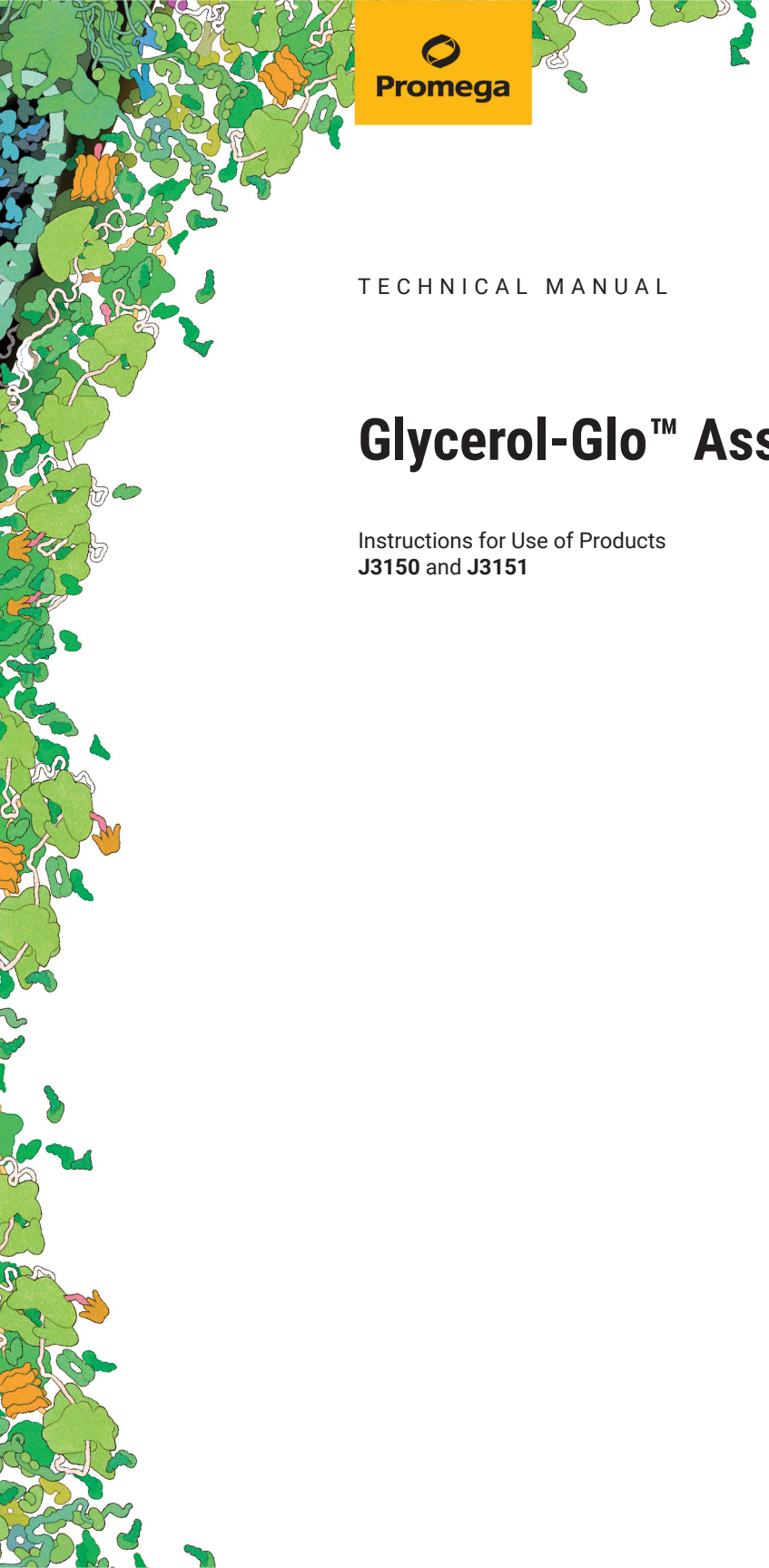

TECHNICAL MANUAL

Glycerol-Glo™ Assay

Instructions for Use of Products
J3150 and **J3151**

Revised 5/23
TM599

Glycerol-Glo™ Assay

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Glycerol-Glo™ Assay[a] provides a luminescent method for measuring glycerol in cell lysates and other biological samples such as cell culture medium, serum and tissue lysates. Glycerol is primarily measured as the product of lipolysis where it is released from triglycerides. Glycerol is also a substrate or product of a variety of other enzymatic or metabolic processes that can be studied with the Glycerol-Glo™ Assay.

