96-well Serum/Plasma Glycerol Kit
Free Glycerol Detection
Cat# SGA-1

INSTRUCTION MANUAL ZBM0043.02

STORAGE CONDITIONS

- **Reagents & Buffers:** 4°C
- **Glycerol Standard:** -20°C
- **Blank assay plates (96-well):** Room Temperature

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INTRODUCTION

This kit is designed to accurately determine the amount of glycerol present in blood serum or plasma of humans, mice, rats, and other animals in a 96-well format for increased throughput analysis. Blood can be collected in plain evacuated tubes or in the presence of common anti-coagulants: sodium citrate, ammonium oxalate and EDTA. **NOTE: Heparin or Heparinized tubes should not be used because this will generate inaccurate readings.** Serum should be separated from clotted blood by centrifugation as soon as possible and may be stored frozen (-20°C) prior to analysis.

PRINCIPLE OF THE ASSAY

Detection of Free Glycerol

Glycerol present in sample 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
Assay Plate, Plate A	96-well assay plate, blank	---	PLATE	2	-----
Dilution Buffer	12 ml	---	BOTTLE	1	4°C
Glycerol Reagent A	Reconstitute with 6.0 ml deionized water prior to use.	---	BOTTLE	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl	CLEAR	EACH	2	-----
Glycerol standard	Glycerol @ 1mM [Dilute with 200 □l Dilution Buffer to make the 200 □M glycerol standard; see page 3 for recommended dilution scheme]	ORANGE	50 □l / VIAL	1	-20°C

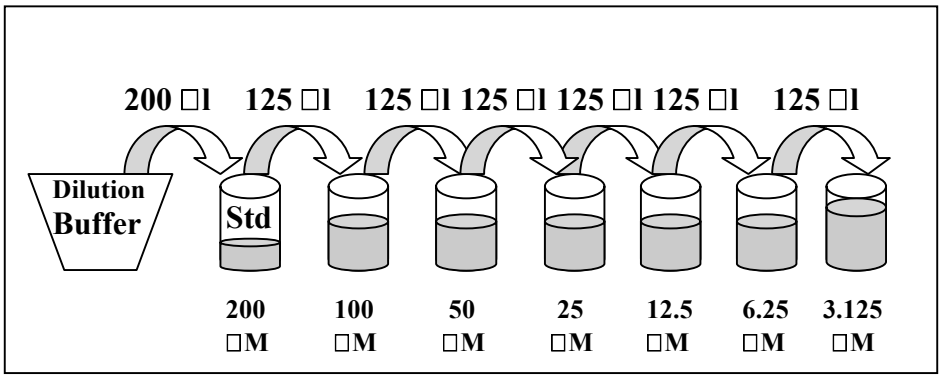
Other equipment/reagents required but not provided with the kit:

- □ Multi-channel Pipet , single channel pipet and pipet tips
- □ Plate reader with a filter of 540 nm
- □ Incubator at 37°C
- □ Large gauge needle
- □ Tubes for diluting standards

ASSAY PROCEDURE

1. Prepare the glycerol standards as follows:

   Briefly spin down the contents of the glycerol standard tube before reconstitution. Pipette 200 □l of Dilution Buffer into the 1 mM glycerol standard tube provided and mix well by vortexing. This produces a diluted stock glycerol standard of 200 □M. Pipette 125 □l of dilution 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 □M stock dilution serves as the highest standard, and the dilution buffer serves as the zero standard.



2. Also at this time prepare the Glycerol Reagent A by adding 6.0 ml room temperature deionized water per bottle and gently invert. DO NOT VORTEX! Use a pipet to ensure that the powder is completely dissolved. 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).

3. Add 20 □l (or 10 - 25 □l) of serum or plasma to a well of a blank plate for assessment of free glycerol. Add 30□l of dilution buffer to each well to total 50 □l including serum or plasma sample. **THIS RESULTS IN A 2.5x DILUTION OF YOUR SAMPLE (20 □l in 50 □l).** Add 50 □l of each standard to empty wells (use another plate, if necessary).

