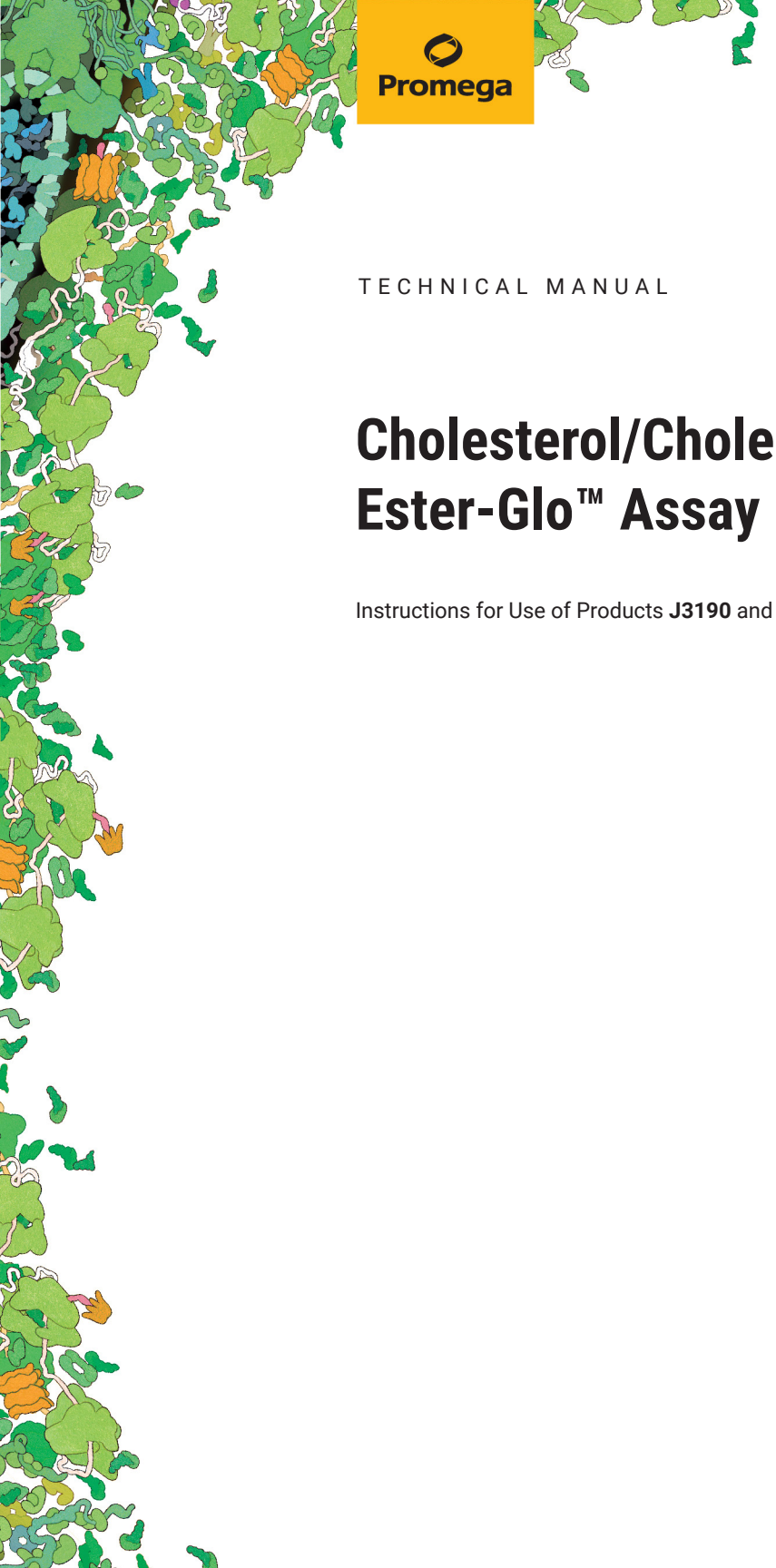

TECHNICAL MANUAL

Cholesterol/Cholesterol Ester-Glo™ Assay

Instructions for Use of Products **J3190** and **J3191**

Revised 6/23
TM601

Cholesterol/Cholesterol Ester-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 Cholesterol/Cholesterol Ester-Glo™ Assay[a] provides a luminescent method for measuring cholesterol and cholesterol esters in cultured cell lysates and other biological samples such as lipoprotein fractions, cell culture medium, serum and tissue homogenates. Cholesterol is an essential lipid involved in steroidogenesis, bile acid synthesis, cell signaling and maintenance of membrane structure. However, excessive levels of cholesterol contribute to inflammation, atherosclerosis, metabolic disease and cancer. In fact, the cholesterol content of lipoproteins is routinely used as an indicator of cardiovascular risk.