The Glycerol-Glo™ Assay measures glycerol in a coupled reaction scheme that links the production of NADH to the activation of a proluciferin that produces light with luciferase (Figure 1).

The Glycerol-Glo™ Assay is part of a bioluminescent metabolite assay platform that offers rapid and sensitive metabolite detection in many sample types compatible with high-throughput applications (1–2). The assay can be performed in 96- and 384-well plates, and the detection reagents are added directly to samples without the need for organic extraction, making it amenable to high-throughput applications (Figure 2).

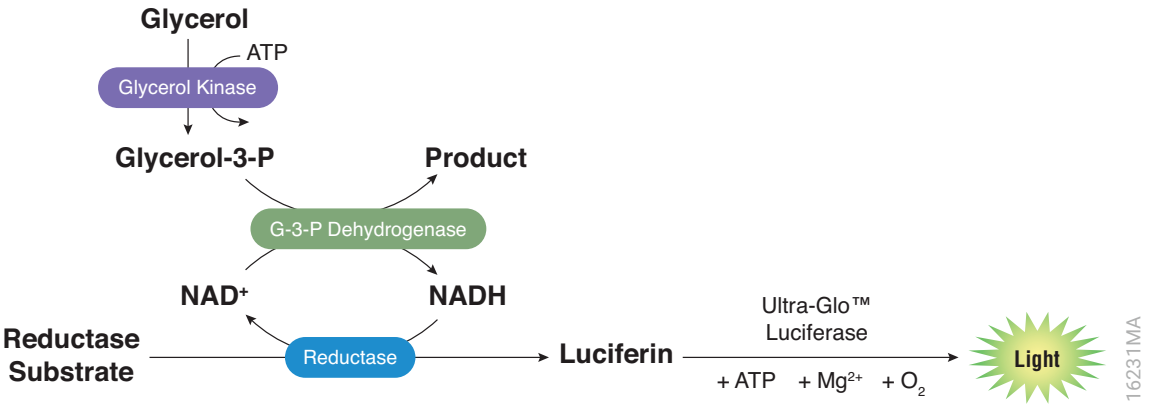

Figure 1. Schematic diagram of the Glycerol-Glo™ Assay principle. Glycerol kinase and glycerol-3-phosphate dehydrogenase are used to generate NADH. In the presence of NADH, reductase enzymatically reduces a proluciferin Reductase Substrate to luciferin. Luciferin is detected in a luciferase reaction using Ultra-Glo™ Luciferase and ATP, and the amount of light produced is proportional to the amount of glycerol in the sample.

