# Adipose Tissue Explant
# Lipolysis Assay Kit: Glycerol Detection
Cat# LIP-6-NC

**INSTRUCTION MANUAL ZBM0046.03**

## STORAGE CONDITIONS

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

**Glycerol Reagent A & Buffers:**

Store at 4°C.

**Use reconstituted Glycerol Reagent A within 7 days.**

**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


- **Zen-Bio, Inc.**
- **3200 Chapel Hill-Nelson Blvd., Suite 104**
- **PO Box 13888**
- **Research Triangle Park, NC 27709**
- **Telephone** (919) 547-0692
- **Facsimile (FAX)** (919) 547-0693
- **Toll Free** 1-866-ADIPOSE (866)-234-7673
- **Electronic mail (e-mail)** information@zen-bio.com
- **World Wide Web** http://www.zenbio.com

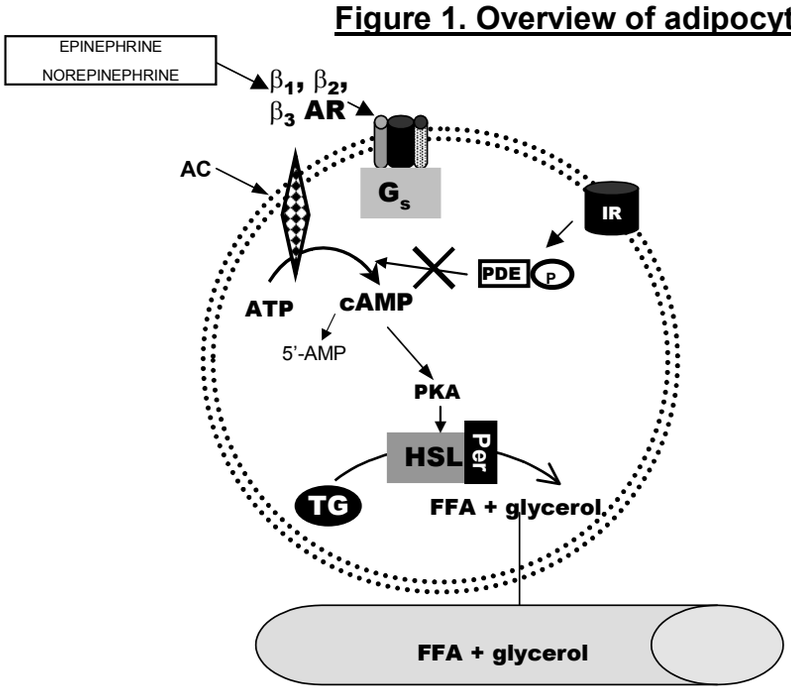
# INTRODUCTION

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 whole mammalian adipose tissue pieces.

**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 |
|---|---|---|---|---|---|
| Blank Assay Plate | 96-well assay plate, blank | --- | PLATE | 1 | ----- |
| Assay Buffer | 100 ml | --- | BOTTLE | 1 | 4°C |
| Wash Buffer | 100 ml | --- | BOTTLE | 1 | 4°C |
| Vehicle | 0.1% DMSO in Assay Buffer | GREEN | 1 ml / VIAL | 2 | -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. Use within 7 days! | | BOTTLE | 1 | 4°C |
| Tray | Clear polyvinyl, 50ml capacity | | 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.5 μl in 5 ml Assay Buffer). | RED | 10 μl / VIAL | 1 | -20°C |

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

- Flat bottom, Tissue culture treated cultureware, 24- or 48 well format
- 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

# ASSAY PROCEDURE

1. 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%.

2. Wash fresh non-enzymatically digested adipose tissue using Wash Buffer. If isolating multiple samples, store removed adipose tissue at room temperature in sterile containers (not provided) containing sufficient phosphate buffered saline (PBS) or Wash Buffer to completely submerge the sample until ready for assay.

3. Place a 24 or 48 well plate on a digital balance and tare the unit. While holding the adipose tissue chunk using tissue forceps, use scissors to cut small uniform pieces (by weight) of adipose tissue directly into each well of the plate. Record weight per well. Suggested weights are 100mg per well for a 24 well plate or 50mg for a 48 well plate.