The Cholesterol/Cholesterol Ester-Glo™ Assay measures cholesterol using a cholesterol dehydrogenase that links the presence of cholesterol to the production of NADH and the activation of a proluciferin that produces light with luciferase (Figure 1). For measuring Cholesterol Ester, the assay includes a cholesterol esterase that will remove the fatty acid from cholesterol esters to produce one molecule of cholesterol per molecule of ester. The amount of cholesterol esters is determined from the difference of cholesterol measured in the absence (free cholesterol) and presence (total cholesterol) of esterase.

The Cholesterol/Cholesterol Ester-Glo™ Assay is part of a bioluminescent metabolite assay platform that offers rapid and sensitve metabolite detection in many sample types (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 convenient for high-throughput applications (Figure 2).

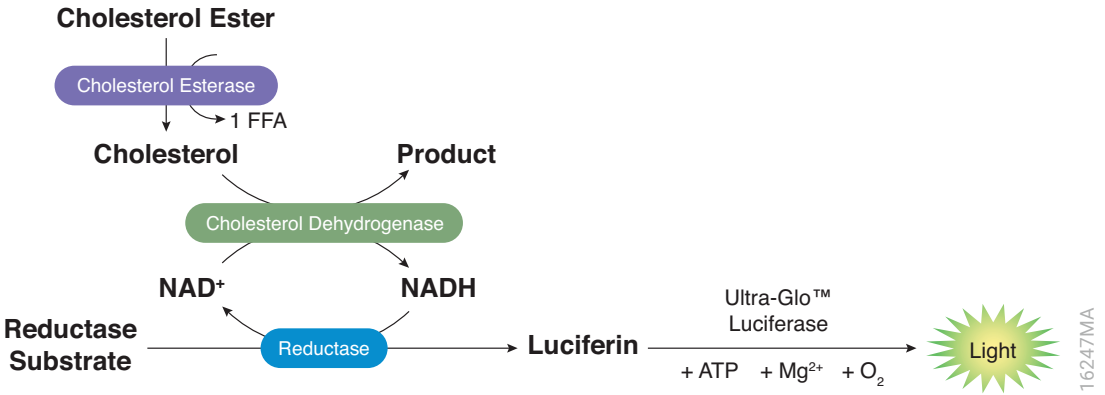

Figure 1. Schematic diagram of the Cholesterol/Cholesterol Ester-Glo™ Assay principle. Cholesterol esterase converts cholesterol esters to cholesterol. Cholesterol Dehydrogenase is used to generate NADH. In the presence of NADH, Reductase enzymatically reduces a pro-luciferin Reductase Substrate to luciferin. 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 cholesterol in the sample.

