Human Adipocyte Lipolysis Assay Kit -Visceral

CAT# LIP-1-OPF

INSTRUCTION MANUAL ZBM0050.01

STORAGE CONDITIONS

- **1 vial omental human preadipocytes, 1.0 X10^6 cells/vial:** liquid nitrogen (LIP-1-OPF)
- **Glycerol Reagent A, media & Buffers:** 4°C
- **Glycerol Standard & Controls:** -20°C

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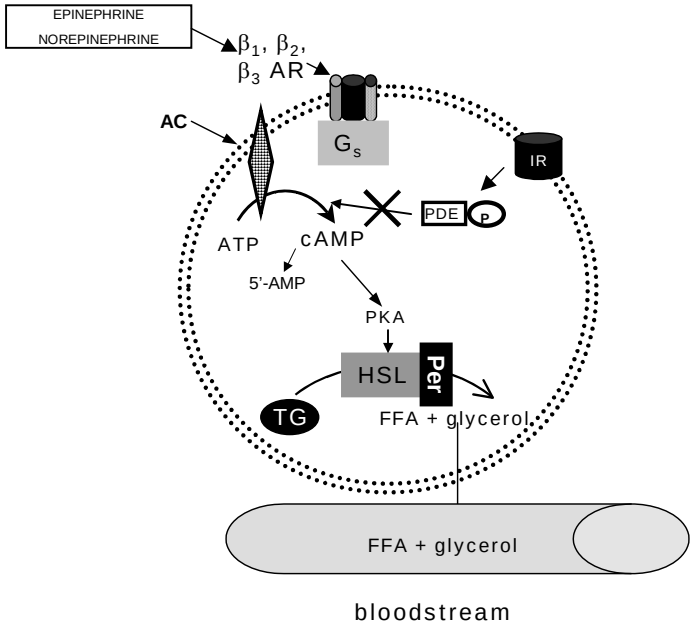
INTRODUCTION

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
AMP	adenosine monophosphate
ATP	adenosine triphosphate
IR	insulin receptor
PDE	phosphodiesterase
Per	perilipins

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 : LIP-1-OPF

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Cells	Cryopreserved human omental preadipocytes, $1.0X10^6$ cells/vial	---	VIAL	1	Liquid nitrogen
Blank Plates	Blank 96 well format plates for plating and/or assay	---	EACH	3	Room temp
Omental Preadipocyte Medium	Preadipocyte Medium, 50.0 ml	CLEAR	BOTTLE	1	4°C
Omental Differentiation Medium	Adipocyte Differentiation Medium, 25.0 ml	CLEAR	BOTTLE	1	4°C
Omental Adipocyte Medium	Adipocyte Maintenance Medium, 25.0 ml	CLEAR	BOTTLE	1	4°C
LIP1 Assay Buffer	100 ml	---	BOTTLE	1	4°C
Wash Buffer	50 ml	---	BOTTLE	1	4°C
Vehicle	0.1% DMSO in LIP1 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
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 (cat# RGTA-10)	Reconstitute with 11.0 ml deionized water prior to use.	BLACK	BOTTLE	1	4°C
Tray, non-sterile	For multi-channel pipetters, clear, non-sterile	---	EACH	2	-----
Glycerol standard	Glycerol @ 1mM [Reconstitute with 400 μl Wash Buffer to make the 200 μM glycerol standard; see page 7 for recommended dilution scheme]	ORANGE	100 μl / VIAL	1	-20°C

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

- Multi-channel pipet , single channel pipet and pipet tips; Tubes for diluting glycerol standards
- Sterile trays for multi-channel pipetters during plating & 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 (100 ASSAY POINTS). YOU MAY USE THIS KIT TO ASSAY A SINGLE 384 OR -96 WELL PLATE OF HUMAN ADIPOCYTES. YOU MUST THEN TRANSFER THE CONDITIONED ASSAY BUFFER TO A 96 WELL FORMAT TO COMPLETE THE ASSAY.

A. DIFFERENTIATION PROCEDURE

Please note: Primary cells can be very sensitive to brands of cultureware. Zen-Bio does not currently recommend the use of Falcon brand plates or flasks. Our scientists are using Nunc, Costar/Corning, or Greiner bio-one Cellstar tissue culture treated plates and flasks. Please contact us if you have any questions.

