ADIPOCYTE LIPOLYSIS ASSAY KIT for 3T3-L1 Cells

CAT# LIP-1-L1; LIP-1-NCL1

INSTRUCTION MANUAL ZBM-8

STORAGE CONDITIONS

- **96-well plate cultured 3T3-L1 preadipocytes** 37°C incubator
- **Glycerol Reagent A & Buffers:** 4°C
- **Glycerol Standard & Controls:** -20°C
- **Long-term storage:** If you ordered a kit with REAGENTS ONLY (cat# LIP-1-NCL1), 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.

For *in vitro* Use Only

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express 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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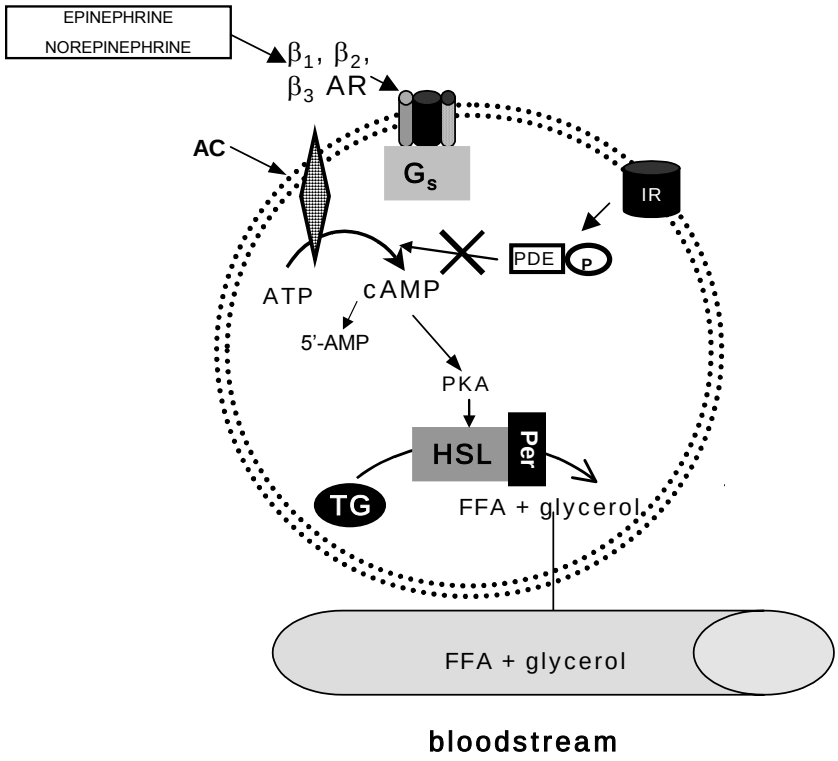
INTRODUCTION:

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 adipocytes.



ABBREVIATIONS:

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

Figure 1. Overview of adipocyte lipolysis

Principle of the assay:

Lipolytic activity is assessed by the measurement of glycerol released into the medium from triglyceride breakdown. 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-ethytl-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
Plate A	96 well plate 3T3-L1 preadipocytes	---	PLATE	1	37°C
Assay Plates, blank	96-well assay plate, blank (for samples + standards)	---	PLATE	2	-----
Preadipocyte Medium	3T3-L1 Preadipocyte Medium (cat# PM-1-L1); 50ml		BOTTLE	1	4°C
Differentiation Medium	3T3-L1 Adipocyte Differentiation Medium (cat# DM-2-L1); 15ml		BOTTLE	1	4°C
Adipocyte Medium	3T3-L1 Adipocyte Maintenance Medium (cat# AM-1-L1); 100ml		BOTTLE	1	4°C
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
	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
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)	RED	10 μl / VIAL	1	-20°C
Glycerol Reagent A	Reconstitute with 11 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 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

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

- Multi-channel pipet , single channel pipet and pipet tips
- Sterile trays for multi-channel pipetters during differentiation of cells
- 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

NOTE:

THIS KIT IS DESIGNED FOR THE ASSAY OF A 96 WELL PLATE. IF YOU WISH TO TEST ANOTHER PLATE FORMAT, PLEASE CONTACT ZEN-BIO TO PURCHASE ADDITIONAL REAGENTS TO COMPLETE YOUR STUDY.

