3T3-L1 Adipocyte Lipolysis Assay Kit
Fluorescent Detection of Glycerol

Cat# LIP-4-L1; LIP-4-NC-L1

INSTRUCTION MANUAL ZBM0045.00

STORAGE CONDITIONS

- **96-well plate cultured 3T3-L1 preadipocytes (LIP-4-L1)** 37°C incubator
- **Fluorescent Glycerol Reagent A & Buffers:** 4°C
- **Glycerol Standard & Controls:** -20°C
- **Long-term storage: If you have received a kit without cells (LIP-4-NC-L1) remove the Fluorescent 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.**

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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- **PO Box 13888**
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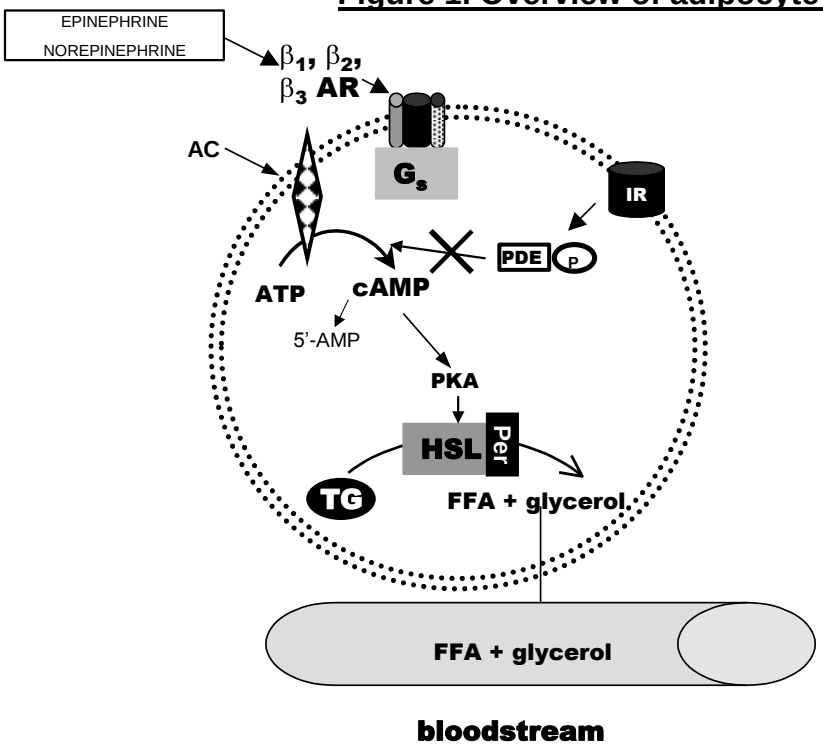
INTRODUCTION

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 1997). 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 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.

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

Assessing lipolytic activity by the measurement of glycerol released into the medium is sufficient protocol since glycerokinase activity is not present in adipocytes. 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 fluorescent product is produced by the peroxidase reaction with H_2O_2. This product has an excitation maximum at 325nm and emission maximum at 420nm with ranges of 315-340nm and 370-470nm, respectively. The increase in fluorescence 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 + Substrate ⟶ Fluorescent product + H_2O

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Adipocytes, Plate A	96 well plate 3T3-L1 preadipocytes (LIP-4-L1 ONLY)	---	PLATE	1	37°C
Blank Assay Plates	96-well white 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
Fluorescent Glycerol Reagent A	Reconstitute with 11 ml Substrate Solution prior to use.		BOTTLE	1	4°C
Substrate Solution	11 ml		BOTTLE	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl		EACH	2	-----
Glycerol standard	Glycerol @ 1mM [Reconstitute with 200 μl Wash Buffer to make the 200 μM glycerol standard; see page 6 for recommended dilution scheme]	ORANGE	50 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
- Fluorescence plate reader with excitation filter 315-340nm and emission filter 370-470nm
- Incubator at 37°C
- Large gauge needle
- Option – Step 5 of Assay Procedure: 96 well plate, blank
- Tubes for diluting glycerol standards

