Glycerol Reagent A

Cat# RGTA-10; RGTA-40

INSTRUCTION MANUAL ZBM0038.03

STORAGE CONDITIONS

Glycerol Reagent A 4°C

Long-term storage: Dry reagent is stable until expiration date on label.
Reconstituted reagent is stable for 7 days at 2 - 8°C.

ALL PRODUCTS ARE FOR RESEARCH USE ONLY. NOT APPROVED FOR HUMAN OR VETERINARY USE OR FOR USE IN DIAGNOSTIC OR CLINICAL PROCEDURES.

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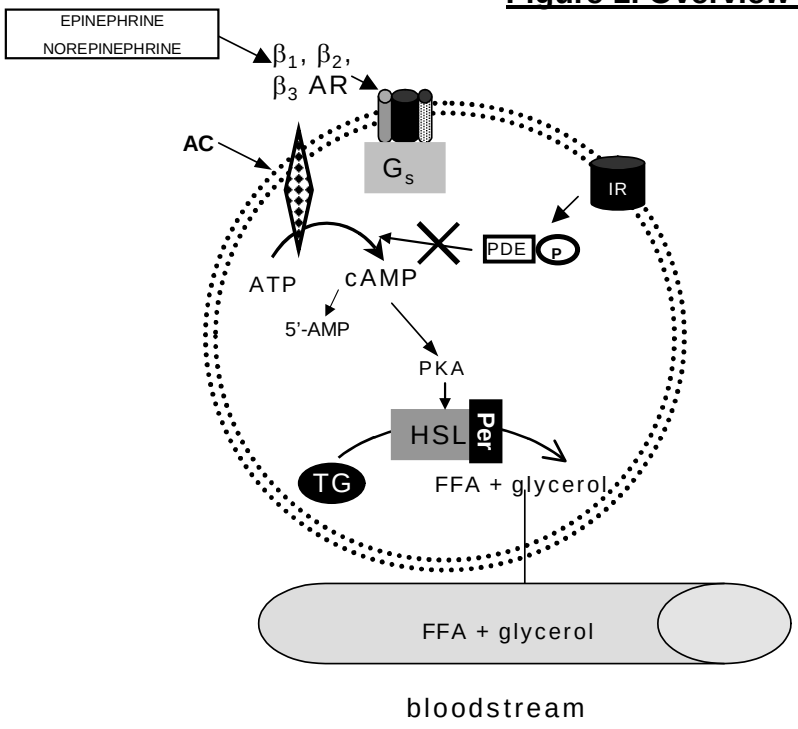
INTRODUCTION

Lipolysis plays a central role in the regulation of energy balance. Lipolysis is the process in which triglycerides (TG) are hydrolyzed into glycerol and free fatty acids. This process releases free fatty acids (FFA) into the bloodstream where they may be either re-esterified by the adipocyte or travel to other tissues and exert other effects throughout the body. Elevated adipocyte lipolysis has been observed in obese and diabetic individuals (Arner 1996). Excessive free fatty acid production is believed to contribute to insulin resistance in skeletal muscle that is observed in obesity. Hormone sensitive lipase is the rate-limiting enzyme catalyzing triglyceride breakdown. Perilipins, one of the PAT (perilipins, adipophilin, TIP47 proteins) family of lipid-associated proteins, are implicated in adipocyte lipolysis by mediating the interaction of HSL with the triacylglycerol molecule (Brasaemle *et al.* 2004; reviewed in, Tansey *et al. 2004.)* The presence of these proteins corresponds to lipolytic stimulation in cultured adipocytes (Braemle *et al.* 2004).

The sympathetic nervous system also plays a key role in the regulation of lipid mobilization. The main lipolytic pathway involves beta-agonists (β-agonists), which activate β-adrenergic receptors via the intracellular G_s proteins in adipocytes. This leads to the activation of adenylate cyclase (AC), which then increases cyclic AMP (cAMP) levels. Elevated cAMP acts as a second messenger to activate hormone sensitive lipase (HSL). HSL, the rate-limiting enzyme regulating adipocyte lipolysis, then catalyzes the hydrolysis of triglycerides and results in the release of glycerol and FFA (increased lipolysis). Phosphodiesterases (PDE) are enzymes that hydrolyze cAMP to 5'-AMP (5 prime adenosine monophosphate). This action results in a decrease in lipolysis. PDE inhibitors increase intracellular cAMP levels. 3-isobutyl-1-methylxanthine (IBMX), a non-specific inhibitor of cAMP phosphodiesterases (PDE), is used as the positive control if your test compounds are suspected PDE inhibitors. Isoproterenol, a non-specific β-adrenergic agonist is used as the positive control if your test compounds affect lipolysis via β-adrenergic receptors (Robidoux *et al*. 2004).