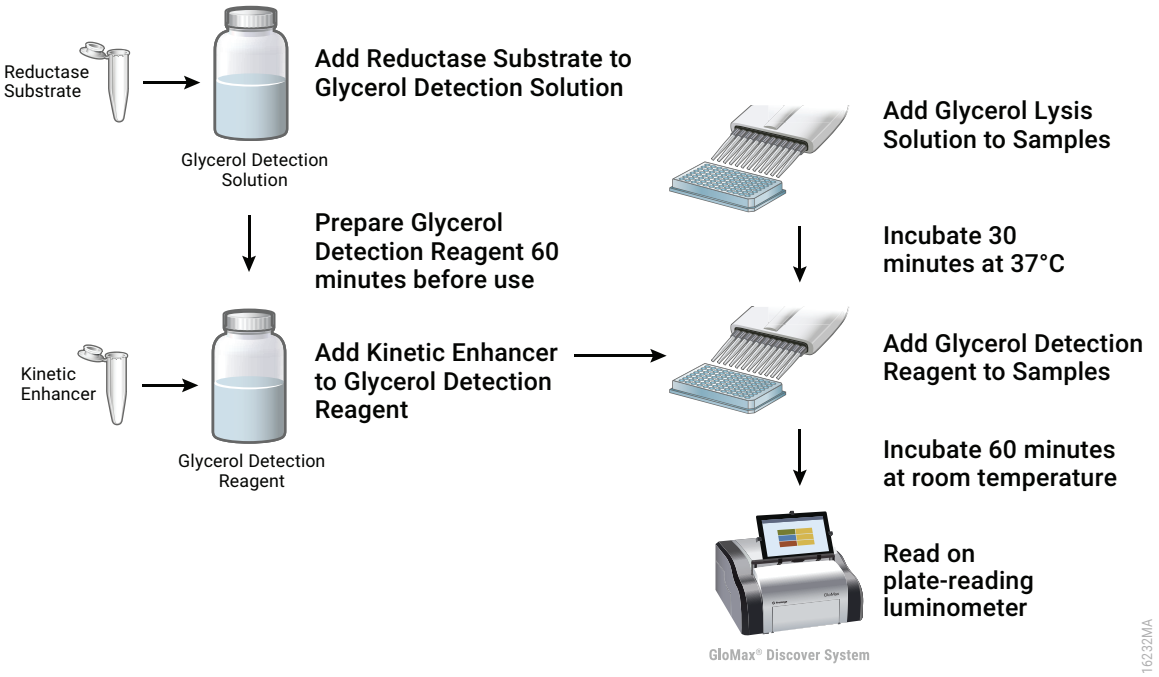

Figure 2. Glycerol-Glo™ Assay reagent preparation and protocol. The protocol above is for measurement of glycerol.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Glycerol-Glo™ Assay	**5ml**	**J3150**

The system contains sufficient reagents to perform 100 reactions in 96-well plates (50µl of sample + 50µl of glycerol detection reagent). Includes:

- 10ml Glycerol Lysis Solution
- 5ml Glycerol Detection Solution
- 55µl Reductase Substrate
- 50µl Kinetic Enhancer
- 500µl Glycerol Standard (20mM)

PRODUCT	SIZE	CAT.#
Glycerol-Glo™ Assay	**50ml**	**J3151**

The system contains sufficient reagents to perform 1,000 reactions in 96-well plates (50µl of sample + 50µl of glycerol detection reagent). Includes:

- 100ml Glycerol Lysis Solution
- 50ml Glycerol Detection Solution
- 2 × 275µl Reductase Substrate
- 500µl Kinetic Enhancer
- 500µl Glycerol Standard (20mM)

Storage Conditions: Store complete kits at less than –65°C. Alternatively, store the Glycerol Detection Solution and Reductase Substrate at less than –65°C, store the Kinetic Enhancer at less than –10°C, and store the Glycerol Lysis Solution and Glycerol Standard at less than +10°C. The kit components can be freeze-thawed three times with no effect on assay performance. As needed, dispense kit components into single-use aliquots to minimize freeze-thaw cycles.

Note: Use personal protective equipment and follow your institution's safety guidelines and disposal requirements when working with biohazardous materials such as cells and cell culture reagents.

3. Measuring Glycerol

3.A. Materials to Be Supplied By the User

- PBS
- 96- or 384-well assay plates (opaque white-walled with white or clear bottoms) compatible with standard plate readers
- single- and multichannel pipettors, tips and reagent reservoirs
- plate-reading luminometer (e.g., GloMax® Discover, Cat.# GM3000)
- water bath

3.B. Reagent Preparation

This protocol is for a reaction with 50µl of a prepared sample and 50µl of glycerol detection reagent in a 96-well plate. This assay can be adapted to other volumes provided the 1:1 ratio of glycerol detection reagent volume to prepared sample volume is maintained (e.g., 20µl of prepared sample and 20µl of glycerol detection reagent in a 384-well format).

Note: Glycerol is a common laboratory reagent present in many biological formulations (e.g., enzyme storage buffers, media, FBS) at concentrations significantly higher than the designed linear range of the Glycerol-Glo™ Assay. For example, 100% glycerol corresponds to a concentration of 13.6M, so even a 0.001% contamination will exceed the upper limit of the assay's linearity (80µM). Therefore, care must be taken to reduce the risk of contamination.