1. Remove cells from liquid nitrogen and place immediately into a 37° C water bath and agitate while in bath. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed. Rinse the vials with 70% ethanol before taking them to the culture hood.
2. Upon thawing, transfer the cells to a sterile conical bottom centrifuge tube containing 10 ml of Preadipocyte Medium (cat # PM-1). Centrifuge: 1,200 rpm (282 X g) / 20°C / 5 minutes. Aspirate the supernatant. TAKE CARE TO NOT ASPIRATE ANY OF THE CELL PELLET.
3. The cell vial contains a minimum of 2.0 x 10^6 viable cells; however, we recommend performing a cell count to determine a more exact number of cells. Resuspend the cell pellet in 2 ml Preadipocyte Medium; dilute an aliquot in 0.4% trypan blue solution. We suggest withdrawing an aliquot of 50 µl of cells and mixing with 100 µl of the trypan blue solution, resulting in a dilution factor of 3. Count live (unstained) cells on a hemacytometer.
4. Plate approximately 40,625 cells / cm^2 using the media volumes from the table below. Refer to the manufacturer's specifications for the specific cultureware brand you are using. See example below.

Table 1. Plating Guide

Brand	Plate Format	Cm^2 per well	Cells per cm^2	Cells per well	Total # Cells per plate	Total # cells for plating	Volume media per well	Total volume for plating
Costar	384	0.06	40,625	2,275	873,600	9.1 x 10^5	30 µl	12.0 ml
Costar	96	0.32	40,625	13,000	1,248,000	1.3 x 10^6	150 µl	15.0 ml

***We recommend preparing slightly larger volumes to allow for loss due to foam and pipet error.**

5. Plate cells in desired format and place in a humidified 37°C incubator with 5% CO_2. Do not agitate the plate, as cells will not plate evenly.
6. Twenty-four hours after plating, check the plates for confluence. If they are not completely confluent, leave for and additional 24 hours maximum before inducing differentiation. If the cells are not confluent after 48 hours, DO NOT INDUCE DIFFERENTIATION (differentiation will be poor). Contact Zen-Bio immediately.

7. To start the differentiation process, aspirate the entire volume of Preadipocyte Medium from all wells. Add the appropriate volume of Adipocyte Differentiation Medium (catalog # DM-2) to the wells (see Table 1. Feeding Volumes). Incubate plate for 7 days at 37^0C and 5% CO_2.
8. After 7 days, cells should be fed by removing some of the media and replacing with fresh Adipocyte Medium (catalog # AM-1) (See Table 2. Feeding Volumes). **Caution**: **Do not dry the wells. Add new medium gently. If using an automatic feeder, set the slowest flow rate possible.**
9. Two (2) weeks after the initiation of differentiation, cells should appear rounded with large lipid droplets apparent in the cytoplasm. Cells are now considered mature adipocytes and are suitable for the lipolysis assay.

Table 2. Feeding Volumes

FORMAT	PLATING	CHANGE PM-1 TO DM-2		CHANGE DM-2 TO AM-1	
PLATE	IN	OUT	IN	OUT	IN
384 well	30 µl/well	30 µl/well	30µl/well l	18 µl/well	24 µl/well
96 well	150 µl/ well	150 µl/ well	150µl/ well	90 µl/ well	120µl/ well

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B. LIPOLYSIS PROCEDURE

1. Make a stock solution using the appropriate vehicle for your test compounds. Dilute your vehicle and compound 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 the 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 0.1%.

Table 3. Lipolysis Assay Volumes

FORMAT	REMOVE MEDIA TO GENTLY ADD WASH BUFFER		CHANGE WASH BUFFER		CHANGE WASH BUFFER TO ASSAY BUFFER	
PLATE	**OUT**	**IN**	**OUT**	**IN**	**OUT**	**IN**
384 well	20 µl/ well	30µl/ well	30µl/ well	30µl/ well	All media	20 µl/ well
96 well	120 µl/ well	200µl/ well	200µl/ well	200µl/ well	All media	150 µl/ well

2. Remove Adipocyte Maintenance medium from each well. Gently add Wash Buffer to all wells. Remove some of the media and Wash Buffer from each well and replace with more Wash Buffer. See Table 3 for volumes.

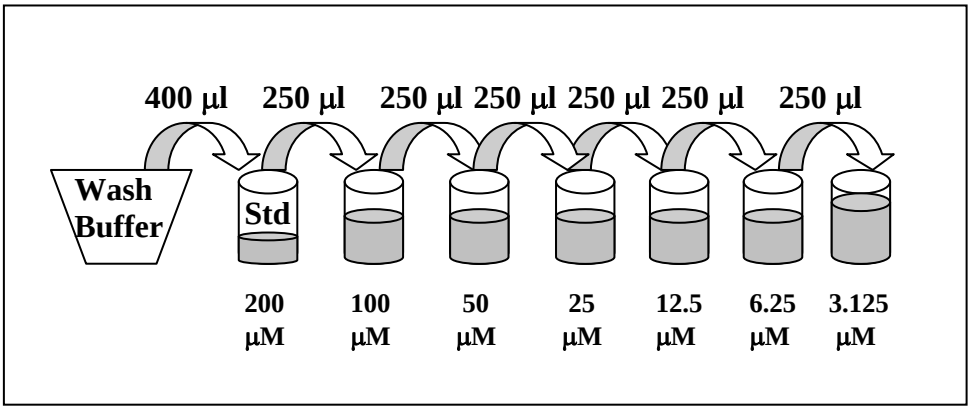
3. Remove all the media and Wash Buffer from the cells from triplicate wells. Treat the cells with 150 µl of the test compounds resuspended in Assay Buffer three (3) wells at a time. [NOTE: for formats other than 96 wells, see column "CHANGE WASH BUFFER TO ASSAY BUFFER" in Table 3 for correct volume]. 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 Glycerol Reagent A, prepare a fresh 96 well plate (not included in kit) containing 50 µl of the compound dilution. 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.