This lipolysis assay kit provides the tool to study chemical compounds that may influence lipolysis in cultured human adipocytes.

Figure 1. Overview of adipocyte lipolysis



ABBREVIATIONS:

AC	adenylate cyclase
AR	adrenergic receptors
G_s	G protein coupled receptor
FFA	free fatty acids
PKA	protein kinase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
IR	insulin receptor
PDE	phosphodiesterase
TG	triglyceride

PRINCIPLE OF THE ASSAY

Glycerol released to the medium 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 quinoeimine 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_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 (ONE OF THE FOLLOWING)

ITEM	DESCRIPTION	UNIT	QTY	STORAGE
Glycerol Reagent A Cat# RGTA-10	RGTA-10 Reconstitute with 11 ml deionized water prior to use. Use within 7 days!	BOTTLE	1	4°C
Glycerol Reagent A Cat# RGTA-40	RGTA-40 Reconstitute with 40 ml deionized water prior to use. Use within 7 days!	BOTTLE	1	4°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
- 96-well assay plates
- Glycerol Standard (Cat# LIP-GLYSTAN)
- Tubes for dilution of standards

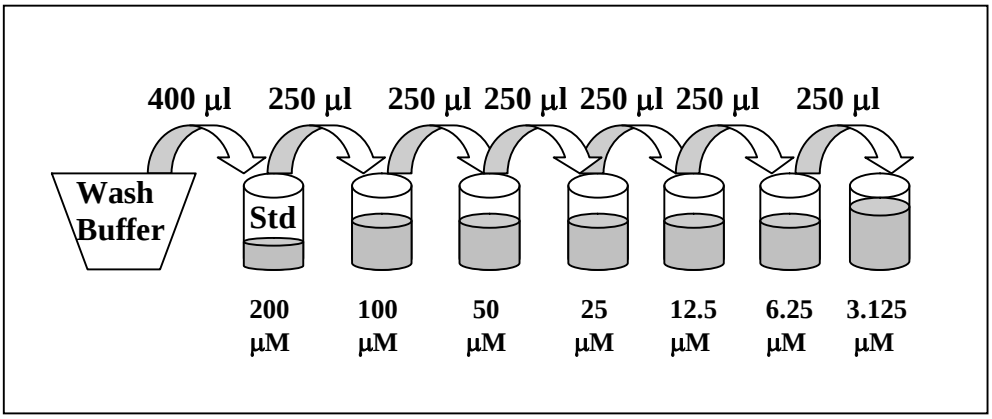
COMPOSITION

ATP	0.375 mM
Magnesium salt	3.75 mM
4-Aminoantipyrine	0.188 mM
Sodium-N-ethyl-N(3-sulfopropyl) m-anisidine	2.11 mM
Glycerol Kinase (microbial)	1250 Units/L
Glycerol phosphate oxidase (microbial)	2500 Units/L
Peroxidase (horseradish)	2500 Units/L
Sodium Azide (added as preservative)	0.05%

ASSAY PROCEDURE

1. One hour prior to the assay, prepare the glycerol standards as follows:

   Briefly spin down the contents of the glycerol standard tube before reconstitution. Pipette 400 µl of Wash 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 wash 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 wash 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 ml room temperature deionized water per bottle and gently invert (40 ml for RGTA-40). DO NOT VORTEX! Use a pipette to ensure that the powder is completely dissolved. 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 7 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).

3. Add an equal volume of test sample and reconstituted Reagent A (For example, 100 µl + 100 µl in 96 well format). Gently, pipet up and down once to mix. Pop the bubbles using a large gauge needle or a clean pipet tip.

