Human Adipogenesis Assay Kit- Glucocorticoid Analogues, 100 point assay kit

Cat#: DIF-GLUC, DIF-GLUC-NC

INSTRUCTION MANUAL ZBM0004.07

STORAGE CONDITIONS

Cryopreserved Subcutaneous Human Preadipocytes

Store in liquid nitrogen IMMEDIATELY upon receipt. No expiration date is applicable; however, the cells must be plated within 1 week of receiving the kit to account for the expiration of the kit components.
Any other storage negates the warranty.

Media, Reagent A, Buffers:

Store at 2 - 8°C. See kit label for expiration date. **Use reconstituted Glycerol Reagent A within 7 days.**

Glycerol Standard & Reagent B Solution:

-20°C New Reagent B Storage Condition.

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ORDERING INFORMATION AND TECHNICAL SERVICES

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- **Durham NC 27713**
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INTRODUCTION

The differentiation assay kits provide the tools to study the compounds that stimulate human adipocyte differentiation or lipogenesis. Such compounds may be PPARγ agonists or a combination of thiazolidinediones and glucocorticoids that are potentially useful in the treatment of diabetes.

This kit is designed to test compounds as potential glucocorticoid analogues, with dexamethasone at 1.0 μM serving as the positive control. This kit contains sufficient reagents to assay 100 assay points in a 96 well format.

ITEMS INCLUDED IN THE KIT

Item	Description	Unit	Volume/ Unit	Quantity	Storage
Cells	Cryopreserved human subcutaneous preadipocytes, 2 million cells/vial	VIAL	---	1	Liquid Nitrogen
PM-1	Preadipocyte Medium	BOTTLE	50 mL	2	4°C/-20°C
DPC	Differentiation, Positive Control	VIAL	1 mL	2	-20°C
DNC	Differentiation, Negative Control	VIAL	1 mL	2	-20°C
DVC	Differentiation, Vehicle Control	VIAL	1 mL	2	-20°C
DM-2	Adipocyte Differentiation Medium	BOTTLE	300 mL	3	4°C/-20°C
MMPC	Maintenance Medium for Positive Control	TUBE	3 mL	2	-20°C
MMNC	Maintenance Medium for Negative Control	TUBE	3 mL	2	-20°C
MMVC	Maintenance Medium for Vehicle Control	TUBE	3 mL	2	-20°C
AM-1	Adipocyte Maintenance Medium	BOTTLE	300 mL	4	4°C/-20°C
Wash Buffer		BOTTLE	50 mL	2	Ambient
Lysis Buffer		BOTTLE	15 mL	2	Ambient
Glycerol Reagent A (cat# RGTA-10)	Reconstitute w/ 11.0 mL deionized water prior to use. **Use reconstituted reagent within 7 days.**	BOTTLE	11 mL	2	4°C
Reagent B	2.5 mL Reagent B Solution	BOTTLE	2.5 mL	2	-20°C
Glycerol Standard	Glycerol @ 1mM [Reconstitute with 400 μL Standards Diluent to make the 200 μM glycerol standard; see page 5 for recommended dilution scheme]	VIAL	100 μL	2	-20°C
Standards Diluent		BOTTLE	2 mL	1	Ambient
Tray	Clear polyvinyl tray for multi-channel pipettes	EACH	---	3	---
Assay Plate, blank	96-well assay plates, blank	PLATE	---	3	---

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

- Single-channel pipette
- Plate reader with a filter of 540 nm
- Multi-channel pipette
- Tubes to dilute standards

ASSAY PROCEDURE

We strongly recommend testing all compounds in triplicate

A. DIFFERENTIATION PROCEDURE

On each day of the procedure, the appropriate medium must be warmed to 37° C prior to use.

Note: This protocol is designed to accommodate a weekday work schedule. Any deviation from the recommended start day of Monday-Thursday may require weekend work.

Day 1: This is the day the cells are plated.

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 for more than 1 minute. 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).

3. Centrifuge: 1,200 rpm (282Xg) / 20°C / 5 minutes. Aspirate the supernatant. TAKE CARE TO NOT ASPIRATE ANY OF THE CELL PELLET.

4. The cell vial contains a minimum of 2 million 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, and dilute an aliquot of that mixture in trypan blue and count live (unstained) cells on a hemacytometer. The cell concentration required for approximately 40,000 cells / cm^2 in the 96 well format with 150 μL /well is 1,300,000 cells in 15 mL Preadipocyte Medium.

5. Plate cells in one of the 96 well plates provided in the kit. Ensure the plate is on a level surface directly after plating cells, being careful not to agitate the plate, otherwise the cells will not plate evenly.

