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

INSTRUCTION MANUAL ZBM0043.05

STORAGE CONDITIONS

- **Reagents & Buffers:** +4°C **Use reconstituted Glycerol Reagent A within 7 days.**
- **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.

PLASMA

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

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 Plates	96-well assay plate, blank	---	PLATE	2	-----
Dilution Buffer	12 ml	---	BOTTLE	1	4°C
Glycerol Reagent A (cat# RGTA-10)	Reconstitute with 11.0 ml deionized water prior to use. **Use reconstituted reagent within 7 days.**	---	BOTTLE	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl	CLEAR	EACH	2	-----
Glycerol standard (cat# LIP-GLYSTAN)	Glycerol @ 1mM [Dilute with 400 µl Dilution Buffer to make the 200 µM glycerol standard; see page 3 for recommended dilution scheme]	ORANGE	100 µl / VIAL	1	-20°C

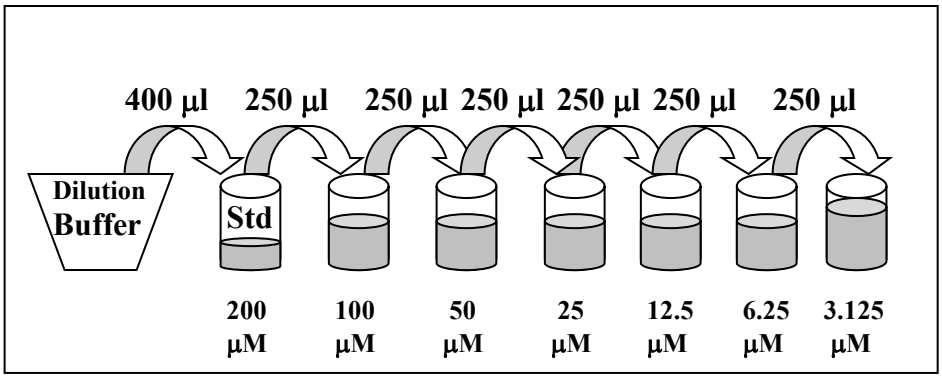
Other equipment/reagents required but not provided with the kit:

- Multi-channel Pipet , single channel pipet and pipet tips
- Plate reader with a filter of 540 nm
- Incubator at 37^{o}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 400 µl 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 µM. Pipette 250 µl 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 µM stock dilution serves as the highest standard, and the dilution 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.

2. 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. 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 µl (or 10 - 25 µl) of serum or plasma to a well of a blank plate for assessment of free glycerol. Add 30µl of dilution buffer to each well to total 50 µl including serum or plasma sample. **THIS RESULTS IN A 2.5x DILUTION OF YOUR SAMPLE (20 µl in 50 µl).** Add 50 µl 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 µl 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 30 minutes.

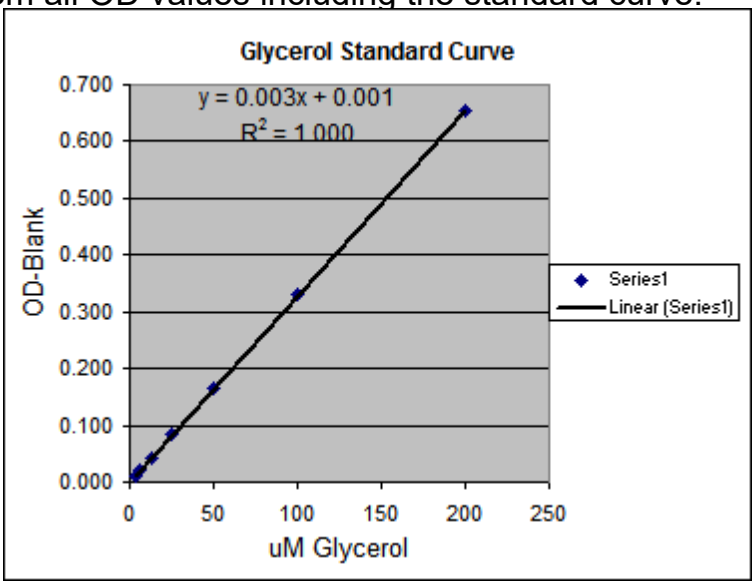
5. The optical density of each well is then measured at 540 nm.

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

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Reconstitute Glycerol Reagent A. Add 50µl/well.

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Incubate **Glycerol Reagent A for 30 minutes at room temperature.**

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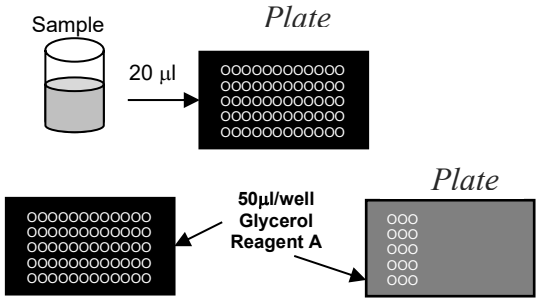
Measure the optical density of each well at 540 nm using a spectrophotometer plate reader.

Reminder: Sample was diluted in Step 3



APPENDIX C. FREQUENTLY ASKED QUESTIONS

1. **Can I buy the reagents separately?**
   a. Yes. The Glycerol Standard, cat# LIP-GLYSTAN and Glycerol Reagent A, cat# RGTA-10 are sold separately.
   b. The Dilution Buffer is not sold separately.