4. Mince tissue for 1 minute using sharp scissors.

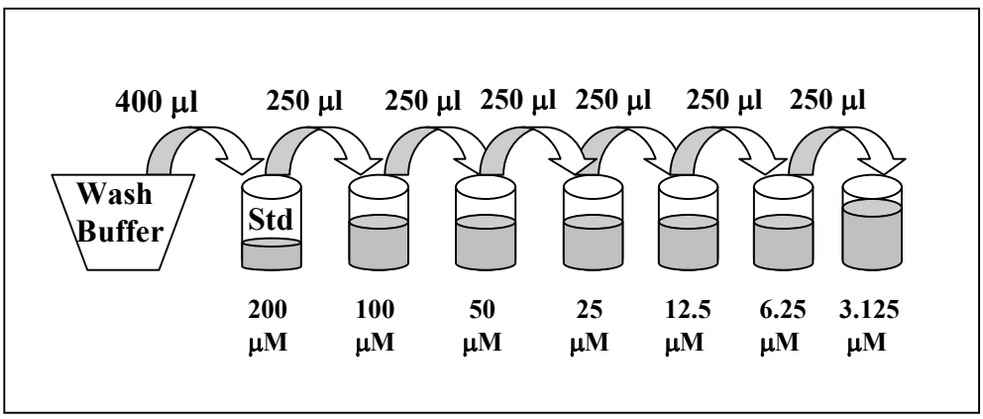
5. Treat each well with 300-500 μl (48 well-24 well) of the test compounds resuspended in Assay Buffer three (3) wells at a time. Treat with the diluted Isoproterenol or optionally, IBMX, as positive control. Use the Assay Buffer alone as one of the vehicle controls. Please be sure to include both the vehicle provided in the kit and your vehicle (if your test compounds are not dissolved in DMSO).

6. OPTION: to determine if the compound alone reacts with the Glycerol Reagent A, prepare a fresh plate (not included in kit) containing 50 μl of the compound. This plate can be incubated at 37$^{o}$C with the treated cells. When performing the assay, add 50 μl of Glycerol Reagent A following the instructions in Steps 10 and 11.

7. Incubate the plates at 37$^{o}$C-humidified incubator for 5-24 hours (for time course experiments the longest time point is usually 24 hours).

8. 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.**

9. Also at this time prepare the Glycerol Reagent A by adding 11 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).

10. At the end of the incubation, a small sample of the conditioned media is removed and transferred to the provided 96 well plate. **The sample may need to be diluted using Assay Buffer.** We suggest a 2-5 fold dilution with a total volume of 100µl per well (i.e. 20µl conditioned medium + 800µl Assay Buffer= dilution factor of 5). Add 100 µl of each glycerol standard to any remaining empty wells the blank 96-well assay plate.

11. 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.

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

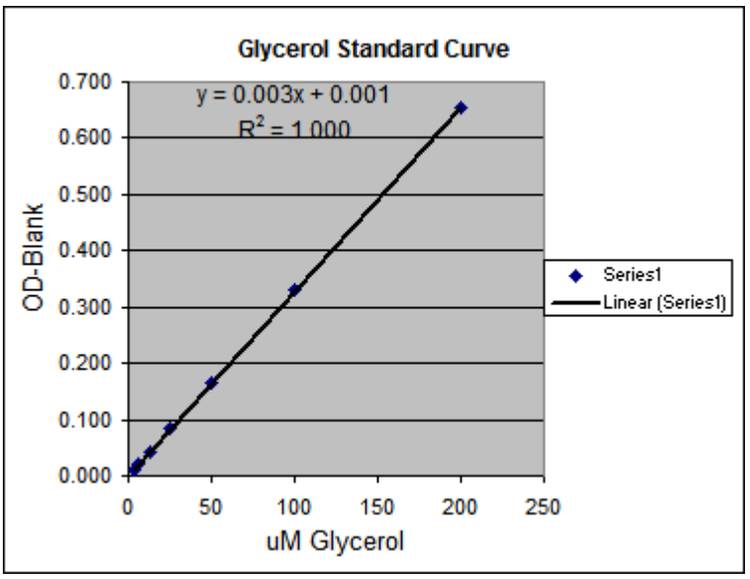
# GLYCEROL STANDARD CURVE

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. Multiply values by the dilution factor (if used).

