 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 ⟶ G-1-P + ADP

G-1-P + O_2 ⟶ DAP + H_2O_2

H_2O_2 + 4-AAP + ESPA ⟶ Quinoneimine dye + H_2O

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Adipocytes, Plate A	Cultured human subcutaneous adipocytes	---	PLATE	1	37°C
Blank Assay Plates	96-well assay plates, blank	---	PLATE	2	-----
Assay Buffer	100 ml	---	BOTTLE	1	4°C
Wash Buffer	50 ml	---	BOTTLE	1	4°C
Vehicle	0.1% DMSO in Assay Buffer	GREEN	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	2	-----
Glycerol standard	Glycerol @ 1mM [Reconstitute with 400 µl Wash Buffer to make the 200 µM glycerol standard; see page 5 for recommended dilution scheme]	ORANGE	100 µl / VIAL	1	-20°C
ALTERNATE : Positive control	3-Isobutyl-1-methylxanthine (IBMX), 100 mM in DMSO Dilute to 100 µM in Assay Buffer before use! (i.e. 1 µl in 1 ml Assay Buffer). **USE ONLY IF YOUR TREATMENT TIME EXCEEDS 5 HOURS.**	RED	10 µl / VIAL	1	-20°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°C
- Large gauge needle
- Option – Step 5 of Assay Procedure: 96 well plate, blank
- Tubes for dilution of standards

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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 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.

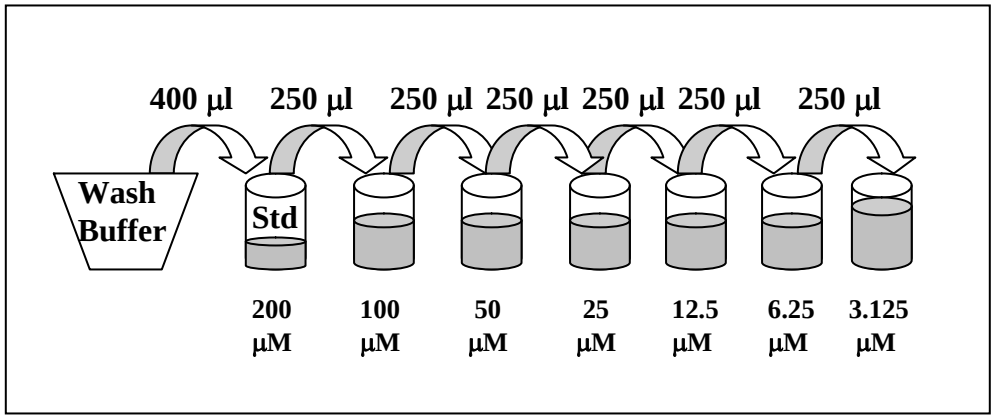
4. Remove all the media and Wash Buffer from the cells from triplicate wells. Treat the cells with 150 µl of the test compounds resuspended in Assay Buffer three (3) wells at a time. Treat with the diluted Isoproterenol or optionally, IBMX (for treatments 5-24 hours), as positive control. Use the Assay 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. 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 10 and 11.

6. Incubate the plates at 37°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. 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. 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).

9. At the end of the incubation, 100 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 100 µl of each glycerol standard to any remaining empty wells in one of the blank assay plates.

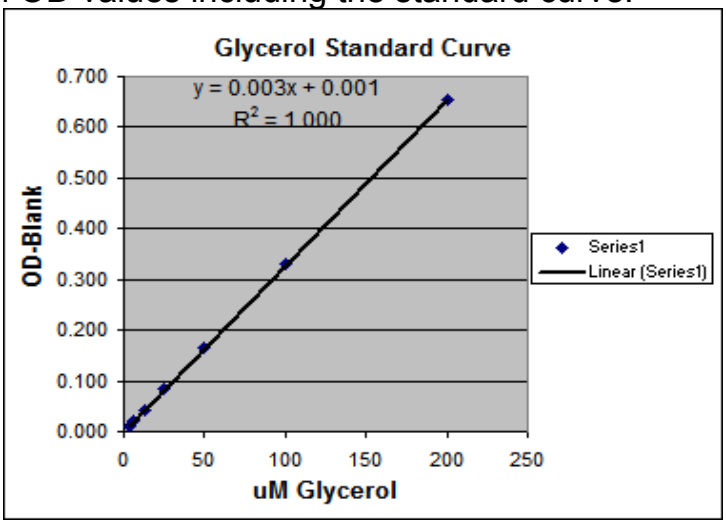
10. Add the reconstituted Glycerol Reagent A solution to one of the disposable trays provided in the kit. Add 100 µl of Reagent A to each well of the assay plates containing samples. Gently, pipet up and down once to mix. Pop the bubbles using a large gauge needle or a clean pipet tip. The plate is then incubated at 25°C (room temperature) for 15 minutes.