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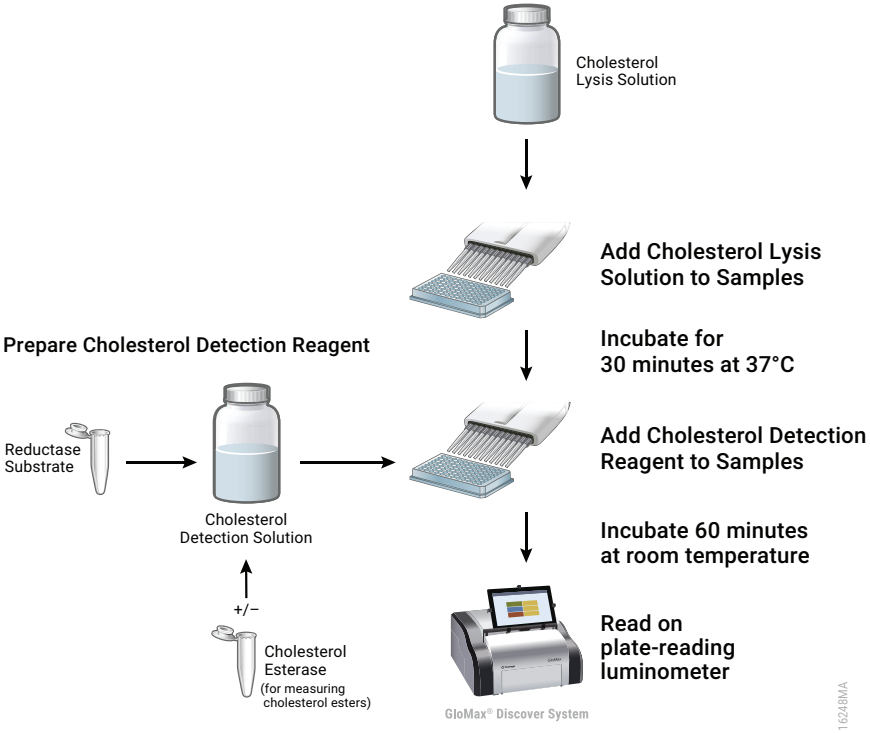

Figure 2. Cholesterol/Cholesterol Ester-Glo™ Assay reagent preparation and protocol. The protocol above is for measurement of total cholesterol. For measurement of free cholesterol, omit cholesterol esterase from the cholesterol detection reagent. Measurements in the absence and presence of esterase are performed in separate wells simultaneously to calculate the amount of cholesterol esters from the difference between free and total cholesterol.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Cholesterol/Cholesterol Ester-Glo™ Assay	**5ml**	**J3190**

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

- 10ml Cholesterol Lysis Solution
- 5ml Cholesterol Detection Solution
- 55µl Reductase Substrate
- 500µl Cholesterol Standard (20mM)
- 50µl Cholesterol Esterase

PRODUCT	SIZE	CAT.#
Cholesterol/Cholesterol Ester-Glo™ Assay	**50ml**	**J3191**

This system contains sufficient reagents to perform 1000 reactions in 96-well plates (50µl of sample + 50µl of cholesterol detection reagent). Includes:

- 100ml Cholesterol Lysis Solution
- 50ml Cholesterol Detection Solution
- 2 × 275µl Reductase Substrate
- 500µl Cholesterol Standard (20mM)
- 500µl Cholesterol Esterase

Storage Conditions: Store complete kits at less than –65°C. Alternatively, store the Cholesterol Detection Solution and Reductase Substrate at less than –65°C, store the Cholesterol Esterase at less than –10°C and store the Cholesterol Standard and Cholesterol Lysis Solution 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 adhere to your institution's safety guidelines and disposal requirements when working with biohazardous materials such as cells and cell culture reagents.

3. Measuring Total and Free Cholesterol

3.A. Materials to Be Supplied By the User

- PBS
- 96- or 384-well assay plates (opaque white-walled with clear or white bottom) compatible with a standard plate reader
- 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 prepared sample and 50µl of cholesterol detection reagent in a 96-well plate. The assay can be adapted to other volumes provided the 1:1 ratio of cholesterol detection reagent volume to sample volume is maintained (e.g., 20µl of sample and 20µl of cholesterol detection reagent in a 384-well format).

1. Thaw all components in a 22°C water bath and mix to ensure homogeneous solutions prior to use. Place the Reductase Substrate and Cholesterol Esterase on ice; all other components can be held at 22°C until use.
2. Determine the amount of cholesterol detection reagent necessary for your current experiment. Use the reagent on the day it is prepared; do not store it for later use.
3. To prepare cholesterol detection reagent, add 10µl of Reductase Substrate per ml of Cholesterol Detection Solution and mix by inversion. Cholesterol detection reagent without Cholesterol Esterase is used to measure free cholesterol.
4. To prepare cholesterol detection reagent with Esterase, add 10µl of Reductase Substrate and 10µl Cholesterol Esterase per ml of Cholesterol Detection Solution and mix by inversion. Cholesterol detection reagent with Esterase is used to measure total cholesterol.

3.C. Assay Protocol

This protocol is for measuring free (without esterase) and total (with esterase) cholesterol. The quantity of cholesterol esters can be calculated as the difference between total and free cholesterol, determined in separate wells simultaneously.

