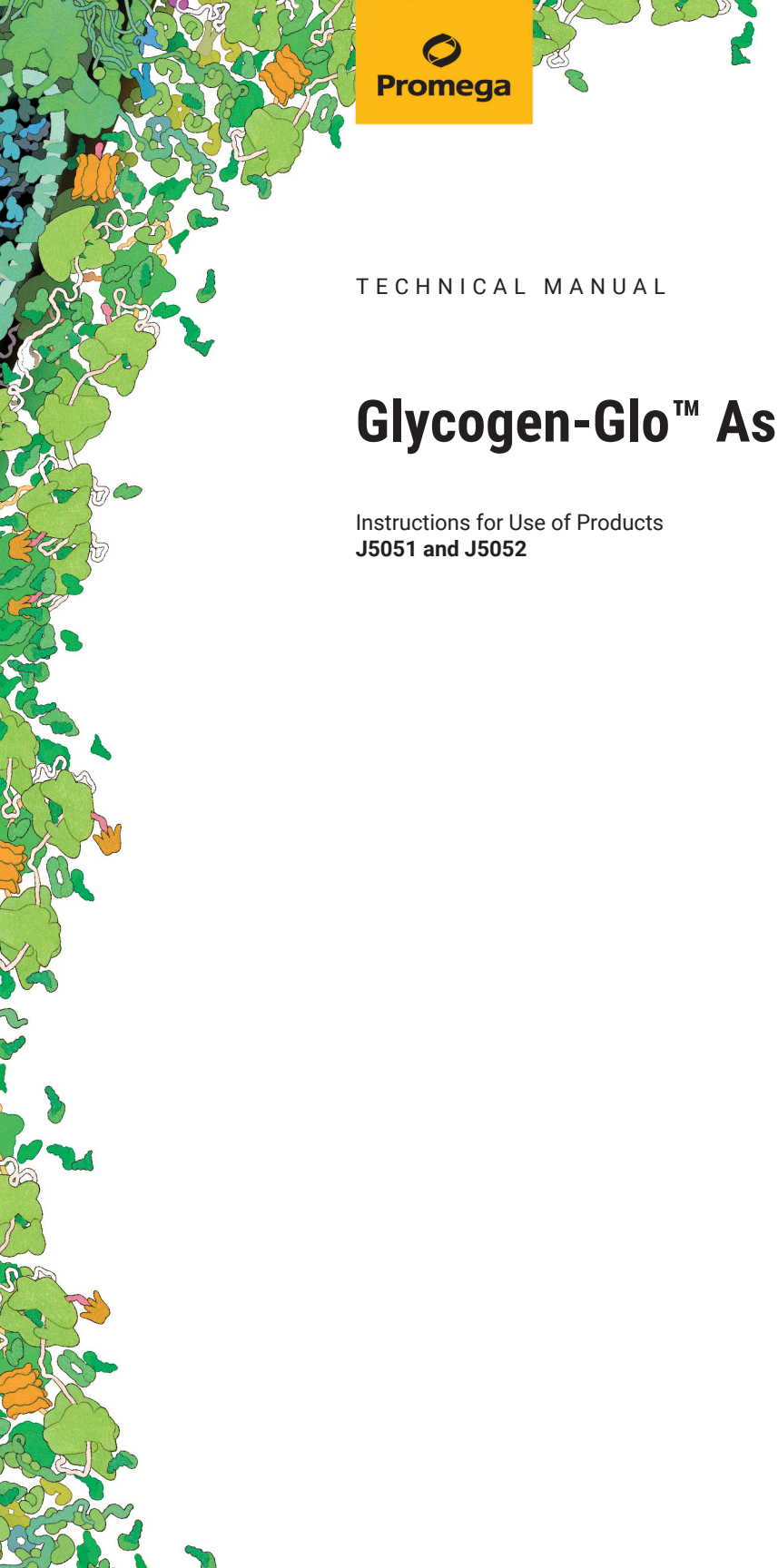

TECHNICAL MANUAL

Glycogen-Glo™ Assay

Instructions for Use of Products
J5051 and J5052

11/23
TM714

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Glycogen-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 Glycogen-Glo™ Assay[a] is a bioluminescent assay for the rapid and sensitive detection of glycogen in biological samples. Glycogen is a large intracellular polymer of glucose synthesized by cells as a means of storing glucose to supply energy needs at both the cellular and organism levels. The glucose can be quickly mobilized to meet cell energy demands without relying on external glucose sources. The liver has large reserves of glycogen, which are used to sustain glucose blood levels and maintain glucose homeostasis throughout the body.

The Glycogen-Glo™ Assay uses two sequential steps to measure glycogen. Glycogen is first digested into glucose units by Glucoamylase enzyme. Then glucose is measured by coupling glucose oxidation and NADH production with a bioluminescent NADH detection system (Figure 1; 1–3). When the glucose detection reagent is added to a sample at a 1:1 ratio, the coupled enzyme reactions start and run simultaneously (Figure 2). The luminescent signal is proportional to the amount of glucose in the sample and increases until all glucose is consumed, at which point a stable luminescent signal is achieved (Figure 3 and Table 1). Samples that contain glucose will require two reactions to determine glycogen levels as described in Section 3.B.

