Cultured Human Adipocyte Differentiation Assay Kit-Glucocorticoid Analogues

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

INSTRUCTION MANUAL ZBM0004.01

STORAGE CONDITIONS

Frozen 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.

Media, Reagents A & B, Buffers:

Store at 2 - 8°C. See kit label for expiration date

Glycerol Standards

-20°C

For Research Use Only
Not For Use In Diagnostic 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

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	Quantity	Item	Storage
Human Preadipocytes Frozen vial	Human subcutaneous preadipocytes	VIAL	1	1	Liquid nitrogen
PM-1	Preadipocyte medium (See Appendix A)	BOTTLE	50ml	2	4°C
DPC	Differentiation, Positive Control (See Appendix A)	VIAL	1 ml	2	4°C
DNC	Differentiation, Negative Control (See Appendix A)	VIAL	1 ml	2	4°C
DVC	Differentiation, Vehicle Control (See Appendix A)	VIAL	1 ml	2	4°C
DMT	Differentiation Medium for Treatments (See Appendix A)	BOTTLE	300 ml	3	4°C
MMPC	Maintenance Medium for Positive Control (See Appendix A)	TUBE	3 ml	2	4°C
MMNC	Maintenance Medium for Negative Control (See Appendix A)	TUBE	3 ml	2	4°C
MMVC	Maintenance Medium for Vehicle Control (See Appendix A)	TUBE	3 ml	2	4°C
MMT	Maintenance Medium for Treatments (See Appendix A)	BOTTLE	300 ml	4	4°C
Wash buffer		BOTTLE	50ml	2	4°C
Lysis buffer		BOTTLE	25ml	2	4°C
Reagent A	Reconstitute w/ 11.0 ml deionized water prior to use	BOTTLE	11ml	2	4°C
Reagent B	Reconstitute w/ 2.5 ml deionized water prior to use	BOTTLE	2.5ml	2	4°C
Glycerol standard	Glycerol @ 1mM [Reconstitute with 200 μl Standards Diluent to make the 200 μM glycerol standard; see page 5 for recommended dilution scheme]	VIAL	50 μl	2	-20°C
Standards Diluent		BOTTLE	2 ml		4°C
Tray	Clear polyvinyl tray for multi-channel pipetters	EACH	3	---	-----
Data sheet	Certificate of Analysis and protocol	EACH	1	---	-----
Plate A	96-well plate for plating and differentiating	PLATE	1	---	-----
Assay Plate, Plate B	96-well assay plate, blank	PLATE	1	---	-----
Assay Plate, Plate C	96-well assay plate, blank (for standards)	PLATE	1	---	-----

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

- Single-channel pipetter
- Multi-channel pipetter
- Plate reader with a filter of 540 nm
- 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 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).
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 2.0 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 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 150µl /well is 1.3 x 10^6 cells in 15 ml Preadipocyte Medium.
5. Plate cells in one of the 96 well plates provided in the kit. Do not agitate the plate, as 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

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*	DMT	MMT

*****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 Differentiation Medium for Treatments (DMT) and Maintenance Medium for Treatments (MMT), 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 Differentiation Medium for Treatment (DMT), 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 Differentiation Medium for Treatment (DMT) containing compounds into appropriate wells.

Day 8: Prepare treatments using the Maintenance Medium Treatment Compounds (MMT). Using a multi-channel pipetter remove media from all wells. Gently feed all wells with 150µl of the appropriate Maintenance Medium (see chart above) 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 37°C water bath.

2. Prepare the Reagent B by adding 2.5ml deionized water per bottle and gently invert. DO NOT VORTEX! Use a pipette to ensure that the powder is completely dissolved. Keep at room temperature. Store in a light protected bottle. Reconstituted Reagent B is stable for 60 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C). Bring Reagent B to room temperature.

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 37°C – 50°C for 20 minutes.