Since cholesterol levels can vary significantly in different samples (see Table 2), samples may need to be diluted to a concentration below 80µM to fit within the linear range of the assay. Twice as much lysis solution as detection solution has been included in the assay for this purpose.

This kit includes a 20mM Cholesterol Standard that can be used to generate a standard curve (Figure 3) to confirm that samples are within the linear range of the assay and to calculate cholesterol concentration. If the sample relative light unit (RLU) values fall outside the linear range of the cholesterol standard curve, the sample dilutions should 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. Since the esterase reaction releases one molecule of cholesterol per molecule of ester, free and total cholesterol can be calculated from the cholesterol standards in the presence and absence of esterase; a cholesterol ester standard is not required.

3.C. Assay Protocol (continued)

1. For each sample, standard or background control, prepare wells for measurement with and without esterase as needed.

   For Medium, Serum and Homogenized Tissue Samples:

   i. Dilute samples in Cholesterol Lysis Solution to bring their cholesterol concentrations below 80µM. Transfer 25µl of sample, standard or background control to a 96-well plate. Refer to Table 2 for dilution recommendations.

   ii. Add 25µl of Cholesterol Lysis Solution, shake briefly and incubate for 30 minutes at 37°C.

   For Adherent Cells and 3D Cultures:

   i. Remove medium from cells in a 96-well plate. Wash cells twice with 100µl of PBS.

   ii. Add 50µl of Cholesterol Lysis Solution, shake briefly and incubate for 30 minutes at 37°C.

   iii. If needed, dilute samples in Cholesterol Lysis Solution to bring their cholesterol concentrations below 80µM. Transfer 50µl of any diluted samples, standards or background controls to empty wells in a 96-well white-walled assay plate.

2. Add 50µl of cholesterol detection reagent with or without Esterase as prepared in Section 3.B to all wells.

3. Shake the plate for 30–60 seconds by hand or at a low rpm on a plate shaker.

4. Incubate at room temperature for 1 hour.

5. Record luminescence using a plate-reading luminometer such as the GloMax® Discover.

   Note: The light signal continues to increase until all cholesterol is consumed and the signal plateaus. At any time point the signal is directly proportional to cholesterol concentration.

6. Calculate free and total cholesterol by comparison of the luminescence of samples and standards assayed under the same conditions (See Figure 3).

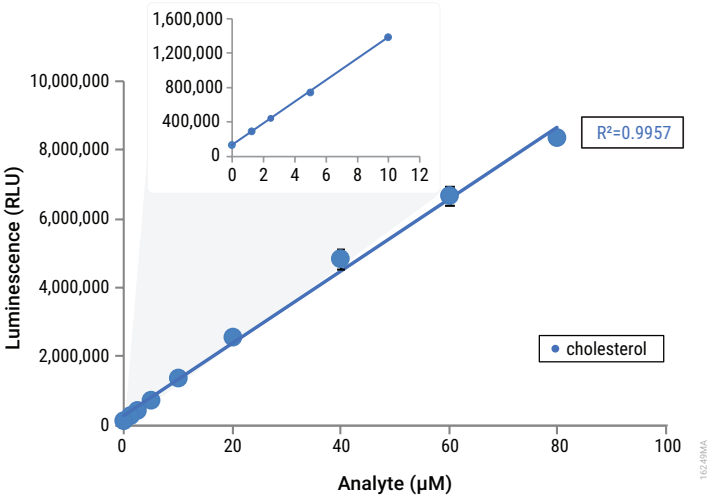

Figure 3. Cholesterol standard curves. Dilutions of the provided Cholesterol Standard (20mM, in ethanol) were prepared in Cholesterol Lysis Solution, beginning with a 2µl aliquot of Cholesterol Standard into 498µl of Cholesterol Lysis Solution. (Refer to Table 1 for concentrations of Cholesterol Standard used in standard curve.) A 50µl aliquot of cholesterol detection reagent was added to 50µl of each standard in triplicate and the luminescence was read after 1 hour. Concentration was plotted against average RLU at each standard point and a linear curve was fit. The Cholesterol/Cholesterol Ester-Glo™ Assay can detect less than 1µM cholesterol and has an upper limit of 80µM cholesterol. See Sections 4.A and 4.B for other ways to convert luminescence into concentration.