6. Place plate in 37°C incubator, 5% CO_2, 97% humidity. The cells will be maintained in the incubator after each manipulation until Day 14.

GUIDE TO REAGENTS

Treatment Reference Table

Well type	# of wells	Differentiation reagent	Maintenance medium
Positive control	3	DPC	MMPC
Negative control	3	DNC	MMNC
Vehicle control	3	DVC	MMVC
Treatment compounds	87*	DM-2	AM-1

***Include any necessary solvent controls as treatments (see Note below).
See Appendix A for description of reagents

NOTE: Included in this kit are sufficient volumes of Adipocyte Differentiation Medium (cat # DM-2) and Adipocyte Maintenance Medium (cat # AM-1), based on using 10 mL of each medium per compound dilution for a maximum of 29 compounds tested in triplicate (87 wells remaining on a 96-well plate after accounting for 9 control wells). If a compound stock is too concentrated to accomplish the desired dilution, use an appropriate solution (not supplied) to prepare an intermediate concentration that would allow for a final volume of 10 mL.

Also the positive control in this kit, dexamethasone, has a final solvent concentration of 0.005% ethanol. This low concentration does not affect the differentiation of adipocytes so the ethanol is not included in the vehicle control. If the concentration of any solvent for the compounds used is high enough to potentially alter differentiation, please include the solvent alone as a treatment. We do not recommend treating the cells with solutions exceeding 1% of any solvent, as higher concentrations may be toxic to the cells.

Day 2:

Begin the differentiation procedure using the 4 types of Differentiation reagents (see summary chart above and Appendix A). Plan to do all treatments and controls in triplicate. A blank plate map is included in these instructions to record the well treatments.

Using the Adipocyte Differentiation Medium (cat # DM-2), prepare treatments. Refer to the note above when preparing compound solutions.

When all treatments are prepared, remove Preadipocyte medium from control wells. We recommend doing the treatments in small groups so the cells do not dry out. Pipet 150 μL each Differentiation Positive Control, (DPC), Differentiation Negative Control (DNC), and Differentiation Vehicle Control (DVC) into appropriate wells. Remove media from experimental wells and pipet 150 μL each Adipocyte Differentiation Medium (cat # DM-2) containing compounds into appropriate wells.

Day 8:

Prepare the treatments using Adipocyte Maintenance Medium (cat # AM-1) to dilute the appropriate compound. Using a multi-channel pipette remove media from all wells. Gently feed all wells with 150 μL of the appropriate Maintenance Medium (see Treatment Reference Table) that is provided with this kit.

Day 15:

Cells are now mature. Proceed to part B. The positive control wells should exhibit significantly greater lipid accumulation than the negative control wells or the vehicle control wells. Refer to page 11 for a picture of a typical positive control when the adipocytes are mature.

B. TRIGLYCERIDE ASSAY

1. Warm the Wash Buffer and Lysis buffer in a 37ºC water bath.

2. Thaw Reagent B Solution to room temperature and gently invert. DO NOT VORTEX! Reagent B can be refrozen and stored at -20°C. Avoid repeated freeze/thaw cycles.

3. Remove all media. Using about 15 mL of the Wash Buffer, wash the cells one time with 150 µL Wash Buffer. Label the disposable tray *"Wash Buffer"* and retain for later use.

4. Remove all Wash Buffer. Using a new tray, add 15 µL Lysis buffer. Incubate at 37ºC – 50ºC for 20 minutes.

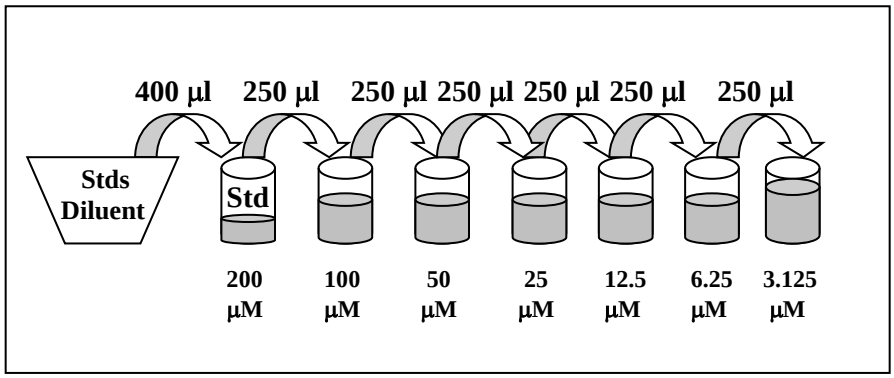
5. After the incubation is complete, visually confirm cell lysis by checking the wells under a microscope. If cells are not fully lysed, incubate for another 10 minutes.