1. Thaw all components in a 22°C water bath and mix to ensure homogeneous solutions prior to use. Place the Reductase Substrate and Kinetic Enhancer on ice; all other components can be held at 22°C until use.
2. Determine the amount of reagents necessary for your current experiment. Use reagents on the day they are prepared; do not store prepared reagents for later use.
3. To prepare glycerol detection reagent, add 10µl of Reductase Substrate per ml of Glycerol Detection Solution and mix by inversion. Prepare this reagent 1 hour before use in order to minimize the assay background. Hold at room temperature.
4. After 1 hour, add 10µl of Kinetic Enhancer per ml of glycerol detection reagent prepared in Step 3 and mix by inversion.

4. Assay Protocol

This kit includes a 20mM Glycerol Standard for generating standard curves (Figure 3) to confirm that samples are within the linear range of the assay and to calculate glycerol concentration. If the sample relative light unit (RLU) values fall outside the linear range of the glycerol standard curve, the sample dilutions must be adjusted and re-assayed. It is important to prepare standards in the same buffers used for preparing samples and to follow the same assay protocol. For example, if samples are prepared in a 1:1 mixture of medium:Glycerol Lysis Solution, then standards must also be in a 1:1 mixture of medium:Glycerol Lysis Solution.

4.A. Assaying Medium, Serum or Homogenized Tissue Samples and Controls

1. Dilute samples in Glycerol Lysis Solution to bring their glycerol concentrations below 80µM. Transfer 25µl of sample, standard or control to a 96-well plate.
2. Add 25µl of Glycerol Lysis Solution, shake briefly and incubate 30 minutes at 37°C.

Note: If measuring glycerol in solution (i.e., no cells need to be lysed), addition of Glycerol Lysis Solution is not necessary. Be sure to also prepare standards and controls without Glycerol Lysis Solution and bring the sample volume to 50µl.

3. Add 50µl of glycerol detection reagent as prepared in Section 3.B to all wells.
4. Shake the plate for 30–60 seconds by hand or at a low rpm on a plate shaker.

4.A. Assaying Medium, Serum or Homogenized Tissue Samples and Controls (continued)

5. Incubate at room temperature for 1 hour.
6. Record luminescence using a plate-reading luminometer.

   Note: The light signal continues to increase until all glycerol is consumed and the signal plateaus. At any time point the signal is directly proportional to glycerol concentration.
7. Calculate glycerol concentration by comparison of the luminescence of samples and standards (see Figure 3).

4.B. Assaying Adherent Cells or 3D Culture Samples and Controls

1. Remove media from cells in a 96-well plate. Wash cells twice with 100µl of PBS.
2. Add 50µl of Glycerol Lysis Solution, shake briefly and incubate 30 minutes at 37°C.
3. **Optional:** If needed, dilute samples in Glycerol Lysis Solution to bring their glycerol concentrations below 80µM. Transfer 50µl of any diluted samples, standards or controls to empty wells in a 96-well plate.
4. Add 50µl of glycerol detection reagent as prepared in Section 3.B to all wells.
5. Shake the plate for 30–60 seconds by hand or at a low rpm on a plate shaker.
6. Incubate at room temperature for 1 hour.
7. Record luminescence using a plate-reading luminometer.

   Note: The light signal continues to increase until all glycerol is consumed and the signal plateaus. At any time point the signal is directly proportional to glycerol concentration.
8. Calculate glycerol concentration by comparison of the luminescence of samples and standards (see Figure 3).

4.C. Example Data

Table 1. Glycerol Titration Data.

Glycerol (µM)	80	60	40	20	10	5	2	1	0
RLU (× 10^3)	25,763	20,963	14,770	7,595	4,095	2,123	974	557	153
Standard Deviation (× 10^3)	380	23	7	56	103	31	1	4	3
Coefficient of Variation	1.5%	0.1%	0.0%	0.7%	2.5%	1.4%	0.1%	0.8%	1.8%
S/B	167.9	136.6	96.2	49.5	26.7	13.8	6.3	3.6	1.0
S/N	9,512.1	7,729.2	5,429.0	2,764.0	1,463.9	731.6	304.7	149.8	—

Note: Coefficient of variation (CV) is 100 × standard deviation/RLU. Signal-to-background ratio (S/B) is the mean signal from samples divided by the mean signal from negative controls. Signal-to-noise ratio (S/N) is the net signal (mean signal minus mean negative control) divided by the standard deviation of the negative control.

