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

INSTRUCTION MANUAL ZBM0043.04

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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ORDERING INFORMATION AND TECHNICAL SERVICES

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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$_2$O$_2$). A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl)m-anisidine (ESPA) with H$_2$O$_2$, which shows an absorbance maximum at 540nm. The increase in absorbance at 540nm is directly proportional to glycerol concentration of the sample.

\[
\text{GLYCEROL + ATP} \rightarrow \text{G-1-P + ADP}
\]
\[
\text{G-1-P + O}_2 \rightarrow \text{DAP + H}_2\text{O}_2
\]
\[
\text{H}_2\text{O}_2 + 4\text{-AAP + ESPA} \rightarrow \text{Quinoneimine dye + H}_2\text{O}
\]

ITEMS INCLUDED IN THE KIT

<table>
<thead>
<tr>
<th>ITEM</th>
<th>DESCRIPTION</th>
<th>Cap Color</th>
<th>UNIT</th>
<th>QT Y</th>
<th>STORAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Plates</td>
<td>96-well assay plate, blank</td>
<td>---</td>
<td>PLATE</td>
<td>2</td>
<td>-----</td>
</tr>
<tr>
<td>Dilution Buffer</td>
<td>12 ml</td>
<td>---</td>
<td>BOTTLE</td>
<td>1</td>
<td>4°C</td>
</tr>
<tr>
<td>Glycerol Reagent A (cat# RGTA-10)</td>
<td>Reconstitute with 11.0 ml deionized water prior to use. <strong>Use reconstituted reagent within 7 days.</strong></td>
<td>---</td>
<td>BOTTLE</td>
<td>1</td>
<td>4°C</td>
</tr>
<tr>
<td>Tray</td>
<td>For multi-channel pipettes, clear polyvinyl</td>
<td>CLEAR</td>
<td>EACH</td>
<td>2</td>
<td>-----</td>
</tr>
<tr>
<td>Glycerol standard (cat# LIP-GLYSTAN)</td>
<td>Glycerol @ 1mM [Dilute with 400 μl Dilution Buffer to make the 200 μM glycerol standard; see page 3 for recommended dilution scheme]</td>
<td>ORANGE</td>
<td>100 μl / VIAL</td>
<td>1</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

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 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.

   ![Diagram of dilution series](image)

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

<table>
<thead>
<tr>
<th>μM glycerol</th>
<th>OD</th>
<th>OD blank</th>
<th>Avg OD blank</th>
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<tbody>
<tr>
<td>0</td>
<td>0.044</td>
<td>0.041</td>
<td>0.043</td>
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<tr>
<td>3.125</td>
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<td>6.25</td>
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<td>12.5</td>
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<tr>
<td>50</td>
<td>0.205</td>
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<td>100</td>
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<tr>
<td>200</td>
<td>0.698</td>
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Slope 0.003
Intercept 0.001
R² 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 R² value should be equal or greater than 0.98 for the standard curve to be valid. Any R² 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.
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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.

Reconstitute Glycerol Reagent A. Add 50μl/well.

Incubate 15 minutes @ room temperature.

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

Reminder: Sample was diluted in Step 3

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APPENDIX C. FREQUENTLY ASKED QUESTIONS

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