ASSAY PROCEDURE

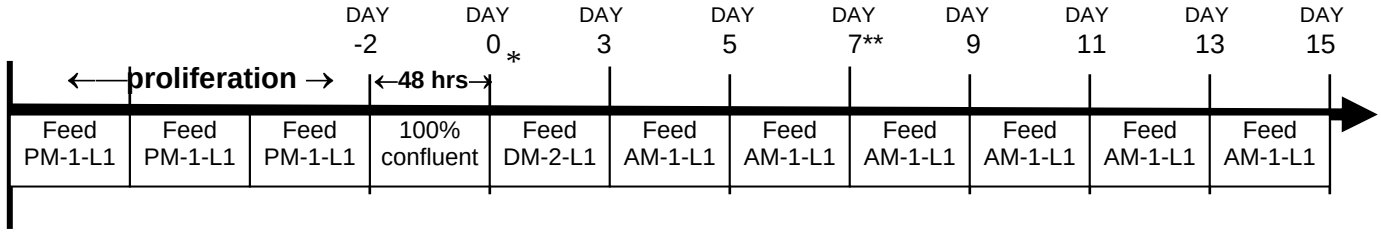
A. DIFFERENTIATION PROCEDURE

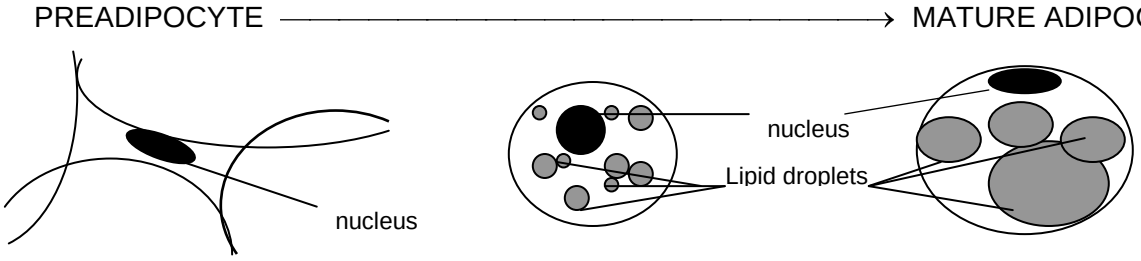
1. Preadipocytes are plated sub-confluent in 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) and shipped the next day via overnight delivery.
2. Incubate cells until they are 100% confluent (in about 4-5 days). Cells will need to be fed every other day with PM-1-L1 during this time. See Table 1 for feeding volumes.
3. Once the cells are confluent, incubate an additional 48 hours before initiating differentiation.
4. Two days after the cells have been confluent, remove the Preadipocyte Medium (cat# PM-1-L1) and replace with an appropriate volume 3T3-L1 Differentiation Medium (cat# DM-2-L1; see table 1 below for recommended volumes). Incubate for 3 days.
5. Remove the 3T3-L1 Differentiation Medium and replace with 3T3-L1 Adipocyte Maintenance Medium. Incubate for 2-3 days.
6. Feed cells every 2-3 days using 3T3-L1 Adipocyte Maintenance Medium until ready for assay. 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation (see Table 1 and 3T3-L1 Growth and Differentiation Feeding Schedule)

Table 1. Feeding Volumes

Format	Change PM-1-L1 to PM-1-L1		Change PM-1-L1 to DM-2-L1		Change DM-2-L1 to AM-1-L1		Change AM-1-L1 to AM-1-L1	
	OUT	IN	OUT	IN	OUT	IN	OUT	IN
96 well plate	90µl/well	90µl/well	150µl/well	150 µl / well	90 µl /well	120µl /well	90 µl /well	120µl /well
48 well plate	300 µl /well	300 µl /well	500µl /well	500 µl /well	300 µl /well	400 µl /well	300 µl /well	400 µl /well
24 well plate	0.6 ml/well	0.6 ml/well	1.0 ml/well	1.0 ml/well	0.6 ml/well	0.8 ml/well	0.6 ml/well	0.8 ml/well
12 well plate	1.2 ml/well	1.2 ml/well	2.0 ml/well	2.0 ml/well	1.2 ml/well	1.6 ml/well	1.2 ml/well	1.6 ml/well
6 well plate	1.8 ml/well	1.8 ml/well	3.0 ml/well	3.0 ml/well	1.8 ml/well	2.4 ml/well	1.8 ml/well	2.4 ml/well
T-75 flask	12 ml/flask	12 ml/flask	20 ml/flask	20 ml/flask	12 ml/flask	16 ml/flask	12 ml/flask	16 ml/flask
T-25 flask	4.2 ml/flask	4.2 ml/flask	7 ml/flask	7 ml/flask	4.2 ml/flask	5.6 ml/flask	4.2 ml/flask	5.6 ml/flask

3T3-L1 Growth and Differentiation Feeding Schedule





* Once the cells are 100% confluent, incubate an additional 48 hours before initiating differentiation.