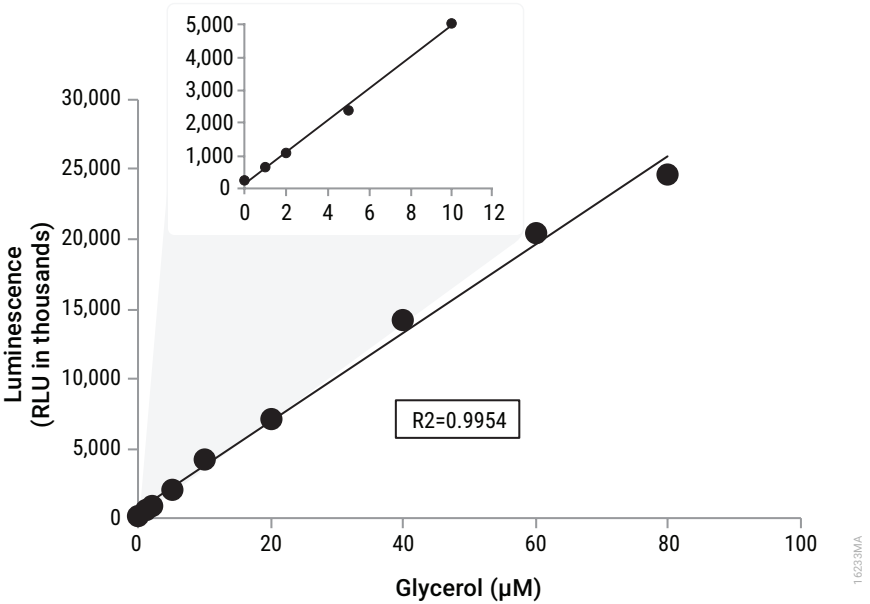

Figure 3. Glycerol standard curves. Dilutions of the provided Glycerol Standard (20mM) were prepared in Glycerol Lysis Solution, beginning with a 2µl aliquot of Glycerol Standard into 498µl of Glycerol Lysis Solution. (Refer to Table 1 for concentrations of Glycerol Standard used in standard curve.) A 50µl aliquot of glycerol detection reagent was added to 50µl of each standard in triplicate and luminescence was read after 1 hour. Concentration was plotted against average relative light units (RLU) at each standard point and a linear curve was fit. The Glycerol-Glo™ Assay can detect less than 1µM glycerol and has an upper limit of 80µM glycerol. See Section 5.A for an alternate way to convert luminescence into concentration.

5. Example Experiments

5.A. Lipolysis in Adipocytes

Glycerol release from cells is easily measured with the Glycerol-Glo™ Assay. An example of glycerol release is lipolysis in adipocytes (Figures 4 and 5). In the absence of stimulation, adipocytes released approximately 15µM glycerol in 1 hour. In the presence of the lipolysis inducer isoproterenol, the amount of glycerol increased to approximately 112µM in 1 hour, representing an approximately 7.5-fold increase in lipolysis. The amount of glycerol released varies with cell number, time of incubation, the identity and concentration of the lipolysis inducer and the metabolic state of the adipocytes. Also note that, in this example, the amount of glycerol released exceeded our 80µM limit of linearity, so a dilution was required prior to addition of the detection reagent.

5.A. Lipolysis in Adipocytes (continued)

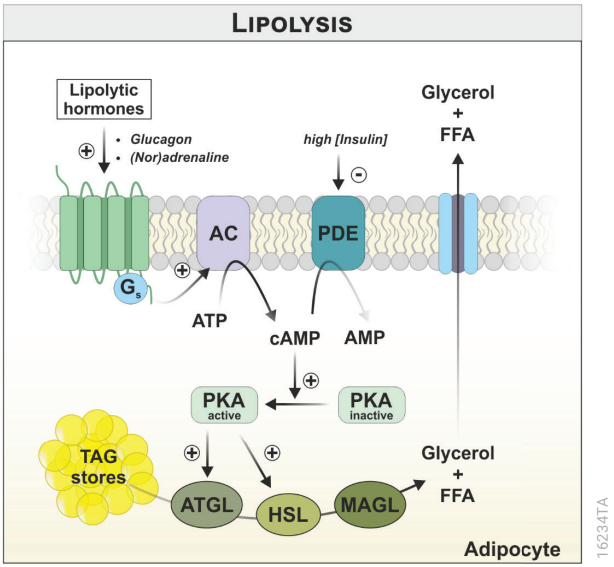

Figure 4. Lipolysis in adipocytes. Lipolytic hormones stimulate an enzymatic cascade through adenylate cyclase (AC) and protein kinase A (PKA) that leads to activation of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL). These enzymes convert triglyceride (TAG) stores into glycerol and free fatty acids (FFA).