The assay is sensitive and will detect <1µg/ml glycogen (limit of detection (L.O.D.) 20ng/ml; Table 1). It is linear to 20µg/ml glycogen or 110µM glucose (Figure 3 and Table 1). If the starting sample has >20µg/ml glycogen, dilute so that it is within the linear range of the assay.

The Glycogen-Glo™ Assay is versatile and compatible with many sample types. We recommend up-front sample preparation to inhibit endogenous enzyme activity and to degrade endogenous NAD(P)H. To simplify sample preparation, we provide a protocol that includes a strong acid for sample lysis, enzyme inactivation and NAD(P)H degradation. The workflow is compatible with 96- and 384-well plate formats, does not require sample centrifugation or spin columns and is well-suited for rapidly analyzing multiple samples.

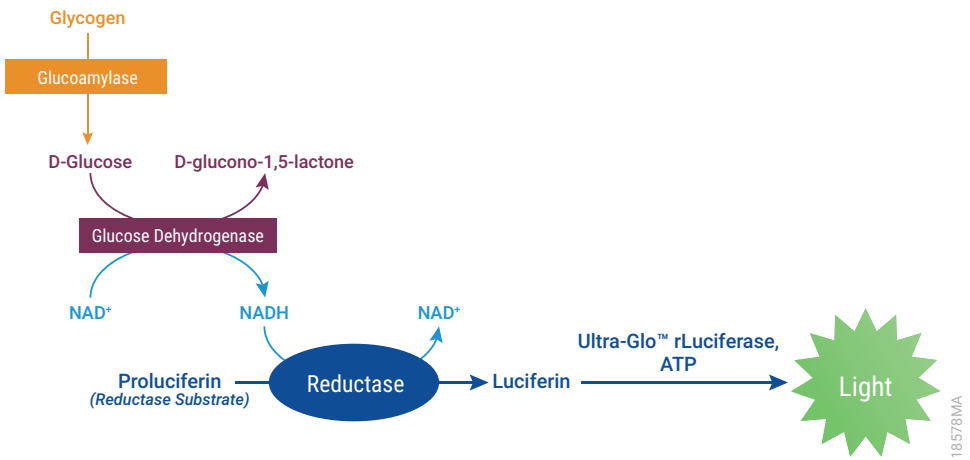

Figure 1. Schematic diagram of the Glycogen-Glo™ Assay principle. Glucoamylase catalyzes the conversion of glycogen to glucose. Glucose dehydrogenase catalyzes the oxidation of glucose with concomitant reduction of NAD+ to NADH. In the presence of NADH, Reductase enzymatically reduces proluciferin Reductase Substrate to luciferin. Luciferin is detected using Ultra-Glo™ Recombinant Luciferase, and the amount of light produced is proportional to the amount of glycogen and glucose in the sample.