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<br>• Use Glycerol Reagent A before the expiration date |
| No response to positive control | • Use a new pipet tips for each well during transfer of conditioned medium.<br>• Do keep samples at room temperature until ready for assay |
| 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.<br>• Make sure adipose tissue sizes are as uniform as possible. |

# FREQUENTLY ASKED QUESTIONS

1. **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.
2. **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 500-750 µl assay medium with treatments per well. Remove 50 µl for each time point. Complete the assay using an equal volume Glycerol Reagent A.
3. **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.
4. **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 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 in the plate to room temperature BEFORE adding the Glycerol Reagent A and completing the assay.

# APPENDIX A: PLATE MAPS _____

## 24 WELL

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | 1 | 2 | 3 | 4 | 5 | 6 |
| **B** | 7 | 8 | 9 | 10 | 11 | 12 |
| **C** | 13 | 14 | 15 | 16 | 17 | 18 |
| **D** | 19 | 20 | 21 | 22 | 23 | 24 |

## 48-WELL

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| **A** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| **B** | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| **C** | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| **D** | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 |
| **E** | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| **F** | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 |

# 96-WELL

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

# APPENDIX B: PROCEDURE FLOWCHART

Isolate fresh adipose tissue. Store at room temperature in Wash Buffer or PBS.

↓

Make all test compound dilutions in Assay Buffer.

↓

Add 300-500 μl treatments/controls to 3 wells at a time.


↓

OPTION: Add 50 μl/well compounds to a fresh plate without cells.

↓

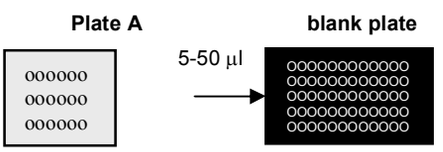
Incubate 5-24 hours at 37°C.

↓

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

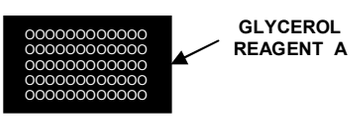
↓

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




↓

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




↓

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.

# REFERENCES


1. Arner P (1996) *Diabetes Rev* 4(4):450-463.
2. Botion LM & Green A. *Diabetes* (1999) 48:1691-1697
3. Brasaemle DL, Dolios G, Shapiro L, Wang R. (2004) *J Biol Chem* 279(45): 46835-42.
4. Cooper DMF, Schlegel W, Lin MC, Rodbell M. (1979) *J Biol Chem* 254(18):8927-8931.
5. Dyck DJ Can J Appl Physiol (2000) 25(6):495-523.
6. Kordik CP & Reitz AB. *J Medicinal Chem* (1999) 42(2):181-201.
7. Rieusset J, Chambrier C, Bouzakri K, Dussere E, Auwerx J, Riou J-P, Laville M, Vidal H. *Diabetologia* (2001) 44:544-554.
8. Robidoux J, Martin TL, Collins S. (2004) *Ann Rev Chem* 253: 7570-7578.
9. Scriba D, Aprath-Husmann I, Blum WF, Hauner H. *Eur J Endocrinol* (2000) 143:439-445
10. Snyder PB *Emerging Therapeutic Targets* (1999) 3(4): 587-599.
11. Tansey JT, Sztalryd C, Hlavin EM, Kimmel AR, Londos C. (2004) *IUBMB Life* 56(7): 379-85.



株式会社ケー・エー・シー
試薬事業部
〒661-0978　兵庫県尼崎市久々知西町2丁目1-20

（お問合せ先）
TEL：06-6435-9747　FAX：06-6435-9748
URL：http://www.kacnet.co.jp/
E-mail：cs-info@kacnet.co.jpC