6. Add 135 µL warm Wash Buffer and mix the lysates by pipetting up and down three times.

7. Add 20 µL Reagent B to each well. It is not necessary to mix at this time, however, gently tap the plate to help mix the reagents. Incubate the plate at 37ºC for 2 hours.

8. Bring Reagent A and the glycerol standards to room temperature during this time. The Wash Buffer can also be kept at room temperature at this point. Warm the Standards Diluent to 37°C. Prepare the standard curve as follows:

   Pipette 400 µL of the Standards Diluent 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 Diluent 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 Diluent 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 Reagent A by adding 11.0 mL deionized water per bottle and gently inverting. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. Keep at room temperature when in use. 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 7 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).

10. To a blank 96 well plate, add 80 µL Wash Buffer to each well needed for the assay (NOTE: do not add Wash Buffer to the wells used for the standard curve).

11. Working with one row or column at a time, mix the lysates very well using a multi-channel pipet. Immediately transfer 20 µL per well of the lysates to the corresponding well of the plate containing the Wash Buffer. **This results in a Dilution Factor of 5.**

12. Prepare the standard curve. Pipet 100 µL of each standard into a well. (NOTE: Eight wells are necessary for the curve. If there are remaining wells on the assay plate, you can utilize the remaining wells. If not, a second plate is included in this kit).

13. Using the third tray, add 100 µL Reagent A to samples and standards. Mix by pipetting up and down one time. Incubate at room temperature for 15 minutes.

14. Read at 540 nm using a microtiter plate reader.

GLYCEROL STANDARD CURVE

This kit is designed to show relative lipid accumulation of experimental treatments compared to controls. The assay is based on the equation

1 M Triglyceride yields 1M glycerol + Free Fatty Acids

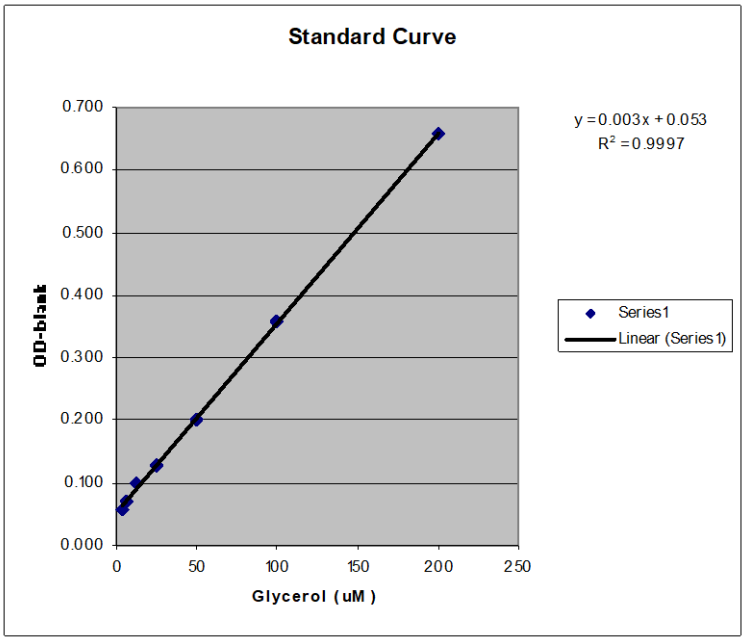
The reagent measures the concentration of glycerol released after lysing the cells and hydrolyzing the triglyceride molecules. The triglyceride concentration can then be determined from the glycerol values.

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 0 μM standard from all OD values including the standard curve.

Glycerol (uM)	OD	OD	OD-blank	OD-blank	Avg OD-blank
0	0.048	0.048			0.048
3.125	0.059	0.058	0.011	0.01	0.059
6.25	0.07	0.07	0.022	0.022	0.070
12.5	0.098	0.098	0.05	0.05	0.098
25	0.127	0.13	0.079	0.082	0.129
50	0.2	0.205	0.152	0.157	0.203
100	0.353	0.362	0.305	0.314	0.358
200	0.649	0.667	0.601	0.619	0.658

slope	0.003
intercept	0.053
R^2	0.9997



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 – 0.0006)/0.0014 where 0.0014= slope of the line and 0.0006= 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.

Solve for the Total Glycerol concentration (i.e. total triglyceride concentration) for each OD. **Remember to include the Dilution Factor in the equation**.
Data is expressed as μM Glycerol.