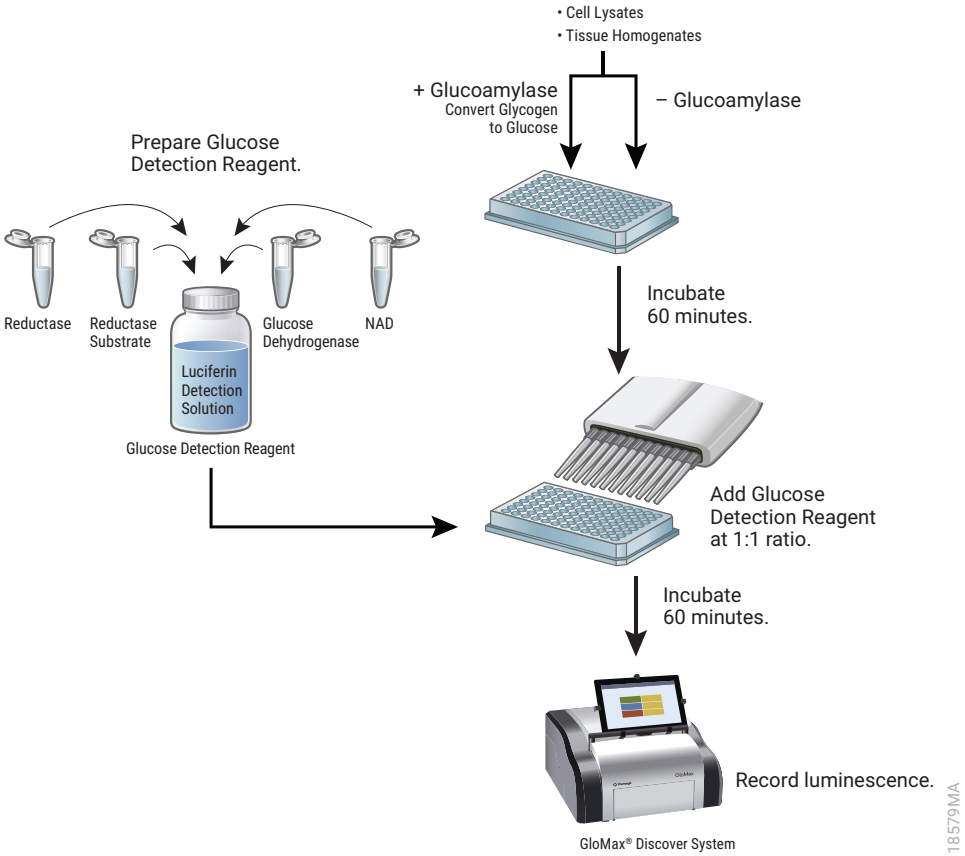

Figure 2. Glycogen-Glo™ Assay reagent preparation and protocol.

1. Description (continued)

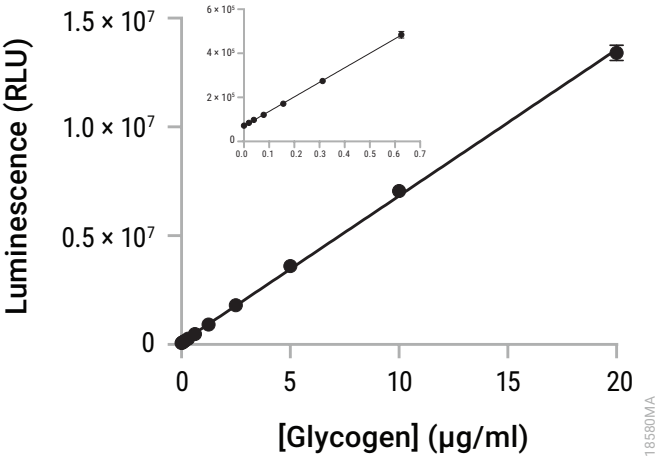

Figure 3. Glycogen titration curve. Twofold serial dilutions of glycogen, 0.02–20µg/ml, were prepared in phosphate-buffered saline (PBS). The negative control was PBS without glycogen. Aliquots of the prepared dilutions (25µl) were transferred to a 96-well plate and the assay was performed following the protocol in Section 3.D. Luminescence was measured in relative light units (RLU) using a GloMax® Discover System (Cat.# GM3000). Each data point represents the average of four replicates. Error bars are ±1 standard deviation.

Table 1. Glycogen Titration Data. The signal-to-background ratio (S/B) was calculated by dividing mean luminescence for samples by the mean luminescence for the negative control (no glycogen). The signal-to-noise ratio (S/N) was calculated by dividing net luminescence (mean luminescence for the sample minus mean luminescence for the negative controls) by the standard deviation of the negative control.

Glycogen (µg/ml)	0	0.02	0.04	0.08	0.16	0.31	0.62	1.25	2.5	5	10	20
Average Luminescence (RLU × 10^3)	71	84	98	121	171	274	484	923	1,811	3,605	7,052	13,410
Standard Deviation (RLU × 10^3)	3.5	4.9	5.1	5.9	7.4	11.6	14.9	24.9	39.7	88.9	180	359
Coefficient of Variation (%)	4.9	5.9	5.3	4.9	4.3	4.2	3.1	2.7	2.2	2.5	2.5	2.7
S/B	1.0	1.2	1.4	1.7	2.4	3.8	6.8	12.9	25.2	50.3	98.4	187
S/N	—	3.6	7.4	14.0	28.6	58	118	244	499	1,013	2,001	3,824

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Glycogen-Glo™ Assay	**5ml**	**J5051**

The system contains sufficient reagents to perform 100 reactions in 96-well plates. Includes:

- 5ml Luciferin Detection Solution
- 55µl Reductase
- 55µl Reductase Substrate
- 30µl NAD
- 200µl Glucose Dehydrogenase
- 25µl Glucoamylase
- 3ml Glucoamylase Buffer
- 50µl Glycogen, 2mg/ml
- 50µl Glucose, 10mM
- 15ml 0.3N HCl
- 15ml Tris Buffer

PRODUCT	SIZE	CAT.#
Glycogen-Glo™ Assay	**50ml**	**J5052**

The system contains sufficient reagents to perform 1,000 reactions in 96-well plates. Includes:

- 50ml Luciferin Detection Solution
- 275µl Reductase
- 275µl Reductase Substrate
- 275µl NAD
- 2 × 1ml Glucose Dehydrogenase
- 150µl Glucoamylase
- 30ml Glucoamylase Buffer
- 50µl Glycogen, 2mg/ml
- 50µl Glucose, 10mM
- 15ml 0.3N HCl
- 15ml Tris Buffer