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
R2	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

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

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	• Make sure to starve the cells for 5-7 days BEFORE initiating treatment. • DO NOT use IBMX as the positive control if you are incubating for less than 5 hours.
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.

2. **When do I need to use the IBMX positive control?** If you use the 3-5 hour incubation described in this manual, you will not need to use the IBMX as your positive control. The IBMX positive control is designed for treatments ranging from 5-24 hours. The IBMX alternate control may be used in addition to the Isoproterenol positive control if your treatment time will exceed 5 hours.

3. **Can I buy the reagents separately?** The Glycerol Standard, cat# LIP-GLYSTAN and Glycerol Reagent A, cat# RGTA-10 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.

5. **I have more samples plus standards to run than can fit on 1 96 well plate. Can I compare data obtained from multiple plates?** The lipolysis kit is designed for the assay of a single plate. You may purchase 2 kits of the same lot number. You may then use one plate that includes the blank, vehicle(s), and positive and negative controls. The second plate may then be used for the remainder of your samples assayed. In order to obtain comparable data, both plates must be

assayed on the same day using kits and cells from the same lot number. An additional blank assay plate is provided for the assay of glycerol standards.

6. **I do not have time to pop the bubbles and read the plate. Can I freeze the conditioned media in one of the assay plates provided with the kit? How long can I store the samples?** Yes. The conditioned media can be immediately stored at -80°C for a maximum of 7 days. Bring the conditioned media in the plate to room temperature BEFORE adding the Glycerol Reagent A and completing the assay.

7. **I need to use another plate format than the 96 well. Do you have a kit I can use?** Yes. We offer a lipolysis assay kit containing a vial of subcutaneous preadipocytes, media to differentiate the cells and the reagents to complete the lipolysis assay. Catalog # LIP-1-SF is suitable for 384-, 96-, 24-, or 12-well plate format. You must then transfer the conditioned assay buffer to a 96 well format to complete the assay.

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

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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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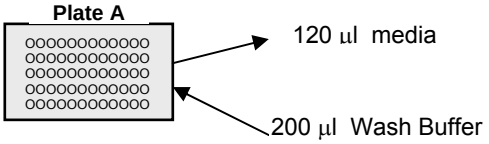
ON DAY OF ASSAY

Make all test compound dilutions in Assay Buffer.

↓

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



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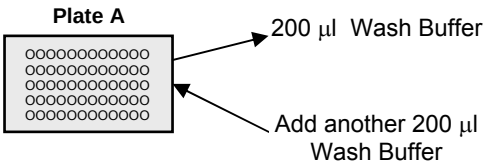
Remove 200 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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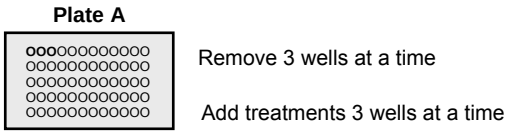
Remove all media & Wash Buffer. Add 150 µl treatments/controls to 3 wells at a time. OPTION: Add 100 µl/well compounds to a fresh plate without cells.



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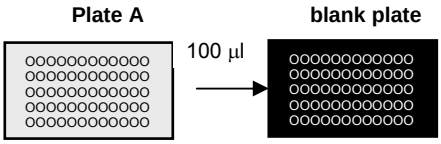
Incubate 3-5 hours at 37°C.

↓

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/well glycerol standards to empty wells.



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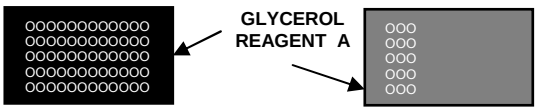
Add 100 µl/well reconstituted Glycerol Reagent A to the plate (including the glycerol standards at 100µl/well) and optional plate without cells.



An additional blank assay plate may be necessary for the assay of glycerol standards if al 96 wells are used.

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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.