4. Add the reconstituted Glycerol Reagent A solution to one of the disposable trays provided in the kit. Add 50 □l of Reagent A to each well of the glycerol plate. 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.

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

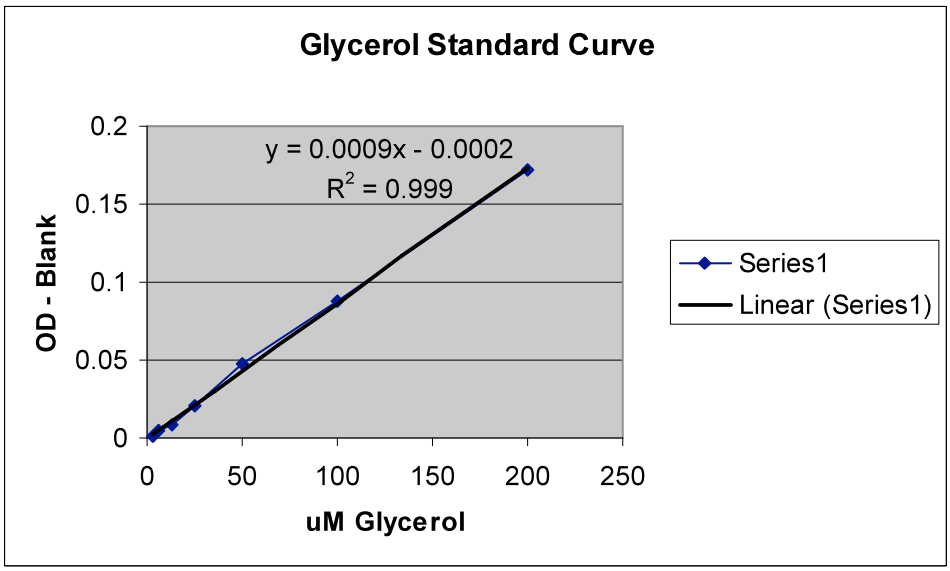
GLYCEROL STANDARD CURVE

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 0□M standard from all OD values including the standard curve.

uM Glycerol	OD	OD - blank
0	0.04	
3.125	0.041	0.001
6.25	0.045	0.005
12.5	0.049	0.009
25	0.061	0.021
50	0.087	0.047
100	0.128	0.088
200	0.212	0.172

Slope	0.0009
Intercept	-0.0002
r^2	0.999



y = observed O.D. minus the blank
x = concentration of glycerol in □M
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 –(-) 0.0002)/0.0009 where 0.0009= slope of the line and -0.0002= 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 □M)) 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 than 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 □M glycerol.

REMEMBER TO ACCOUNT FOR THE DILUTION FACTOR IN STEP 3.

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

Glycerol Detection

Add 20 □l/well test sample and 30 □l/well dilution buffer to one of the blank assay plates provided. Add 50 □l/well diluted standard curve to empty wells.

↓

Reconstitute Glycerol Reagent A. Add 50□l/well.

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Incubate 15 minutes @ room temperature.

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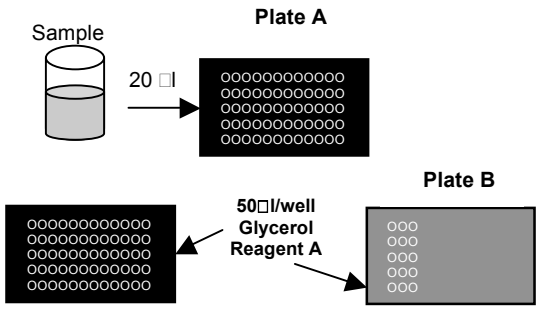
Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.
Reminder: Sample was diluted in Step 3



APPENDIX C. FREQUENTLY ASKED QUESTIONS

1. **Can I buy the reagents separately?** The Glycerol Standard, cat# LIP-GLYSTAN and Glycerol Reagent A, cat# RGTA-6 are sold separately. The Dilution Buffer is not sold separately.