Storage Conditions: Store the Glycogen-Glo™ Assay at less than –65°C. Alternatively, store the Reductase Substrate at less than –65°C protected from light, and all other components at –30°C to –10°C. The 0.3N HCl and Tris Buffer can be stored at +2°C to +10°C or at room temperature. Do not freeze-thaw the kit components more than three times. 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 Glycogen

Materials to Be Supplied By the User

- phosphate-buffered saline (PBS; e.g., Sigma Cat.# D8537 or GIBCO™ Cat.# 14190)
- 96-well assay plates (opaque white-walled with white or clear bottom; e.g., Corning® Cat.# 3903 or 3912)
- luminometer (e.g., GloMax® Discover System, Cat.# GM3000)

3.A. Sample Preparation

Metabolism is a dynamic process. Work quickly while collecting and preparing samples.

The Glycogen-Glo™ Assay can be used to measure glycogen in samples such as cells and tissues. This requires preparing cell lysates and tissue homogenates. For sample preparation we recommend using the 0.3N HCl (acid) and Tris Buffer (450mM Tris, pH 8.0) supplied with this kit. Acid treatment lyses cells, rapidly stops metabolism, inhibits endogenous enzyme activity and destroys endogenous NAD(P)H dinucleotides. When dealing with difficult-to-lyse samples, such as 3D cultures, Triton® X-100 can be added to 0.3N HCl to a final concentration of 0.2%.

After acid treatment is complete, Tris Buffer is added to the sample. This will raise the pH slightly but will not neutralize the acid treatment of the sample. The combination of 0.3N HCl and Tris Buffer was optimized to achieve pH 5 once the Glucoamylase Buffer is added. This is the required pH for efficient glucoamylase activity.

Prepared samples can be assayed immediately following the protocol in Section 3.D or stored at –20°C. If needed, an aliquot of the sample can be removed for protein measurement (see Section 5.A).

Acid treatment is compatible with multiwell plate workflows and can be directly added to cells plated in 96- or 384-well plates. Although cells lysed with acid might appear not completely lysed when viewed under a microscope (Figure 4), metabolites are efficiently released.

We do not recommend using detergent lysis without acid because many dehydrogenases remain active in detergent lysed samples, significantly increasing the Glycogen-Glo™ Assay background.

Perchloric treatment or KOH treatment, recommended by other kits, is not compatible with the Glycogen-Glo™ Assay and should not be used.

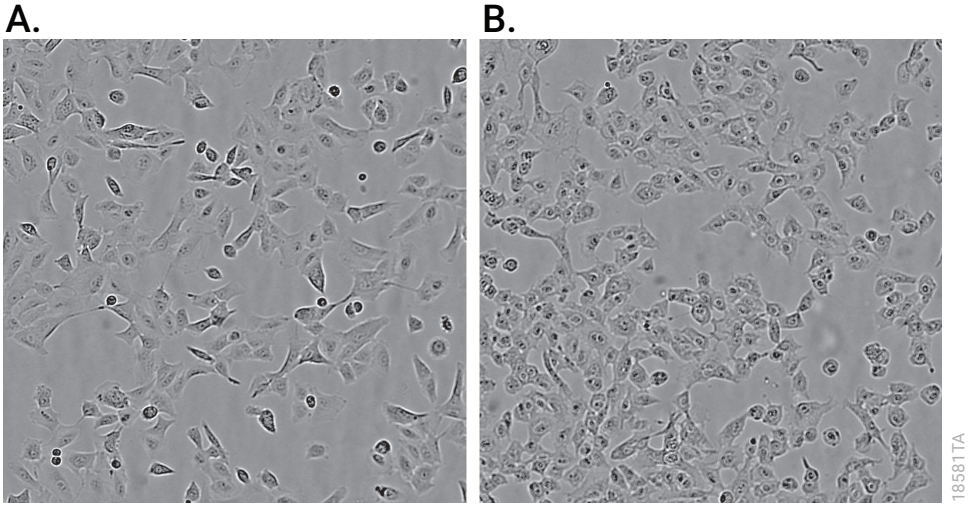

Figure 4. Cell imaging after acid addition. A549 cells were plated at 25,000 cells/well in DMEM (GIBCO™ Cat.# A14430) and 10% dialyzed FBS (GIBCO™ Cat.# A3382001) in a 96-well plate (Greiner Cat.# 655094) overnight. Cells were washed three times with 100µl of PBS. PBS (**Panel A**) or PBS premixed with 0.3N HCl at 1:0.5 volume ratio to lyse cells (**Panel B**) was added to each corresponding well. The plate was shaken and wells were imaged at 10X with a Tecan Spark® Cyto instrument.

Table 2. Recommended Sample Preparation.