Table 1. Cholesterol Titration Data.

Cholesterol (µM)	80	60	40	20	10	5	2	1	0
RLU (thousands)	8,343	6,685	4,825	2,537	1,389	745	444	293	134
STDEV (thousands)	229	277	285	31	35	8	7	2	3
CV	2.7%	4.1%	5.9%	1.2%	2.5%	1.0%	1.6%	0.7%	2.5%
S/B	62.5	50.1	36.1	19.0	10.4	5.6	3.3	2.2	1.0
S/N	2,416.5	1,928.5	1,381.2	707.4	369.6	180.1	91.5	46.8	–

Note: Coefficient of variation (CV) is 100 × STDEV/RLU. Signal-to-background ratio (S/B) is 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.

3.C. Assay Protocol (continued)

Table 2. Recommendations for Sample Preparation.

Sample Type	Expected Cholesterol Concentration in Sample	Preparation Recommendations
Cell culture medium (extracellular)	Variable	•Dilute medium samples in Cholesterol Lysis Solution
Cell lysates (intracellular)	Variable	•Remove media from cells and wash with PBS •Add Cholesterol Lysis Solution •Further dilution may be necessary
Differentiated adipocytes	10–100s of µM	• Remove media from cells and wash with PBS • Add Cholesterol Lysis Solution • Further dilution may be necessary
Tissues	e.g., 100s of µM in a 25 mg/ml mouse liver homogenate	• Homogenize tissue with Tissue Tearor or other homogenization device • Add Cholesterol Lysis Solution • Further dilution (e.g., 10-fold) may be necessary
Plasma and serum	100s of µM	• Dilute plasma or serum samples (e.g., 10-fold or more) in Cholesterol Lysis Solution
Purified lipoproteins	mM levels in mg/ml amounts of lipoprotein	• Dilute lipoproteins (e.g., 200-fold) in Cholesterol Lysis Solution

4. Example Experiments

4.A. Measuring Cholesterol and Cholesterol Esters in Lipoproteins

It is important to note that if the amount of free cholesterol is significantly greater than the amount of esterified cholesterol, it may not be possible to detect the ester. We have found this to be true with a number of cancer cell lines. In contrast, lipoproteins have significant amounts of cholesterol esters, and it is easy to detect them.

In Figure 4, esterified and unesterified cholesterol concentrations were measured using a single standard for quantification. Signals from a lipoprotein sample (sample RLU), a cholesterol standard (standard RLU) of known concentration (STD) and a negative control (background RLU) containing no cholesterol were applied to the following formula:

$$[\text{Cholesterol}] = \frac{\text{standard} \times (\text{sample RLU} - \text{background RLU})}{(\text{standard RLU} - \text{background RLU})}$$

Considering the data in Figure 4, and 250- or 500-fold dilutions of high density lipoprotein (HDL) or low density lipoprotein (LDL), respectively, this formula yields free and total cholesterol concentrations of 2.2mM and 6.8mM for HDL and 7.4mM and 17.7mM for LDL, respectively. The difference between free and total corresponds to cholesterol ester concentrations of 4.6mM in HDL and 10.3mM in LDL.