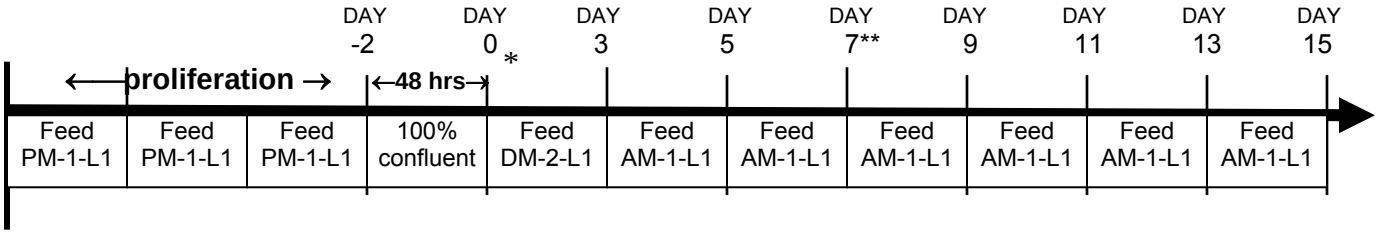
PREADIPOCYTE DIFFERENTIATION

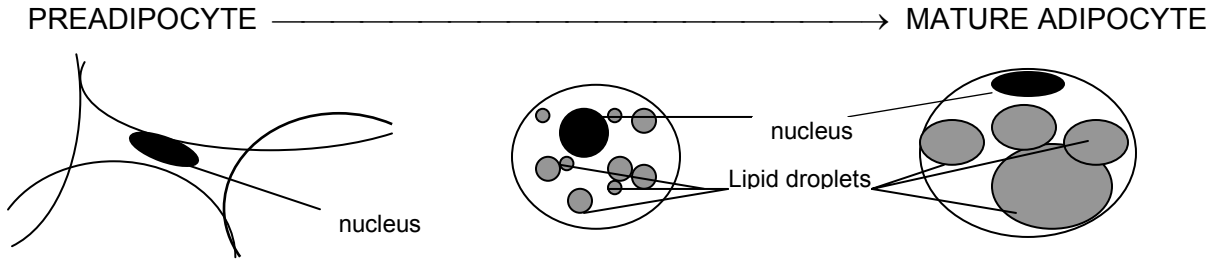
1. Preadipocytes are plated sub-confluent in 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) and shipped the next day via overnight delivery.
2. Incubate cells until they are 100% confluent (in about 4-5 days). Cells will need to be fed every other day with PM-1-L1 during this time.
3. Once the cells are confluent, 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 above 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 Figure 1 below and Figure 2. 3T3-L1 Growth and Differentiation Feeding Schedule)

Table 1. Feeding Volumes

Format	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
96 well plate	150µl/well	150 µl / well	90 µl /well	120µl /well	90 µl /well	120µl /well
48 well plate	500µl /well	500 µl /well	300 µl /well	400 µl /well	300 µl /well	400 µl /well
24 well plate	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	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	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	20 ml/flask	20 ml/flask	12 ml/flask	16 ml/flask	12 ml/flask	16 ml/flask
T-25 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

ADIPOCYTE LIPOLYSIS PROTOCOL

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 140 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 IBMX and Isoproterenol as positive controls. 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.

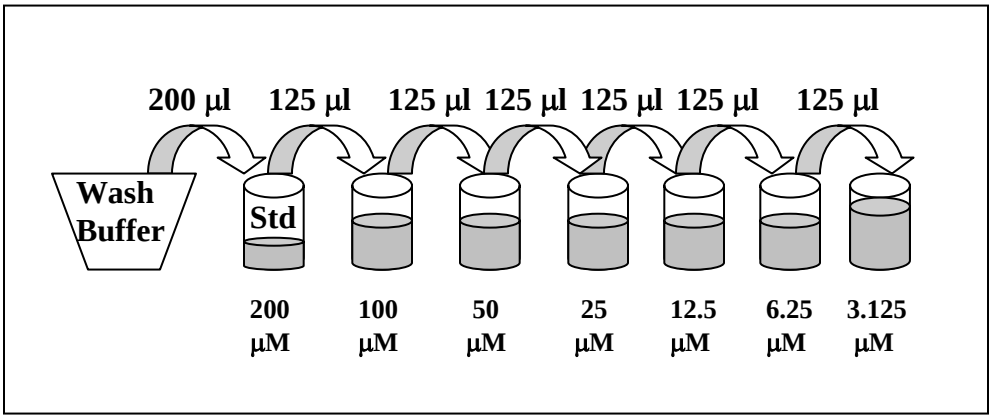
4. OPTION: to determine if the compound alone reacts with the Glycerol Reagent A, prepare a fresh plate (not included in kit) containing 100 µl of the compound. This plate can be incubated at 37^{o}C with the treated cells. When performing the assay, add 100 µl of Glycerol Reagent A following the instructions in Steps 10 and 11.

5. Incubate the plates at 37^{o}C-humidified incubator for 5 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 Glycerol Reagent A by adding 11ml room temperature deionized water per bottle and gently invert. DO NOT VORTEX! Use a pipet to insure 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 Glycerol Reagent A is stable for 60 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 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 Plate B or use Plate C for the standards.

9. 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 Plate B and Plate C (if used). 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.