Sample	Glycogen Concentration in Sample	Recommendations
Cell lysates (intracellular)	1–10µg/ml for 20,000 cells lysed in 60µl	• Cells in PBS • Add 0.3N HCl (1/2 of the sample volume). • Add Tris Buffer (1/2 of the sample volume).
Tissues	10µg/ml for 5–15mg of liver homogenized in 1ml and diluted 50- to 100-fold	• Tissues in PBS • Add 0.3N HCl (1/2 of the sample volume). • Add Tris Buffer (1/2 of the sample volume).

3.B. Considerations for Samples that Contain Glucose

The Glycogen-Glo™ Assay uses two sequential steps to measure glycogen. Glycogen is first digested into glucose units by the enzyme, Glucoamylase, followed by the detection of released glucose. The signal from glycogen is only generated in the presence of Glucoamylase (Figure 5). Light signals from glycogen samples are typically ≥70% of the signals from glucose samples at the same concentration (Figure 5). This suggests effective digestion of glycogen into glucose monomers, which can simplify data interpretation and calculations.

Samples that contain both glycogen and glucose require two reactions to determine the glycogen concentration. The first reaction is with the enzyme Glucoamylase, to measure total glucose resulting from digested glycogen plus any starting glucose in the sample. The second reaction is with Glucoamylase Buffer only (no Glucoamylase) to detect any glucose present in the sample prior to digestion. The difference in signal between the two reactions represents the glycogen contribution in the sample.

Cell lysate samples should be tested to determine if glucose is present (Section 4.A). Tissue samples contain glucose and will require two reactions (Section 4.B).

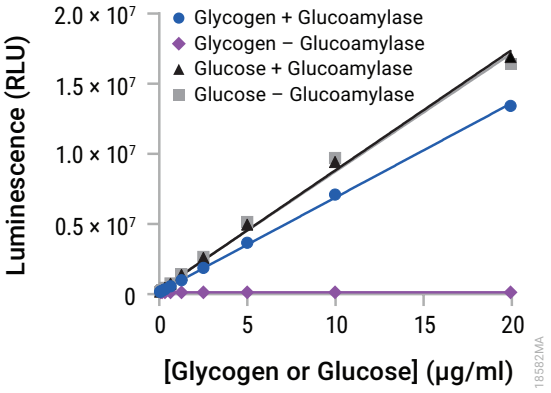

Figure 5. Glycogen and glucose titration curves with and without glucoamylase. Twofold serial dilutions of glycogen, 0.02–20µg/ml, were prepared in PBS. The negative control was PBS without glycogen. In parallel, twofold dilutions of glucose were also prepared starting at 20µg/ml, which corresponds to 110µM. Aliquots of the prepared dilutions (25µl) were incubated with Glucoamylase enzyme for glycogen digestions or with Glucoamylase Buffer only. The assay was performed following the protocol in Section 3.D. Luminescence was measured in relative light units (RLU) using a GloMax® Discover System. Each data point represents the average of four replicates. Error bars are ± 1 standard deviation.

3.C. Reagent Preparation

This protocol is for a reaction with 25µl of sample and 25µl of glucoamylase digestion solution, followed by the addition of 50µl of glucose detection reagent in a 96-well plate. The assay can be adapted to other volumes provided the 1:1:2 ratio of sample volume to glucoamylase digestion solution volume to glucose detection reagent volume is maintained (e.g., 12.5µl of sample and 12.5µl of glucoamylase digestion solution plus 25µl of glucose detection reagent in a 384-well format). To use a different assay format, scale the volumes of samples, controls, glycogen standards and reagents, accordingly.

When calculating the volume of glucoamylase digestion solution and glucose detection reagent to prepare, consider the following:

- Include glycogen positive controls and negative controls in your calculations. For negative controls, use wells containing sample buffer only for determining assay background. Information on preparing and using appropriate positive and negative controls for the Glycogen-Glo™ Assay can be found in Section 3.E.
- We recommend preparing additional reagent to compensate for pipetting error.
- Prepare only the volume of glucoamylase digestion solution and glucose detection reagent needed for each experiment. Unused glucoamylase digestion solution and glucose detection reagent cannot be stored.

1. Thaw all components at room temperature. Once thawed, keep the Glucoamylase Buffer and Luciferin Detection Solution at room temperature. Place all other components on ice. Mix thawed components prior to use.
2. Prepare the glucoamylase digestion solution by diluting Glucoamylase 200-fold into Glucoamylase Buffer as shown below.

Component	Volume Per Reaction	Volume Per 100 Reactions
Glucoamylase Buffer	25µl	2.5ml
Glucoamylase	0.125µl	12.5µl

3. Mix by gently vortexing or inverting 5 times.
4. Immediately before use, prepare the glucose detection reagent by combining components as shown below.

Component	Volume Per Reaction	Volume Per 100 Reactions
Luciferin Detection Solution	50µl	5ml
Reductase Substrate	0.25µl	25µl
Reductase	0.25µl	25µl
NAD	0.25µl	25µl
Glucose Dehydrogenase	2.0µl	200µl

5. Mix by gently inverting 5 times.

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3.D. Protocol

When performing the Glycogen-Glo™ Assay be sure to use plates that are compatible with your luminometer. See Section 5.C for more information on assay plates.