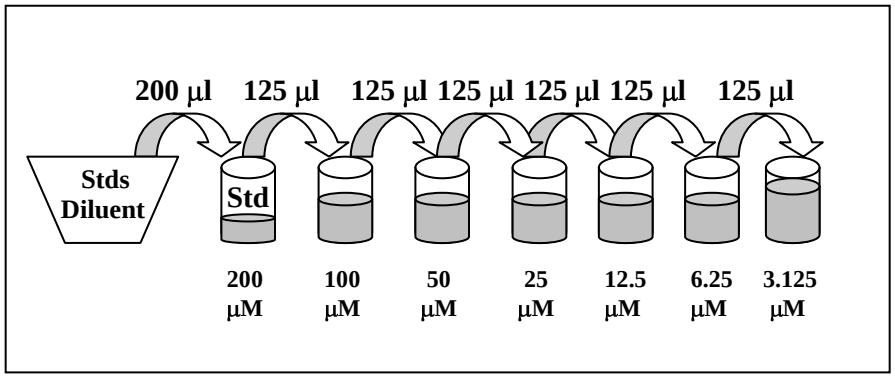
5. After the incubation is complete, visually confirm cell lysis by checking the wells under a microscope. If cells are not fully lysed, incubate for another 10 minutes.

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 37°C 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 200 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 125 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.



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. 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. 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.

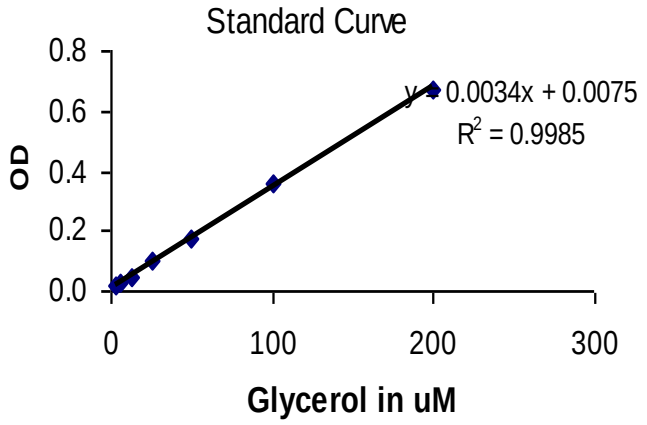
Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 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 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.

NOTE: Any OD values that are negative after the blank is subtracted should be considered to be 0 for the OD value.

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 20μl 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	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Penicillin • Streptomycin • Amphotericin B
Differentiation, Positive Control (DPC) agonist, dexamethasone	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPARγ agonist • Dexamethasone
Differentiation, Negative Control (DNC) agonist, dexamethasone, TNF-α	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPARγ agonist • Dexamethasone • TNF-α
Differentiation, Vehicle Control (DVC) agonist, no dexamethasone	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPARγ agonist

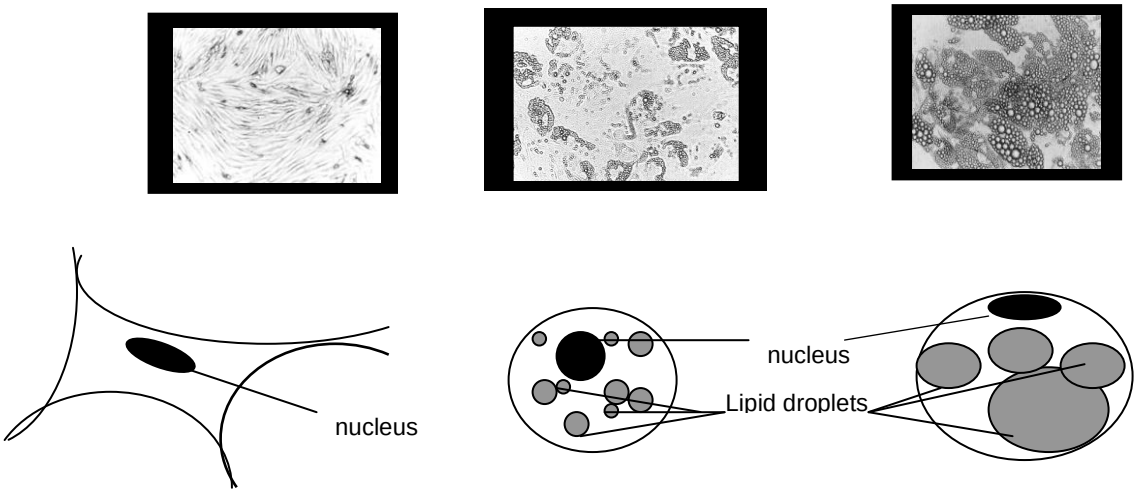
Differentiation Medium for Treatment compound (DMT) agonist, compound	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Insulin • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPARγ agonist • Compound
Maintenance Medium Positive Control (MMPC) dexamethasone	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Insulin • Penicillin • Streptomycin • Amphotericin B • Dexamethasone
Maintenance Medium Negative control (MMNC) dexamethasone TNF-α	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Insulin • Penicillin • Streptomycin • Amphotericin B • Dexamethasone • TNF-α
Maintenance Medium Vehicle control (MMVC) no dexamethasone	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Insulin • Penicillin • Streptomycin • Amphotericin B
Maintenance Medium Treatment compounds (MMT) compound	• DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Insulin • 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.

