Triglyceride Assay Kit-Bulk 5 Plate Kit
Cat# TG-5-RB

INSTRUCTION MANUAL (ZBM-14)

STORAGE CONDITIONS

- **Reagents A & B, Buffers:**
  Store at 2 - 8°C.
  Glycerol Standard
  -20°C

For Research Use Only
Not For Use In Diagnostic Procedures

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ORDERING INFORMATION AND TECHNICAL SERVICES

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Triglyceride Assay Kit-Bulk 5 Plate Kit

(Cat# TG-5-RB)

Items included in the kit:

The contents of this kit are sufficient for the assay of up to 5 96 well plates. The protocol is designed for assay of cells in a 96-well format. For other formats, please adjust the volumes added to each well according to the surface area of the well/flask you are using. See your cultureware manufacturer's technical information for the specifications.

Item	Instructions	Amount	QTY	Storage
Wash Buffer		250ml	1	4°C
Lysis Buffer		125ml	1	4°C
Reagent A	Reconstitute w/ 40 ml deionized water. Reconstitute w/ 11 ml deionized water. Combine and mix before use.	40ml 11ml	1 1	4°C
Reagent B	Reconstitute w/ 10 ml deionized water. Reconstitute w/ 2.5 ml deionized water. Combine and mix before use.	10ml 2.5ml	1 1	4°C
Glycerol standard	Glycerol @ 1mM [Reconstitute with 200 µl Standards Diluent to make the 200 µM glycerol standard; see manual for recommended dilution scheme]	50 µl	5	-20°C
Standards Diluent		10 ml	1	4°C

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

- Single-channel pipetter
- Multi-channel pipetter
- Plate reader with a filter of 540 nm

PROTOCOL

1. Warm the Wash buffer and Lysis buffer in a 37°C water bath.

2. Prepare the Reagent B by adding 10ml and 2.5 ml deionized water to the labeled bottles and gently invert. DO NOT VORTEX! Use a pipet to insure that the powder is completely dissolved. Keep at room temperature. Store in a light protected bottle. Reconstituted Glycerol Reagent B is stable for 60 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).

3. Remove all media from mature human adipocytes. 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 per well. Incubate at 37°C - 50°C for 20 minutes.

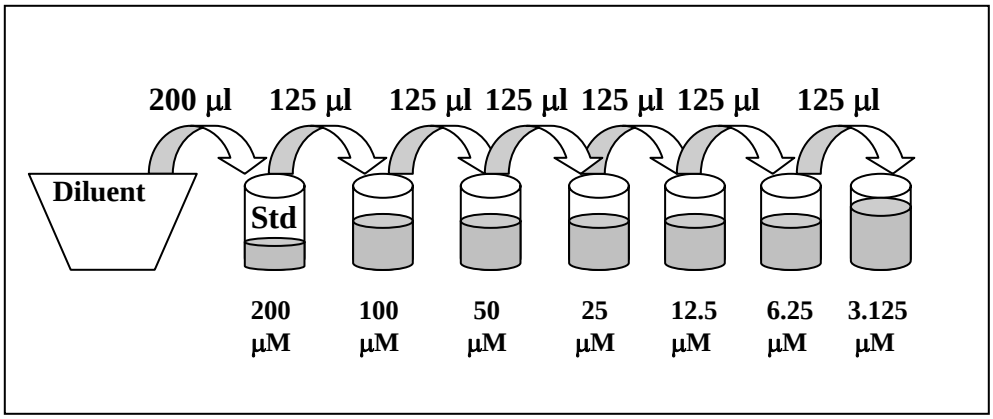
5. After the incubation is complete, visually confirm cell lysis by checking the wells under a microscope.

6. Add 135 µl wash buffer to each well.

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

8. One hour prior to the assay, bring the glycerol standard and Reagent B to room temperature. 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 ml and 40 ml deionized water the labeled bottles and gently invert. DO NOT VORTEX! Use a pipet to insure 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.

DATA ANALYSIS

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.

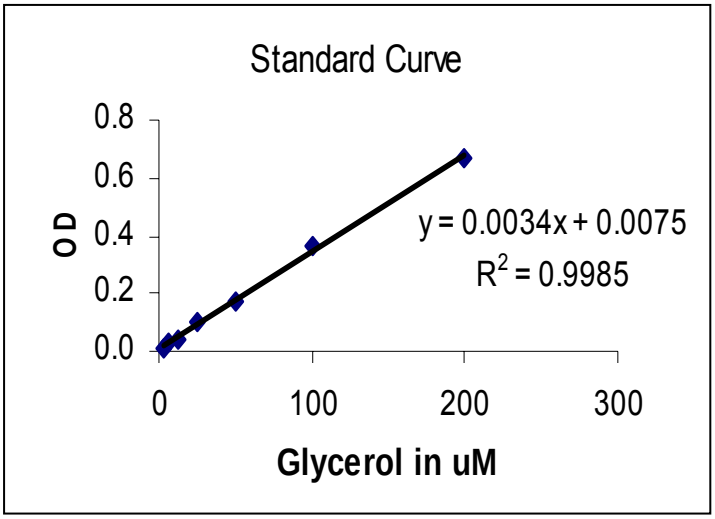
Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

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. Also 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 more dilute solution of the condition medium at the time of 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 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 : Plate layout _____

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

Appendix B Triglyceride Assay Flowchart

Remove all media from wells

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Wash with 150µl wash buffer
Remove all wash buffer from wells

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Add 15µl lysis buffer

↓

Incubate 20 minutes at 37 – 50°C
Verify cell lysis

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Add 135µl wash buffer
Add 20µl Reagent B
Gently tap plate to mix reagents. Incubate 2 hours at 37 °C

↓

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

↓

Add 80 µl wash buffer to a new plate.
Mix lysates and transfer 20 µl lysates to the wash buffer wells.

↓

Transfer 100 µl of each standard to a new plate

↓

Add 100 µl Reagent A to samples and standards

↓

Incubate 15 minutes
Room Temperature

↓

Measure O.D. 540 nm