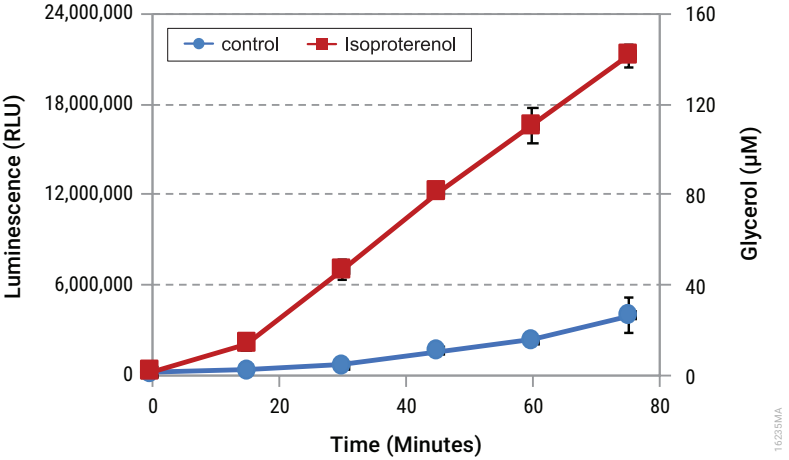

Figure 5. Glycerol release from adipocytes. Adipocytes were differentiated from 3T3L1-MBX fibroblasts (see Section 5.B of *Glucose Uptake-Glo™ Assay Technical Manual* #TM467 for recommendations on differentiation of fibroblasts into adipocytes). Medium was removed and the cells were washed twice with PBS. RPMI was added containing 2% fatty acid free BSA and 5µM triacsin C; the BSA binds fatty acids and the triacsin C inhibits lipogenesis, both of which aid in promoting extracellular glycerol accumulation. The medium also contained 0 or 10µM isoproterenol to create control or stimulated conditions. Aliquots of medium were removed over time, diluted 1:1 with Glycerol Lysis Solution and assayed for glycerol.

The glycerol concentrations in Figure 5 were calculated using a single standard approach, rather than a full standard curve (Figure 6). The luminescence of each lipolysis sample (sample RLU) was converted into glycerol concentration using the luminescence of a glycerol standard (standard RLU) of known concentration (STD) and the luminescence of a negative control (background RLU) containing no glycerol and the following formula:

$$[\text{Glycerol}] \text{ of sample} = \frac{[\text{Glycerol Standard}]\ (\mu M) \times (\text{sample RLU} - \text{background RLU})}{(\text{Standard RLU} - \text{background RLU})}$$

For the data in Figure 5 at 1 hour, this formula yields unstimulated and stimulated glycerol concentrations of 7.5µM and 56µM, respectively. Since the samples were diluted twofold, the glycerol concentrations of the unstimulated and stimulated medium samples are 15µM and 112µM, respectively.

5.A. Lipolysis in Adipocytes (continued)

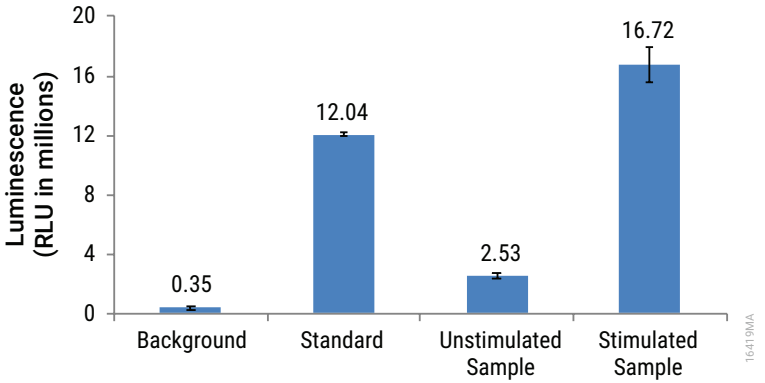

Figure 6. Calculation of glycerol concentration using a single glycerol standard. The sample data is from the 1 hour time point of Figure 5; see Figure 5 for experimental details. The standard concentration in this experiment was 40µM. The RLU value (in millions) is listed at the top of each bar.