This protocol is for a reaction with 25µl of sample and 25µl of Glucoamylase, plus 50µl of glucose detection reagent in a 96-well plate.

1. Prepare samples using the appropriate method for your sample type. See Sections 3.A and 4 for more information.
2. Arrange samples and controls for analysis.
    a. **For glycogen analysis:** Transfer 25µl of each sample, positive control (glycogen standards in the same buffer as the samples) and negative control (buffer only) into a well of a 96-well plate.
    b. **(Optional) For endogenous glucose analysis:** Transfer an additional 25µl of each sample into a second well (See Section 3.B.).
3. Prepare the glucoamylase digestion solution as described in Section 3.C.
4. Perform glycogen digestion or control reactions.
    a. **For glycogen analysis:** Add 25µl of glucoamylase digestion solution to each well prepared in Step 2.a.
    b. **(Optional) For endogenous glucose analysis:** Add 25µl Glucoamylase Buffer only (with no Glucoamylase) to the second well prepared in Step 2.b.
5. Mix by gently shaking the plate for 30–60 seconds.
6. Incubate for 60 minutes at room temperature.
7. Prepare the glucose detection reagent as described in Section 3.C. Ensure that the reagent is at room temperature prior to use.
8. Add 50µl of glucose detection reagent to each well.
9. Mix by gently shaking the plate for 30–60 seconds.
10. Incubate for 60–90 minutes at room temperature.

    Note: The light signal will increase until all glucose is consumed and the signal plateaus. At any time point, the signal is directly proportional to the glucose concentration.
11. Record luminescence using a plate-reading luminometer as directed by the manufacturer.

3.E. Assay Controls and Data Analysis

There is a linear relationship between luminescence signal and glycogen concentration, and many luminescent measurements can be described simply in terms of relative light units (RLU). The data can be analyzed as the change in RLU between the experimental controls and test conditions. When comparing changes in luminescence, wells containing buffer only should be included as negative controls and can be subtracted as assay background.

Different buffers can affect light output. Therefore, standards and negative controls (buffer only) should be prepared using the same buffers as the samples.

To calculate glycogen concentration and determine if your samples are within a linear range of the assay, a standard curve using a titration of 2mg/ml Glycogen, included in the kit, can be used (see Table 1). If the sample RLU values fall outside of the linear range of the glycogen standard curves, the sample dilutions should be adjusted and re-assayed.

Alternatively, in place of running a full standard curve, 2 to 4 concentrations of glycogen standard can be used and glycogen concentrations in samples can be calculated based on RLU from those concentrations. We recommend including a high concentration (10 or 20µg/ml), a low concentration (0.2 or 0.4µg/ml) and concentrations that are near your sample concentration. These points can be adjusted based on the glycogen concentration expected in your sample. For the most accurate results, include standards on the same assay plate as the test samples.

The Glycogen-Glo™ Assay also includes 10mM Glucose. While the efficiency of converting glycogen to glucose is typically ≥70%, when samples contain a significant amount of starting glucose (e.g., detected in the reactions with only Glucoamylase Buffer), the glucose concentration can be determined separately using a glucose standard curve (Figure 5). To determine the amount of glycogen in these samples, subtract the glucose concentration (in µg/ml) from the glycogen + glucose concentration (in µg/ml) that was determined in the reactions with glucoamylase digestion solution. Diluting the Glucose, 10mM standard 90-fold results in a standard that is 110µM, which equates to 20µg/ml (glucose molecular weight is 180.2).

4. Example Protocols and Data for Various Sample Types

This section includes example protocols that were used to generate data depicted in this Technical Manual. Optimization may be required, depending on sample type and experimental conditions.

4.A. Mammalian Cells

Metabolism is a dynamic process guided by fuel availability. Levels of glycogen are greatly affected by the glucose concentration in the cell culture medium. The formulations of commonly used cell culture media such as DMEM and RPMI 1640 contain different amounts of small metabolites, including glucose, glutamine, amino acids and other components, which need to be considered when studying metabolism. Supplementing the culture medium with 5–10% of fetal bovine serum (FBS) is a standard practice when culturing mammalian cells and will also contribute variable levels of metabolites. Using defined medium, for example DMEM (GIBCO Cat.# A1443001) lacking major fuel sources such as glucose, glutamine and pyruvate, adding those components back at the desired concentrations, and supplementing with dialyzed serum (e.g., GIBCO Cat.# A3382001) allows better control for studying metabolic changes.

4.A. Mammalian Cells (continued)

Measuring glycogen in cells requires cell lysate preparation. The Glycogen-Glo™ Assay can be used for monitoring changes in intracellular glycogen levels in cells plated in 96- or 384-well plates. Cell processing steps have been simplified to permit in-well lysis, eliminating the need for cell collection, centrifugation and spin columns. Alternatively, cells can be collected in bulk, lysed and samples can be transferred to 96- or 384-well plates for glycogen measurements.