FREQUENTLY ASKED QUESTIONS

1. **Can I buy the reagents separately?** The only reagents sold separately are Glycerol Reagent A (cat# RGTA-10) and the glycerol standard for the Triglyceride Assay kit (cat# TG-GLYSTAN).
2. **Can I use another plate format besides 96 well?** This kit is designed for the assay of A 96 well plate (100 assay points). We do not have a protocol for other formats.
3. **Can I use this kit to measure total triglyceride in other cell lines and other human and non-human cells?** Yes. The assay is not species specific. As long as the sample concentration is in the linear range, this kit should be able to detect it.
4. **My cells did not lyse. What can I do?** If cells are not fully lysed, incubate for another 10 minutes at 37°C - 50°C. Sometimes mixing by pipetting up and down several times is necessary for full lysis.
5. **I do not have time to complete the assay. Can I freeze the samples?** Yes. The cell lysates can be stored at -80°C for a maximum of 7 days. Mix the thawed lysates in the plate by pipetting up and down several times. Allow all reagents and samples to reach room temperature BEFORE adding the Wash Buffer and Glycerol Reagent A to complete the assay.

TROUBLESHOOTING

Problem	Suggestions
High background or the triglyceride reagent turns a darker color before the assay begins.	Use clean tray and tips Change pipet tips frequently
Edge effects	Ensure a saturated humidity in the incubator to prevent evaporation from the outside wells
Inconsistent OD reading	Be careful when pipetting to avoid bubbles. If bubbles persist, burst the bubbles using a large gauge needle prior to reading and read the plate again. Mix the lysates well before transferring the 10 μL to the Wash Buffer plate.

REFERENCES

1. Green, H. and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. *Cell* 1, 113-116.
2. Hauner, H., et al., (1989) *J. Clin. Invest*. (84), 1663-1670.
3. Kuri-Harcuch W, Wise LS, Green H. (1978) Interruption of the adipose conversion of 3T3 cells by biotin deficiency: differentiation without triglyceride accumulation. *Cell* **14:**53-58.

APPENDIX A: COMPOSITION OF REAGENTS

Reagent	Components
Preadipocyte Medium (Catalog # PM-1)	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Penicillin • Streptomycin • Amphotericin B
Differentiation, Positive Control (DPC) agonist, dexamethasone	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate • Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B • 3-isobutyl-1-methylxanthine (IBMX) • PPARγ agonist • Dexamethasone
Differentiation, Negative Control (DNC) agonist, dexamethasone, TNF-α	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate • Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B • 3-isobutyl-1-methylxanthine (IBMX) • PPARγ agonist • Dexamethasone • TNF-α
Differentiation, Vehicle Control (DVC) agonist, no dexamethasone	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate • Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B • 3-isobutyl-1-methylxanthine (IBMX) • PPARγ agonist
Adipocyte Differentiation Medium (Catalog # DM-2) *(continued 1/2)*	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate

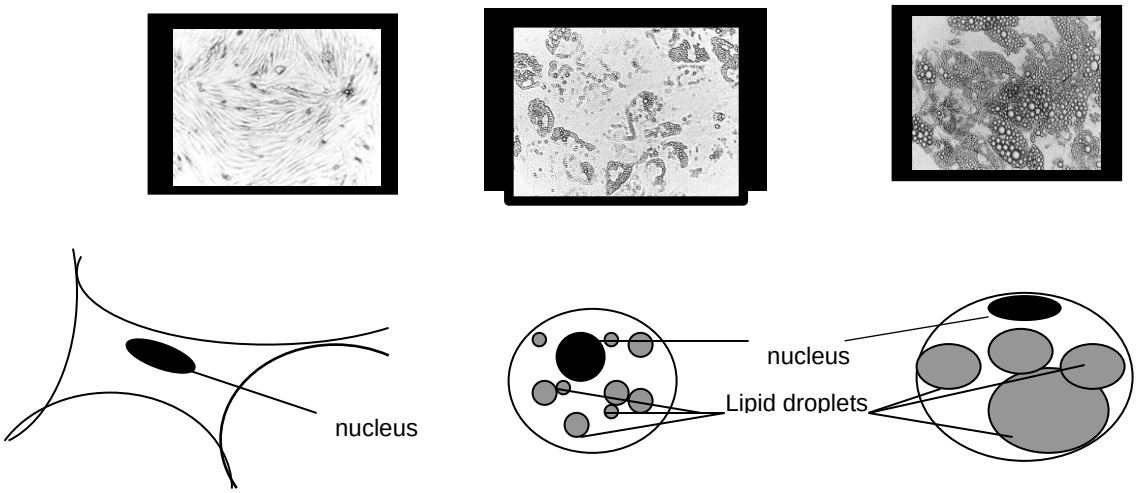
Adipocyte Differentiation Medium (Catalog # DM-2) *(continued 2/2)*	• Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B • 3-isobutyl-1-methylxanthine (IBMX) • PPARγ agonist • Compound
Maintenance Medium Positive Control (MMPC) dexamethasone	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate • Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B • Dexamethasone
Maintenance Medium Negative control (MMNC) dexamethasone TNF-α	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate • Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B • Dexamethasone • TNF-α
Maintenance Medium Vehicle control (MMVC) no dexamethasone	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate • Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B
Maintenance Medium Treatment Catalog # (AM-1)	• DMEM/ Ham's F-12 (1:1, v/v), 3.15 g/L (17.5 mmol/L) D-glucose • HEPES pH 7.4 • Fetal Bovine Serum (FBS; USA Origin) • Biotin • Pantothenate • Human Insulin, recombinant • Penicillin • Streptomycin • Amphotericin B • Compound