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INCUBATE 24 HOURS 37°C

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

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

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DAY 15
FEED CELLS APPROPRIATE
MAINTENANCE MEDIUM

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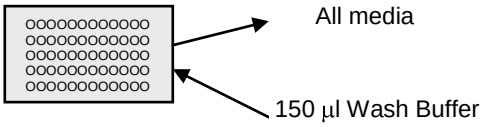
MOVE ON TO
TRIGLYCERIDE ASSAY PROTOCOL

APPENDIX E: TRIGLYCERIDE ASSAY FLOWCHART__

Remove all media from wells.



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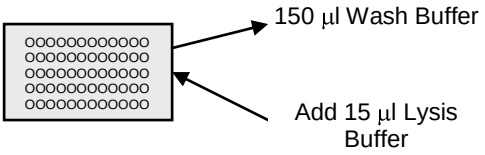
Wash with 150 µl Wash Buffer
Remove all Wash Buffer from wells
and add 15 µl Lysis Buffer.



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Incubate 20 minutes at 37 – 50°C

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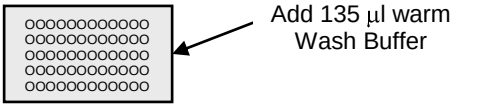
Verify lysis and add 135 µl warm
Wash Buffer. Mix by pipetting up and
down 3 times.



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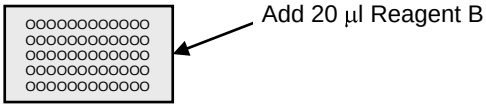
Add 20 µl Reagent B and incubate 2
hours at 37 °C.



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

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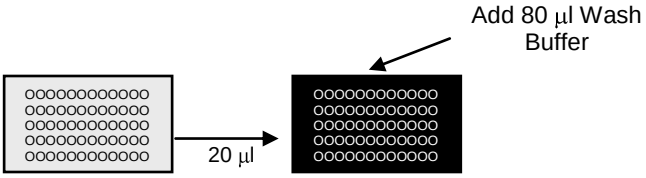
Add 80 µl Wash Buffer to a new plate.
Mix lysates and transfer 20 µl lysates
to the wells containing Wash Buffer.



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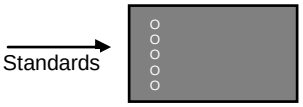
Transfer 100 µl of each standard to a
new plate.



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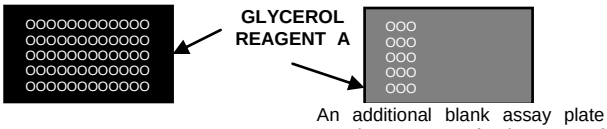
Add 100 µl Reagent A to samples
and standards.



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

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Incubate 15 minutes at 25°C (room
temperature). 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.