** 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation

B. LIPOLYSIS 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%.

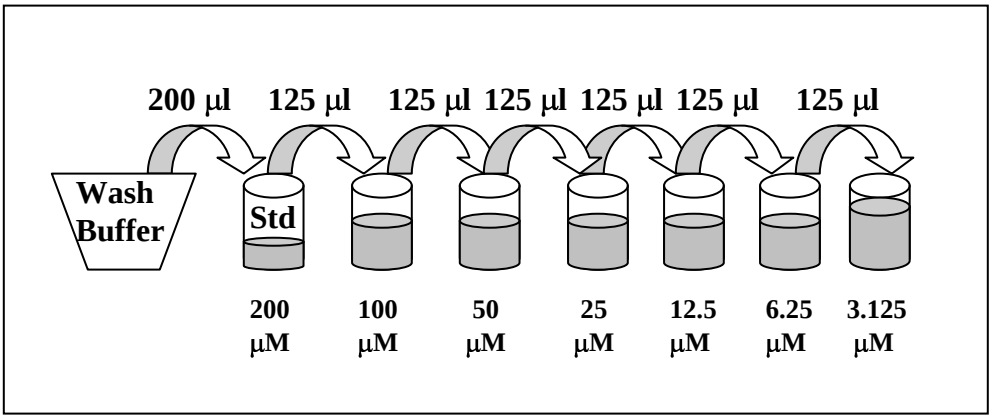
2. Remove 120 µl medium from each well. Gently add 200 µl Wash Buffer to all wells. Remove 200 µl of the media and Wash Buffer from each well and replace with another 200 µl Wash Buffer.

3. 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.

4. OPTION: to determine if the compound alone reacts with the Fluorescent 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 Fluorescent Glycerol Reagent A following the instructions in Steps 10 and 11.

5. Incubate the plates at 37^{o}C-humidified incubator for 3 hours (for time course experiments the longest time point is usually 24 hours).
6. 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 200 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 125 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.



7. Also at this time prepare the Fluorescent Glycerol Reagent A by adding 11ml room temperature Substrate Solution per bottle and gently invert. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. Store at room temperature. 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 Fluorescent Glycerol Reagent A is stable for 14 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).

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

9. Add the reconstituted Fluorescent 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 blank assay plate provided in the kit. Gently, pipet up and down once to mix. Pop the bubbles using a large gauge needle or a clean pipet tip. Protect the plate from light and incubate at 25°C (room temperature) for 10 minutes.

10. The fluorescence of each well is then measured by excitation at 315-340nm and emission at 370-470nm.

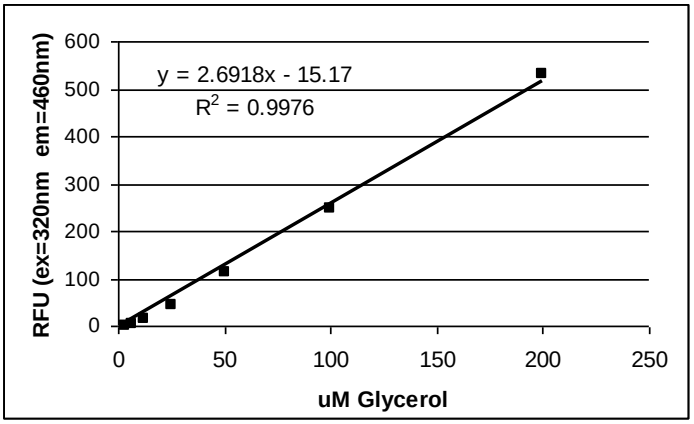
GLYCEROL STANDARD CURVE

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the RFU value of the 0μM standard from all RFU values including the standard curve.