Two reactions are needed to measure glycogen in cell lysates. One reaction measures total glucose resulting from digested glycogen plus any starting glucose in the lysate. The second reaction measures glucose in the lysate prior to digestion.

In our experience, intracellular glucose levels are very low because glucose is rapidly metabolized to glucose-6-phosphate. Therefore, any glucose detected in this step may represent residual glucose from the medium and can be reduced by additional wash steps. If the glucose has been determined to be undetectable or insignificant, the second reaction measuring only glucose can be omitted from the protocol.

The glucose levels in cell culture media are very high and can range from 5–25mM. Washing is required to reduce the levels to <1µM to obtain assay sensitivity. Residual glucose can also contribute to well-to-well variability.

Example protocol for measuring glycogen in cells plated in 96-well plates.

1. Plate 5,000–50,000 cells per well in 96-well tissue culture plates. Add compounds to the cells if treatment is part of the experimental design. Incubate plates for the desired timeframe.
2. After incubation, remove and discard the medium and wash the cells five times with 200µl of cold PBS.

   Notes:

   a. Washes are necessary to remove glucose present at high concentrations in the medium.
   b. Work quickly to minimize changes in glycogen metabolism.
3. Add 30µl of PBS to the washed cells.
4. Add 15µl of 0.3N HCl (e.g., 1/2 sample volume) to lyse cells. Alternatively, combine PBS (Step 3) with 0.3N HCl, then add to the washed cells.
5. Mix by gently shaking the plate for 5 minutes.
6. Add 15µl of Tris Buffer (e.g., same volume as 0.3N HCl, Step 4).
7. Mix by gently shaking the plate for 30–60 seconds.
8. Arrange samples and controls for analysis:

   a. **For glycogen analysis:** Transfer 25µl of each sample, positive control (glycogen standards in the same buffer as the samples) and negative control (buffer only) into a well of a 96-well plate.

   b. **(Optional) For endogenous glucose analysis:** Transfer an additional 25µl of each sample into a second well (See Section 3.B).
9. Prepare the glucoamylase digestion solution as described in Section 3.C.

10. Perform glycogen digestion or control reaction:
    a. **For glycogen analysis:** Add 25µl of glucoamylase digestion solution to each well prepared in Step 8.a.
    b. **(Optional) For endogenous and residual glucose analysis:** Add 25µl of Glucoamylase Buffer only (no Glucoamylase) to the second well prepared in Step 8.b.
11. Mix by gently shaking the plate for 30–60 seconds.
12. Incubate for 60 minutes at room temperature.
13. Add 50µl of glucose detection reagent prepared as described in Section 3.C to each well.
14. Mix by gently shaking the plate for 30–60 seconds.
15. Incubate for 60–90 minutes at room temperature.
16. Record luminescence.

Figure 6 shows an example of measuring glycogen in adherent A549 cancer cells using the described protocol. The data show that the intracellular glycogen was measured with >twofold signal above background with 5,000 cells plated per well and that the signal proportionally increased with increasing cell numbers. The wells without Glucoamylase had low background signal, indicating minimal starting glucose in the samples.

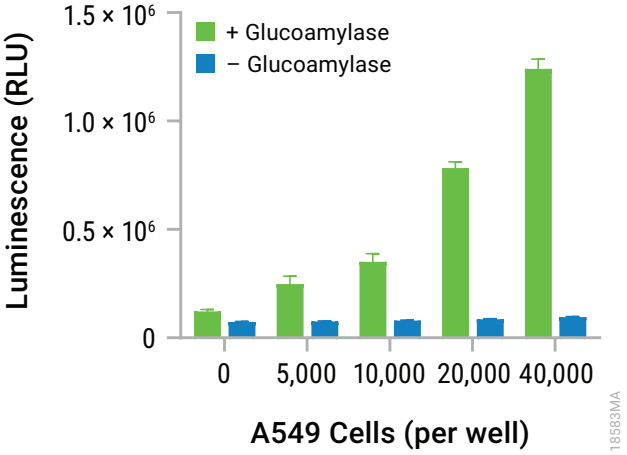

Figure 6. Intracellular glycogen. A549 cells in 100µl of F-12 medium supplemented with 10% serum were plated at 5,000, 10,000, 20,000 and 40,000 cells per well. After an overnight incubation, the medium was removed, cells were washed five times with 200µl cold PBS, and glycogen was measured as described in the example protocol. The average luminescence for four replicates is shown in relative light units (RLU). Error bars are ± 1 standard deviation.

4.A. Mammalian Cells (continued)

Example protocol for measuring glycogen with collected or suspension cells in a tube.