4. The plate is then incubated at 25°C (room temperature) for 15 minutes.

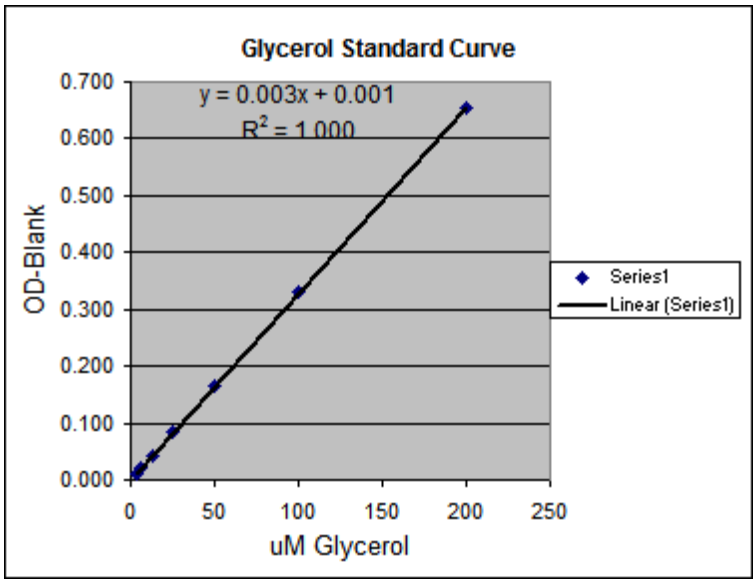
5. The optical density of each well is then measured at 540 nm.

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.

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 O.D. values that fall below the value for the 3.125 μM standard should be considered 0 μM glycerol. 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.

Data are expressed as μM glycerol released.

OPTION: express data as Fold induction over appropriate vehicle

$$\text{Fold induction} = \frac{\mu\text{M glycerol SAMPLE}}{\mu\text{M glycerol VEHICLE}}$$

LIMITATIONS

Glycerol Reagent A is linear up to 200 μM glycerol. If the sample glycerol concentration exceeds this value, dilute sample and retest. Multiply result by the dilution factor to compensate for dilution.

PRECAUTIONS

Glycerol Reagent A is for "in vitro research use only". Not for household, clinical or diagnostic use. Normal precautions used in handling laboratory reagents should be followed.

Glycerol reagent A contains sodium azide, which is HIGHLY TOXIC. May cause heritable genetic damage. Very toxic by inhalation, skin contact or if swallowed. Sodium azide is readily absorbed through the skin. Contact with acids yields very toxic gases. Keep container tightly closed and wear suitable protective clothing and gloves. If you feel unwell or in case of accident, seek medical advice immediately.

Avoid contact with metals. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Avoid azide accumulation. Refer to Material Safety Data Sheets for any updated risk, hazard or safety information.

TROUBLESHOOTING

Problem	Suggestions
High background or the glycerol reagent A turns purple before the assay begins.	• Change pipet tips frequently • Use clean tray and tips. Glycerol contamination in trays, tubes, tips, glassware will interfere with the assay. • Use Glycerol Reagent A before the expiration date
No response to positive control	• Do not add the compounds and controls too fast. The cells can float if a solution is added too fast. • Make sure to starve the cells for 5-7 days BEFORE initiating treatment.
Spectrophotometer does not have 540 nm filter	• Test the response using wavelengths ranging from 520-560 nm. Choose the wavelength that demonstrates linear standard curve from 0-200 μM glycerol.
Inconsistent OD reading	• The Assay Buffer contains bovine serum albumin (BSA). Be careful when pipetting to avoid bubbles. If bubbles persist, burst the bubbles using a large gauge needle and read the plate again.

REFERENCES

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ORDERING INFORMATION

Description	Catalog #
Glycerol Reagent A, 11 ml	RGTA-10
Glycerol Reagent A, 40 ml	RGTA-40

Optional Products

Glycerol Standard, 1mM, 100µl	LIP-GLYSTAN
Lipolysis Assay Kit (REAGENTS ONLY) (Includes Glycerol Standards Set, positive controls, wash & assay buffers for lipolysis assay)	LIP-1-NC
Lipolysis Assay Kit (REAGENTS + CELLS) (Includes Glycerol Standards Set, positive controls, wash & assay buffers for lipolysis assay, 96 well plate of cultured human adipocytes)	LIP-1
Lipolysis Assay Bulk Kit (REAGENTS ONLY) (Includes Glycerol Standard, positive controls, wash and assay buffer for lipolysis assay)	LIP-1RB

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