Zero (blank) = 24.1

μM Glycerol	RFU	OD - blank
3.125	24.2	0.1
6.25	26.5	2.4
12.5	38.4	14.3
25	66.0	41.9
50	135.8	111.7
100	270.2	246.1
200	554.6	530.5



slope =	2.6918
intercept=	-15.17
r^2=	0.9976

y = observed RFU. minus the blank
x = concentration of glycerol in μM
To calculate x for each y, (i.e. to change the observed RFU into glycerol concentration) use the following equation:
y=(slope) times (x) plus intercept
y=mx+b so x=(y-b)/m
x=(y + 15.17)/2.6918 where 2.6918= slope of the line and -15.17= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

Any RFU 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

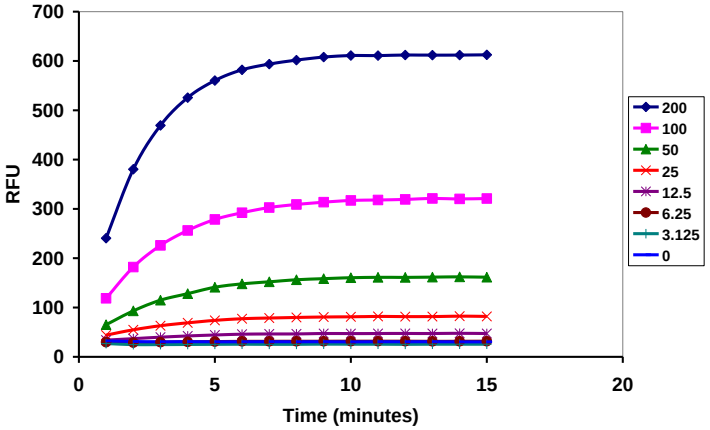
$$\text{Fold induction} = \frac{\mu\text{M glycerol SAMPLE}}{\mu\text{M glycerol VEHICLE}}$$

FLUORESENT ASSAY INFORMATION

The assays were performed using a Thermo Electron Corporation Fluoroskan Ascent FL with a 320nm excitation filter and either a 390nm or 460nm emission filter.

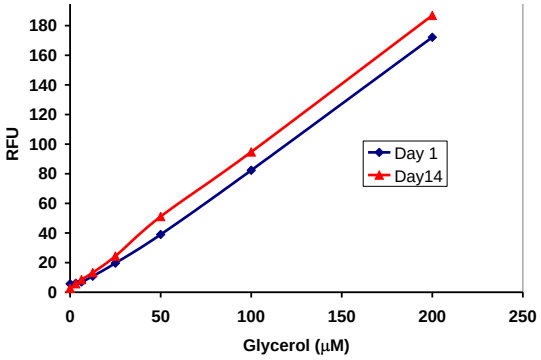
Time Course of Reaction

Fluorescent Glycerol Reagent A (50µl) was added to glycerol standards (50µl) ranging from 3µM to 200µM and fluorescence emission at 460nm followed for 15 minutes after reagent addition. The reaction is complete after 10 minutes and remains stable thereafter. We recommend reading the fluorescence signal after 10 minutes incubation at room temperature.



Stability of Reagent A

Fluorescent Glycerol Reagent A was tested for its stability at 4°C by analyzing the signal generated by glycerol standards over a two week period. 50µl of each standard was analyzed using 50µl fluorescent Reagent A. Fluorescence was determined after a 15 minute room temperature incubation using a 320nm excitation and 390nm emission filter. The reconstituted Reagent A is stable for at least two weeks at 4°C.



Emission Filters

The fluorescence signal from glycerol standards was determined at 2 emission wavelengths, 390nm and 460nm using a dual kinetic mode. The signals were determined following a 15 minute room temperature incubation using an opaque white plate. The overall signal is increased at 460nm compared to 390nm, however, both emission wavelengths work equally as well.

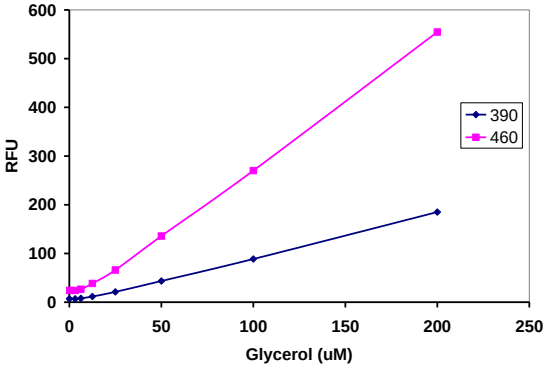
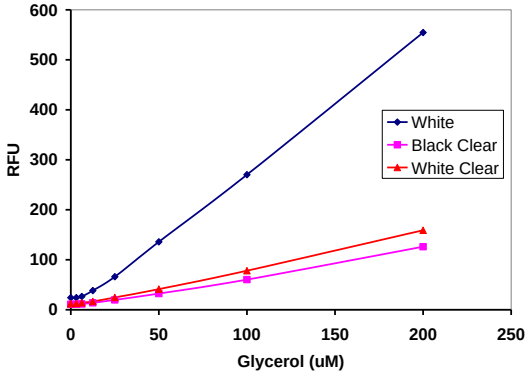