5.B. Measurement of Other Enzyme Activities

The Glycerol-Glo™ Assay was designed to measure glycerol produced as the result of in vivo cellular processes. Additionally, it can be used to measure enzyme activity in vitro. For example, phospholipase D hydrolyzes phosphatidylglycerol to glycerol and phosphatidic acid (3). In Figure 7, phospholipase D from *Streptomyces spp.*(Sigma Cat.# P4912) was titrated and a linear correlation was observed between luminescence and enzyme concentration. Any enzyme activity that produces glycerol as a product can be measured with this assay, with a few caveats. If the source of the enzyme to be studied is stored in a glycerol solution, it may not be possible to measure changes in glycerol due to the potential for high background. Moreover, enzymes with significant color can absorb emitted light; such quenching may need to be accounted for when using different amounts of such enzymes.

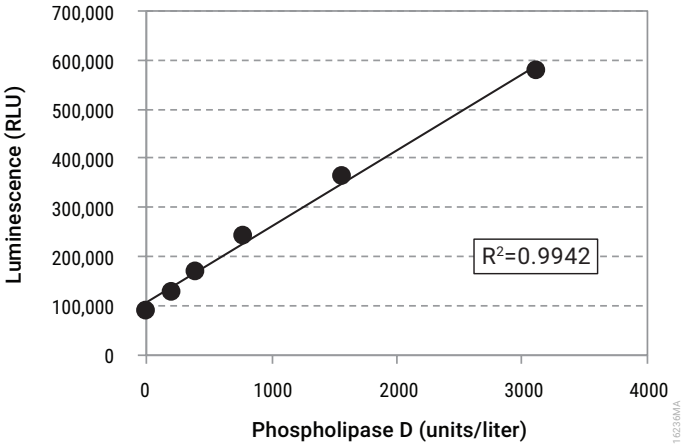

Figure 7. Measurement of phospholipase D activity. A range of phospholipase D (PLD) concentrations was mixed 1:1 with 40µM phosphatidylglycerol in PBS and incubated for 1 hour at room temperature. This solution was then mixed 1:1 with glycerol detection reagent and luminescence was recorded after 1 hour.

6. Appendix

6.A. Signal Stability

After addition of the glycerol detection reagent to a sample, the Glycerol-Glo™ Assay yields a signal that is stable over several hours (Figure 8).

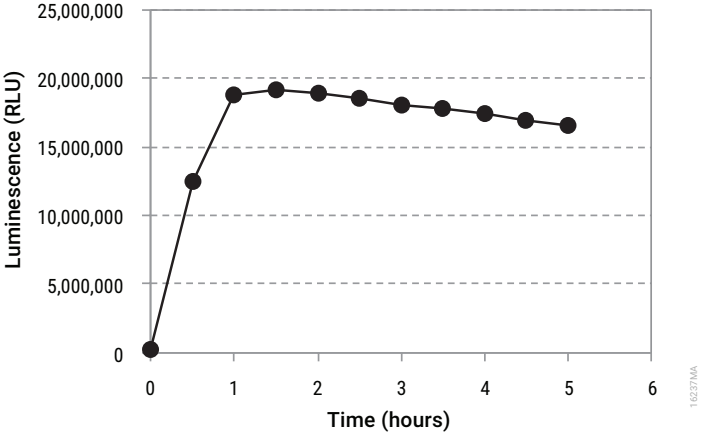

Figure 8. Signal stability of the Glycerol-Glo™ Assay. An equal volume of glycerol detection reagent was added to 40µM glycerol in Glycerol Lysis Solution and the luminescence was recorded over time. After 5 hours, the signal is >50% of the signal at 1 hour, which is convenient for high-throughput applications.