APPENDIX B: PLATE LAYOUT _____

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

APPENDIX C: DIFFERENTIATION PICTURES

PREADIPOCYTE ——————————————————→MATURE ADIPOCYTE



APPENDIX D: DIFFERENTIATION FLOWCHART

DAY 1
PLATE CELLS.

↓

INCUBATE 24 HOURS 37°C

↓

DAY 2
APPLY TREATMENTS
AND CONTROLS
INCUBATE 7 DAYS 37°C

↓

DAY 8
CHANGE CELLS TO APPROPRIATE
MAINTENANCE MEDIUM.
INCUBATE 7 DAYS 37°C

↓

DAY 15
FEED CELLS APPROPRIATE
MAINTENANCE MEDIUM

↓

MOVE ON TO
TRIGLYCERIDE ASSAY PROTOCOL

APPENDIX E: TRIGLYCERIDE ASSAY FLOWCHART__

Thaw Reagent B Solution. Remove all media from wells.

↓

Wash with 150 µl Wash Buffer Remove all Wash Buffer from wells and add 15 µl Lysis Buffer.

↓

Incubate 20 minutes at 37 – 50ºC

↓

Verify lysis and add 135 µl warm Wash Buffer. Mix by pipetting up and down 3 times.

↓

Add 20 µl Reagent B and incubate 2 hours at 37 ºC.

↓

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

↓

Add 80 µl Wash Buffer to a new plate. Mix lysates and transfer 20 µl lysates to the wells containing Wash Buffer.

↓

Transfer 100 µl of each standard to a new plate.

↓

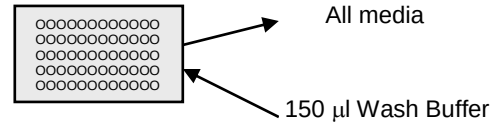
Add 100 µl Reagent A to samples and standards.

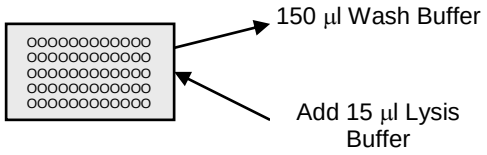
↓

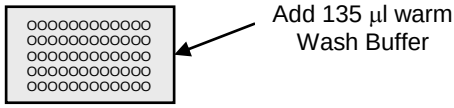
Incubate 15 minutes at 25ºC (room temperature). Pop the bubbles in each well5

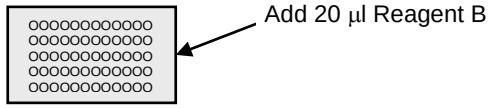
↓

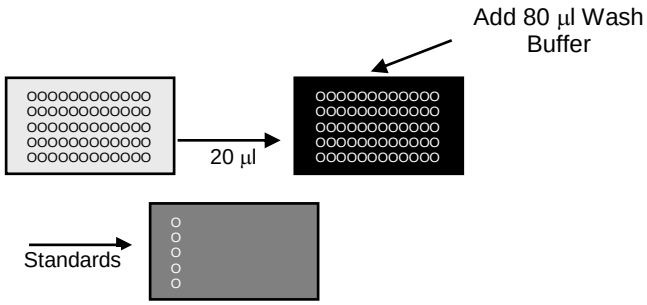
Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

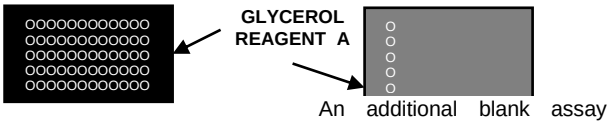












An additional blank assay plate may be necessary for the assay of glycerol standards.