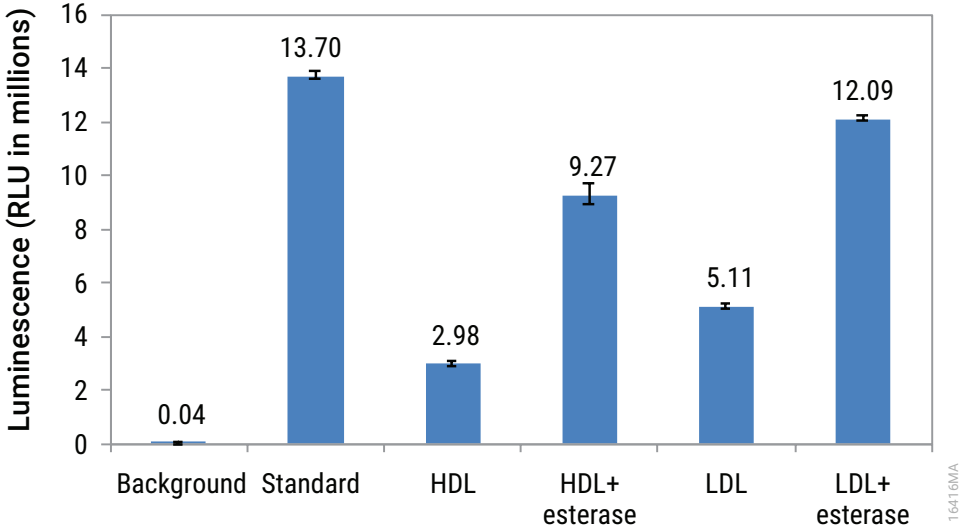

Figure 4. Cholesterol detection in human lipoproteins using a single cholesterol standard. Human high density lipoprotein (HDL, 10mg/ml) and human low density lipoprotein (LDL, 5mg/ml) were purchased from Kalen Biomedical. The HDL and LDL samples were diluted 250- and 500-fold into Cholesterol Lysis Solution, respectively, and 50µl aliquots were assayed per the protocol, with and without esterase. 40µM and 0µM of cholesterol in Cholesterol Lysis Solution were assayed as the standard and background controls, respectively. The RLU value (in millions) is listed at the top of each bar.

4.A. Measuring Cholesterol and Cholesterol Esters in Lipoproteins (continued)

The relative amounts of lipoproteins in serum samples can also be determined by quantifying total cholesterol. An important feature of such an experiment is to have a way to separate the different lipoprotein fractions; an example using PEG8000 is shown in Figure 5.

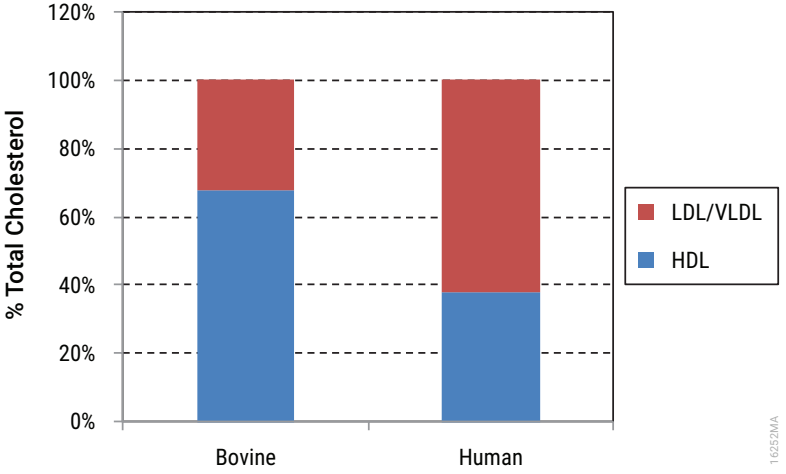

Figure 5. Relative amounts of high vs. low and very low density lipoproteins (VLDL) in sera. Samples of bovine and human sera were mixed 5:2 with 20% PEG8000, incubated for 20 minutes at room temperature and then centrifuged for 30 minutes at 10,000 × *g*. HDL in the supernatant was transferred to a fresh tube and the LDL/VLDL in the precipitate was resuspended in PBS. Each lipoprotein fraction was diluted 100-fold into Cholesterol Lysis Solution and then assayed for total cholesterol (unesterified + esterified) as per the standard protocol.

4.B. Measuring Cholesterol in Mouse Liver Tissue

Cholesterol concentrations in a mouse liver homogenate were measured using a cholesterol standard spike for quantification (Figure 6). Signals from mouse liver samples minus (sample RLU) or plus (sample + spike RLU) of an added amount of cholesterol (spike) and from a negative control (background RLU) containing no cholesterol were applied to the following formula:

$$[\text{Cholesterol}] = \frac{\text{spike} \times (\text{sample RLU} - \text{background RLU})}{(\text{sample + spike RLU} - \text{sample RLU})}$$

Using the data in Figure 6, and accounting for a 10-fold dilution in Cholesterol Lysis Solution, this formula yields a value of 102µM cholesterol in a 25mg/ml mouse liver homogenate.