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 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.

7. 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).

8. At the end of the incubation, 100 µl of the conditioned media is removed and transferred to the corresponding well of a blank 96 well plate. [This is most easily accomplished using a multi-channel pipet for 96 and 384 well formats] Add 100 µl of each glycerol standard to any remaining empty wells in one of the blank 96 well assay plates.

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 the 96 well 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.

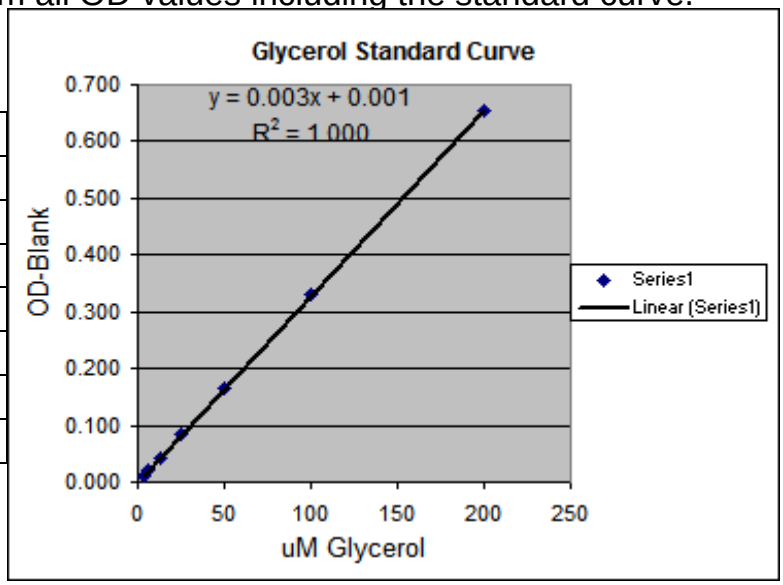
10. The optical density of each well is then measured at 540 nm.

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.

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

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

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. **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. **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. A REAGENTS ONLY kit is available cat# LIP-1-NC. Contact ZenBio to order additional cells or media.

3. **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.

4. **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.

5. **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 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
96 WELL FORMAT

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

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

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

Incubate 3 hours at 37°C.
(OPTION: 5-24 hours using IBMX control)

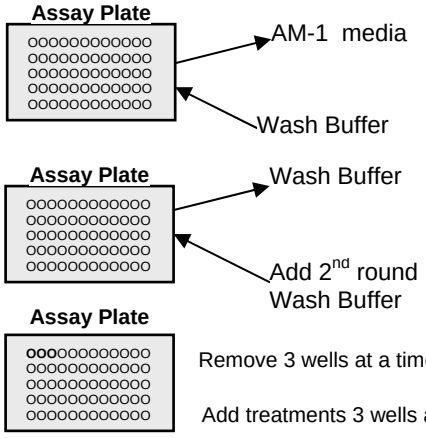
One hour prior to assay, reconstitute Glycerol Reagent A and prepare standards. Keep all at room temp.

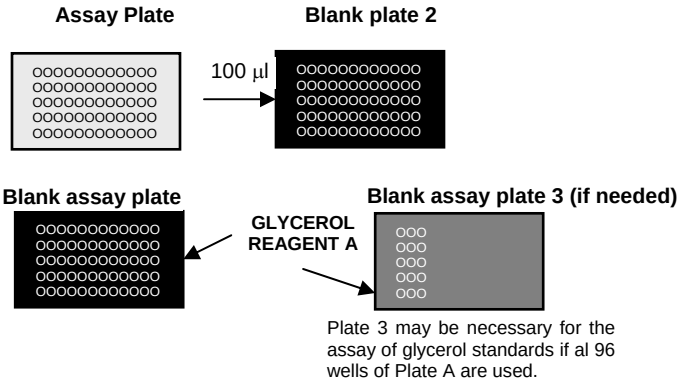
Remove 100 µl/well conditioned media from Plate A to Plate B. Add 100 µl glycerol standards to empty wells

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.

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.





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