Plate Formats

We compared the fluorescence signal generated using glycerol standards in opaque white plates, white plates with clear bottom, or black plates with clear bottom. The assay was performed using 50 µl standards and reagent A and incubating for 15 minutes at room temperature. Fluorescence signal was determined using a 320nm excitation filter and a 460nm emission filter. We recommend using opaque plates to generate larger signals.

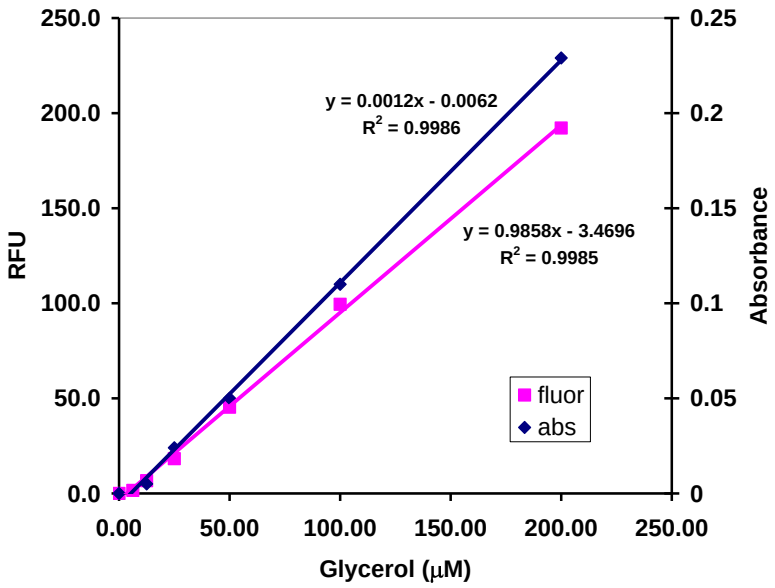


COMPARISON TO ABSORBANCE ASSAY

Our original Glycerol Reagent A (cat# RGTA) assay is an absorbance based assay that researchers have been successfully using for years. The new fluorescence based Fluorescent Glycerol Reagent A (cat# RGTA-FL) assay yields the same results with the one step add and read simplicity of the original, and expands this capability to the fluorescence format.

STANDARD CURVES

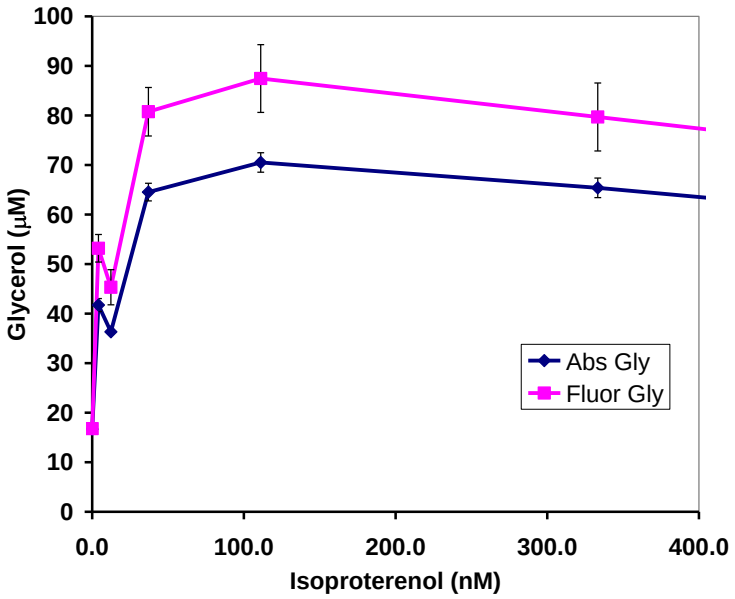
50 µl of either Glycerol Reagent A or Fluorescent Glycerol Reagent A were added to 50 µl of each glycerol standard ranging from 200µM to 3.125µM. The plates were incubated at room temperature for 15 minutes prior to measuring absorbance at 540nm or fluorescence at 390nm (excitation, 320nm). The standard curves for each assay are plotted to the right and are used for determining lipolytic response in the following assay.