Note that the homogenate itself does not interfere with the detection chemistry because the ΔRLU ± 10µM cholesterol is the same in the absence (standard RLU – background RLU = 5.12 million RLU) or presence (sample + spike RLU – sample RLU = 5.30 million RLU) of the diluted homogenate.

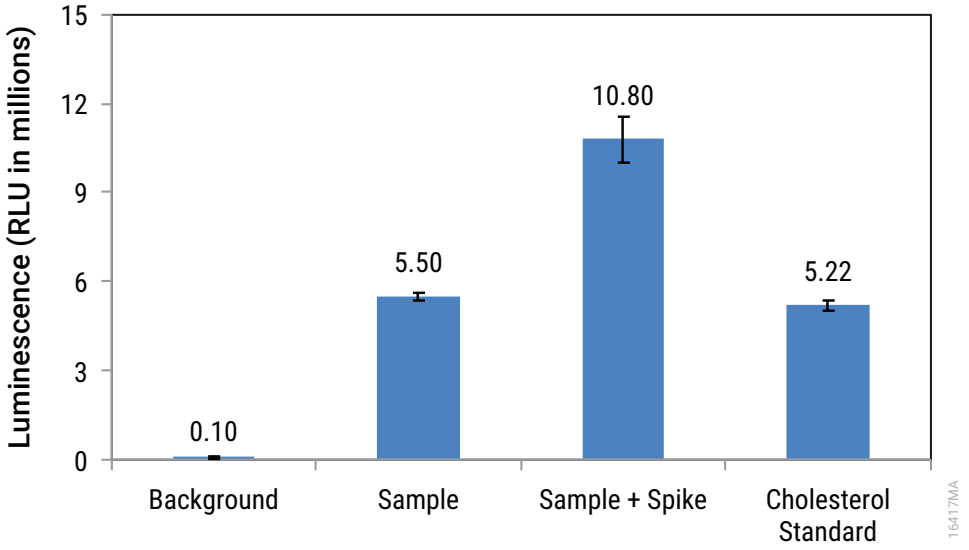

Figure 6. Cholesterol detection in mouse liver using a cholesterol spike. 25mg of mouse liver was placed in 1ml of PBS and homogenized with a Tissue Tearor (BioSpec Cat.# 985370-07) for 30 seconds. A 5µl aliquot of the homogenate was diluted into 45µl of Cholesterol Lysis Solution in the presence (sample + spike) or absence (sample) of an additional 10µM of cholesterol. These diluted samples were incubated for 30 minutes at 37°C and assayed per the standard protocol for free cholesterol. Cholesterol (10µM and 0µM) in Cholesterol Lysis Solution were assayed as the standard and background controls, respectively. The RLU value (in millions) is listed at the top of each bar.

4.C. Extraction of Cholesterol with β-Cyclodextrins

Some researchers choose to manipulate the cholesterol levels in their biological systems prior to starting an experiment by incubation with β-cyclodextrins. These molecules have a hydrophobic core that is roughly the size of cholesterol, and hence they can "extract" cholesterol from cells. However, extraction of too much cholesterol can lead to cell death, so it is important to control the amount of β-cyclodextrin and the time of incubation. In the experiment shown in Figure 7, HCT116 colon cancer cells were incubated with and without methyl-β-cyclodextrin, and samples of the medium were removed over time and assayed with the Cholesterol/Cholesterol Ester-Glo™ Assay. To monitor cell viability, RealTime-Glo™ MT Cell Viability Reagent was added to the cells and luminescence was measured over time. Cytotoxicity was also measured by removing samples of medium over time and assaying for lactate dehydrogenase (LDH) release with the LDH-Glo™ Cytotoxicity Assay.

4.C. Extraction of Cholesterol with β-Cyclodextrins (continued)