6.B. Temperature and Reagent Compatibility

The intensity and stability of the luminescent signal is temperature sensitive. For consistent results, equilibrate the reagents and samples to room temperature before use. Avoid the use of DTT and other reducing agents in the samples to be tested. Reducing agents will react with the Reductase Substrate and increase background.

6.C. Plates and Equipment

Most standard plate readers designed for measuring luminescence are suitable for this assay. Some instruments do not require gain adjustment, while others may require optimization of the gain settings to achieve sensitivity and dynamic range. An integration time of 0.5–1 second per well should serve as guidance. For exact instrument settings, consult your instrument manual. For optimum performance, use opaque, white multiwell plates that are compatible with your luminometer (e.g., Corning Costar® #3917 96-well or Costar® #3570 384-well plates). Luminescence signal is diminished in black plates and increased well-to-well crosstalk is observed in clear plates. The RLU values shown in the figures of this technical manual vary depending on the plates and luminometers used to generate the data. Although relative luminescence output will vary with different instruments, this variation does not affect assay performance.

6.D. Multiplexing and Normalization

The Glycerol-Glo™ Assay can be multiplexed to normalize for changes in cell viability and to account for well-to-well variation. If a sample of medium is removed, the remainder of the sample can be assayed with RealTime-Glo™, CellTiter-Fluor™ or CellTiter-Glo® Cell Viability Assays, following the protocols provided with the respective assays. If multiplexing with intracellular glycerol detection, RealTime-Glo™ and/or CellTiter-Fluor™ reagents can be added to the medium and measured prior to media removal. To determine if a treatment is toxic to cells, the LDH-Glo™ Cytotoxicity Assay may be multiplexed with the Glycerol-Glo™ Assay. A small (2–5µl) sample of medium can be removed to a separate plate for the LDH-Glo™ Assay, and the remaining cells and medium can be used for glycerol detection.

6.E. References

1. Vidugiriene, J. *et al.* (2014) Bioluminescent cell-based NAD(P)/NAD(P)H assays for rapid dinucleotide measurement and inhibitor screening. *Assay Drug Dev. Technol.* **12**, 514–26.
2. Leippe, D. *et al.* (2017) Bioluminescent assays for glucose and glutamine metabolism: High-throughput screening for changes in extracellular and intracellular metabolites. *SLAS Discovery* **22**, 366–77.
3. Morita, S.Y. and Terada, T. (2015) Enzymatic measurement of phosphatidylglycerol and cardiolipin in cultured cells and mitochondria. *Sci. Rep.* **5**, 11737.
4. *Glucose Uptake-Glo™ Assay Technical Manual* #TM467.

6.F. Related Products

Energy Metabolism Assays

Product	Size	Cat.#
Triglyceride-Glo™ Assay	5ml	J3160
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190
Glucose-Glo™ Assay	5ml	J6021
Glucose Uptake-Glo™ Assay	5ml	J1341
Glutamate-Glo™ Assay	5ml	J7021
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Lactate-Glo™ Assay	5ml	J5021
NAD(P)H-Glo™ Detection System	10ml	G9061
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
Mitochondrial ToxGlo™ Assay	10ml	G8000

Other sizes are available.

Viability Assays

Product	Size	Cat.#
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711
CellTiter-Glo® 2.0 Assay	10ml	G9241
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080

Other sizes are available.

Cytotoxicity Assays

Product	Size	Cat.#
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
CellTox™ Green Cytotoxicity Assay	10ml	G8741
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260

Other sizes are available.

6.F. Related Products (continued)

Multiplex Viability and Cytotoxicity Assays

Product	Size	Cat.#
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200

Other sizes are available.

Oxidative Stress Assays

Product	Size	Cat.#
ROS-Glo™ H_2O_2 Assay	10ml	G8820
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611

Other sizes are available.

Detection Instrumentation

Product	Size	Cat.#
GloMax® Discover System	each	GM3000
GloMax® Explorer System	each	GM3500

7. Summary of Changes

The following changes were made to the 5/23 revision of this document:

1. The legend for Figure 3 was updated.
2. Equations were reformatted and miscellaneous text edits made.
3. The font was updated.

[a]U.S. Pat. Nos. 9,273,343 and 9,951,372, European Pat. No. 2751089, Japanese Pat. No. 6067019 and other patents pending.