DOSE RESPONSE TO ISOPROTERENOL

Mature cultured human adipocytes were treated with increasing concentrations of isoproterenol to stimulate lipolysis. Cells were incubated for 3 hours at 37°C as described in the assay procedure. 50µl aliquots of the conditioned media were transferred to two plates for the fluorescence or absorbance assay. 50µl of either Glycerol Reagent A or Fluorescent Glycerol Reagent A were added to each well and the plates incubated at room temperature for 15 minutes. The amount of glycerol released was determined by absorbance at 540nm or fluorescence at 390nm (excitation 320nm) and calculated from the standard curves above.

Both reagents show a robust 4 to 5-fold increase in glycerol release in response to isoproterenol treatment. The fluorescence assay generated a 20% higher values than the absorbance assay.



NOTE: Fluorescent Glycerol Reagent A is NOT a substitute for Glycerol Reagent A and is only to be used in the context of the Lipolysis Assay Kit cat# LIP-4-L1/LIP-4-NC-L1. These 2 reagents are NOT interchangeable.

TROUBLESHOOTING

Problem	Suggestions
High background.	• Change pipet tips frequently • Use Fluorescent 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. • 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 RFU 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. **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 250 µl assay medium with treatments per well. Remove 100 µl for each time point. Complete the assay using an equal volume Fluorescent 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 perform the assay. 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 Fluorescent Glycerol Reagent A 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

ON DAY OF ASSAY

Make all test compound dilutions in Assay Buffer.

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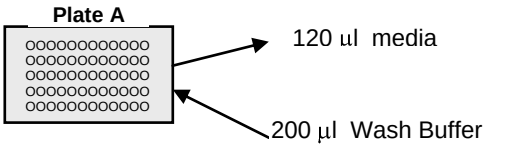
Remove 120 µl media from all wells. Add 200 µl Wash Buffer to all wells.



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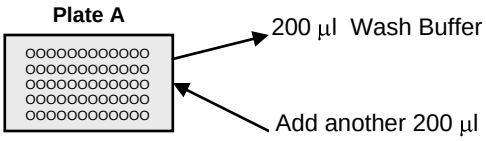
Remove 200 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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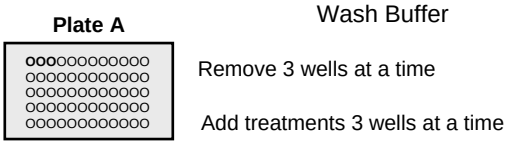
Remove all media & Wash Buffer. Add 150 µl treatments/controls to 3 wells at a time. OPTION: Add 50 µl/well compounds to a fresh plate without cells.



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Incubate 3-5 hours at 37°C.

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

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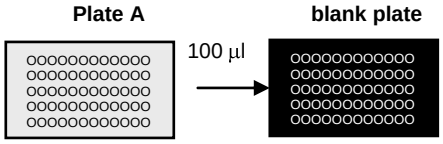
Remove 100 µl/well conditioned media from Plate A to a blank assay plate. Add 100 µl glycerol standards to empty wells



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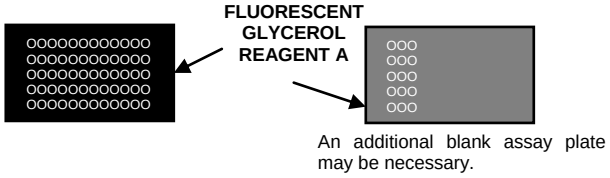
Add 100 µl/well reconstituted Fluorescent Glycerol Reagent A to the plate (including the glycerol standards at 100µl/well) and optional plate without cells.



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Protect from light and incubate at 25°C (room temperature) for 15 minutes. Pop the bubbles in each well.

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Measure the fluorescence of each well using excitation filter 315-340 nm (320 nm) and emission filter of 370-470 nm (460 nm).

REFERENCES

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2. Greenway FL, Bray GA, Heber D. Topical fat reduction Obes Res (1995) 3 Suppl 4:561S-568S.
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