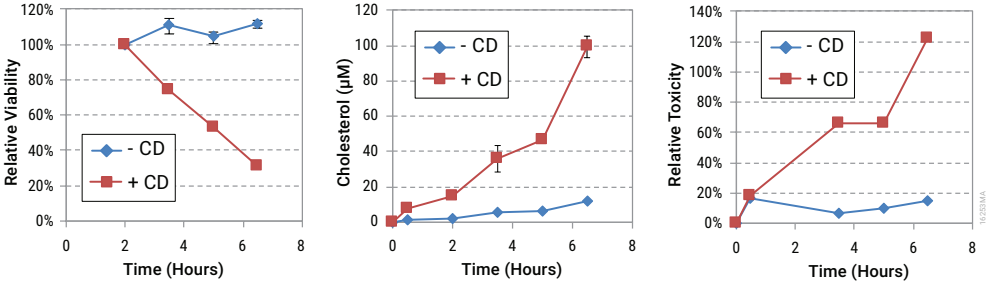

Figure 7. Multiplexing viability and cytotoxicity measurements with the Cholesterol/Cholesterol Ester-Glo™ Assay during extraction of cholesterol by β-cyclodextrin. Twenty-thousand HCT116 colon cancer cells per well were plated in a 96-well plate and incubated overnight. The cells were washed twice with PBS, and 100µl of medium was added containing DMEM, 2% BSA and 1X RealTime-Glo™ Reagent, in the absence (blue) or presence (red) of 20mM methyl-β-cyclodextrin. At the indicated times, luminescence from the RealTime-Glo™ Assay was measured, and aliquots of medium were collected to measure the amount of extracted cholesterol. These aliquots were diluted 20-fold into Cholesterol Lysis Solution for the Cholesterol/Cholesterol Ester-Glo™ Assay and 100-fold into LDH Storage Buffer for the LDH-Glo™ Assay. The latter two assays were performed once all samples were collected per their standard protocols. Viability was normalized to the luminescent value measured at 2 hours, cholesterol was calculated from a standard curve and toxicity was normalized to a maximum release sample (via addition of 0.2% Triton X-100).

5. Appendix

5.A. Signal Stability

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

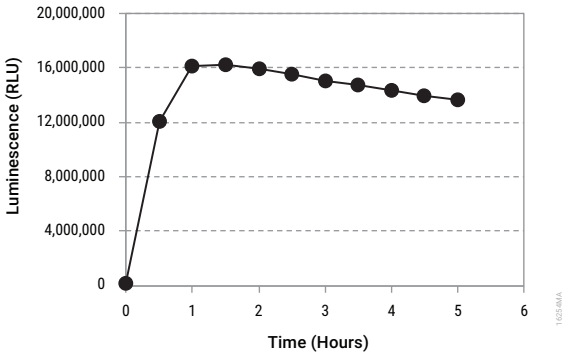

Figure 8. Signal Stability of the Cholesterol/Cholesterol Ester-Glo™ Assay. An equal volume of cholesterol detection reagent was added to 40µM of cholesterol in Cholesterol Lysis Solution and the luminescence was read over time. After 5 hours, the signal was >50% of the signal at 1 hour, which is convenient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

5.B. Cholesterol Solutions

Cholesterol is not very soluble or stable in aqueous solution, and it can adhere to plastic surfaces. Thus, it is important to store cholesterol in a nonaqueous solution and in glass containers. Our Cholesterol Standard is supplied as an ethanol solution in a glass vial. Significant concentrations of detergents are included in our lysis and detection solutions to maintain cholesterol concentrations while performing the assays, but cholesterol is not stable in these solutions over extended periods of time.

5.C. 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.

5.D. 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-walled, 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.

5.E. Multiplexing and Normalization

The Cholesterol/Cholesterol Ester-Glo™ Assay can be multiplexed to normalize for changes in viability and to account for well-to-well variation. If a sample of media is removed for assay, 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 cholesterol 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 Cholesterol/Cholesterol Ester-Glo™ Assay. A small (2–5µl) sample of medium can be removed for the LDH-Glo™ Cytotoxicity Assay to a separate plate, and the remaining cells and medium can be used for cholesterol detection. Refer to Section 4.C for example data.

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5.F. 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**, 514–56.
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. *Glucose Uptake-Glo™ Assay Technical Manual* #TM467.

5.G. Related Products

Energy Metabolism Assays

Product	Size	Cat.#
Glycerol-Glo™ Assay	5ml	J3150
Triglyceride-Glo™ Assay	5ml	J3160
Glucose Uptake-Glo™ Assay	5ml	J1341
Glutamate-Glo™ Assay	5ml	J7021
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glucose-Glo™ Assay	5ml	J6021
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.

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 Instruments

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

6. Summary of Changes

The following changes have been made to the 6/23 revision of this document:

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