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

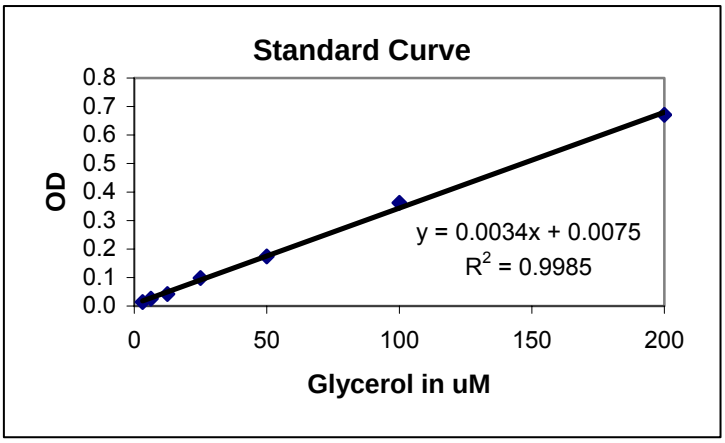
GLYCEROL STANDARD CURVE

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.

Zero
(blank) = .040

μM Glycerol	OD	OD - blank
3.125	0.054	0.014
6.25	0.066	0.026
12.5	0.082	0.042
25	0.138	0.098
50	0.214	0.174
100	0.402	0.362
200	0.711	0.671



slope =	0.0034
intercept=	0.0075
r^2=	0.9985

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 – 0.0075)/0.003 where 0.003= slope of the line and 0.0075= 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 conditioned 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.	• Use clean tray and tips • Change pipet tips frequently • Use Glycerol Reagent A before the expiration date
No response to either positive control	• Visually observe adequate differentiation of the cultured adipocytes prior to 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.

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. We do not recommend modification of the 100 µl required for the assay.

2. **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 or more kits of the same lot number. You may then use one plate that includes the blank, vehicle(s), and positive and negative controls. Additional plates may then be used for the assay of the remainder of your samples. In order to obtain comparable data, all plates must be assayed on the same day using kits from the same lot number. Plate C is provided for the assay of glycerol standards.

3. **I do not have time to pop the bubbles and read the plate. Can I freeze the conditioned media in PLATE B? How long can I store the samples before I complete the assay?** Yes. The conditioned media in PLATE B can be immediately stored at -80°C for a maximum of 7 days. Bring the conditioned media in PLATE B to room temperature BEFORE adding the Lipolysis Reagent 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 : Protocol Flowchart

ON DAY OF ASSAY

Plate A = plate of mature 3T3-L1 adipocytes

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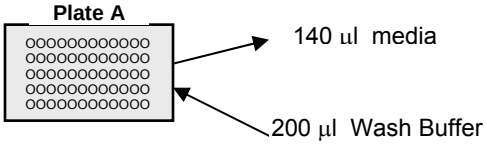
Remove 140 µl media from all wells.
Add 200 µl Wash Buffer to all wells.



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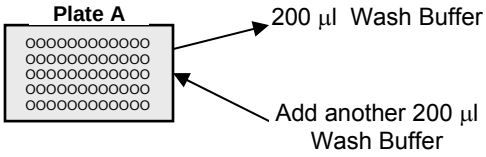
Remove 200 µl media & Wash Buffer. Add another 200 µl Wash Buffer to all wells.



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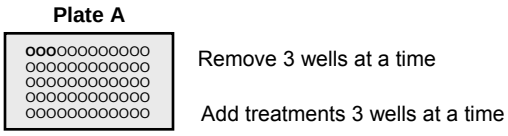
Remove all media & Wash Buffer. Add 150 µl treatments/controls to 3 wells at a time. OPTION: Add compounds to a fresh plate without cells.



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Incubate 5-24 hours at 37°C.

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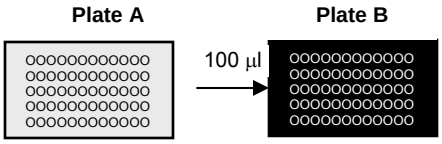
One hour prior to assay, reconstitute Glycerol Reagent A and prepare standards. Keep all at room temp.



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Remove 100 µl/well conditioned media from Plate A to Plate B.

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Add 100 µl/well reconstituted Glycerol Reagent A to Plate B (including the glycerol standards at 100µl/well in the blank assay plate(s) and optional plate without cells.

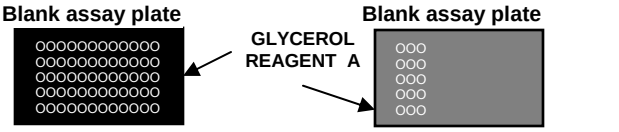

Plate C may be necessary for the assay of glycerol standards if al 96 wells of Plate A are used.

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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.

References:

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