1. Collect cells, wash five times with cold PBS and resuspend in PBS to a concentration of 5×10^4–3.2×10^6 cells/ml.
2. Add 1/2 volume of 0.3N HCl to lyse cells (e.g., add 500µl of 0.3N HCl per 1ml cells in PBS). Mix well and incubate for 5 minutes at room temperature.
3. Add the same volume of Tris Buffer as 0.3N HCl in Step 2. Mix well.

   Note: Aliquots can be removed for protein measurement and stored at or below –20°C (Section 5.A).
4. Arrange samples and controls for analysis.
   a. **For glycogen analysis:** Transfer 25µl of each sample, positive control (glycogen standards in the same buffer as the samples) and negative control (buffer only) into a well of a 96-well plate.
   b. **(Optional) For endogenous glucose analysis:** Transfer a second 25µl of each sample into a second well (see Section 3.B).
5. Prepare the glucoamylase digestion solution as described in Section 3.C.
6. Perform glycogen digestion or control reaction:
   a. **For glycogen analysis:** Add 25µl of glucoamylase digestion solution to each well prepared in Step 4.a.
   b. **(Optional) For endogenous and residual glucose analysis:** Add 25µl Glucoamylase Buffer only (no Glucoamylase) to the second well prepared in Step 4.b.
7. Mix by gently shaking the plate for 30–60 seconds.
8. Incubate for 60 minutes at room temperature.
9. Add 50µl of glucose detection reagent prepared as described in Section 3.C to each well.
10. Mix by gently shaking the plate for 30–60 seconds.
11. Incubate for 60–90 minutes at room temperature.
12. Record luminescence.

Representative data for three cell lines using the described protocol for measuring glycogen with collected or suspension cells in a tube are shown in Figure 7. The data show a linear relationship between light signal and cell density, indicating that glycogen measurements are within the linear range of the assay. Cellular glycogen content varies greatly, depending on cell type and growth conditions (4). The high sensitivity and wide linearity of the assay accommodates measurement of glycogen in cells with low or high glycogen levels.

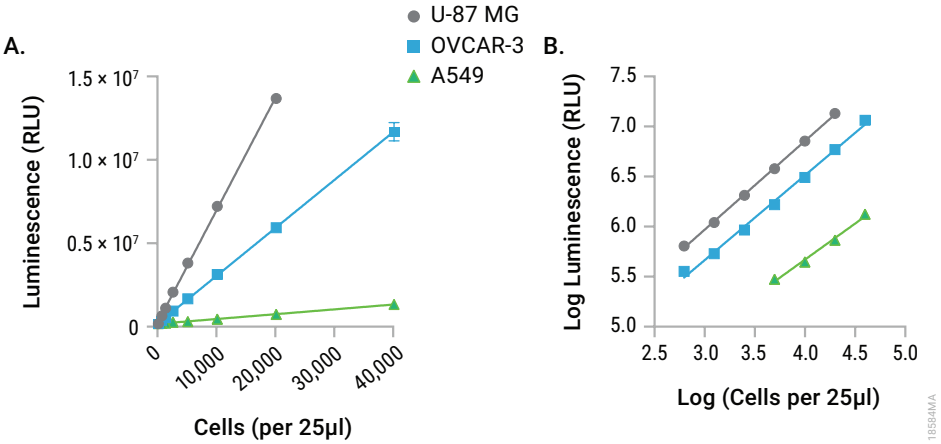

Figure 7. Measuring glycogen in cell lysates. A549, OVCAR-3 and U-87 MG cells were cultured overnight in T75 flasks in their growth medium (F-12, RPMI and EMEM, respectively) supplemented with 10% serum. The cells were trypsinized, collected, washed and counted. The cells were resuspended to 3.2 × 10^6 cells/ml in PBS and lysates were prepared as described in the protocol. To determine the linear range of glycogen measured in cell lysates, twofold serial dilutions were prepared in PBS premixed with 0.3N HCl and Tris Buffer at a 1:0.5:0.5 ratio. Twenty-five microliters (25µl) of each dilution was transferred to a 96-well assay plate and glycogen was measured using the example protocol. Data are the average of three replicates. **Panel A.** Linear-linear plot. **Panel B.** Log-log plot. Error bars are ± 1 standard deviation.

4.B. Tissues

The Glycogen-Glo™ Assay can be used to measure the glycogen concentration in homogenized tissues. To fit into the glycogen assay detection range and avoid assay interference, we recommend homogenizing the tissues at 3–15mg/ml of tissue in PBS premixed with 0.3N HCl. Homogenized tissues can be assayed immediately or stored below –10°C.

Tissues can contain glucose in addition to glycogen. Therefore, two reactions are needed to measure glycogen in tissue homogenates. One reaction is used to measure total glucose resulting from digested glycogen plus any starting glucose in the homogenate. The second is used to measure any glucose in the homogenate prior to glycogen digestion.

Glycogen content of tissues varies widely. Tissues such as liver will contain high levels of glycogen and will require dilutions to fit into the linear range of the assay. We recommend testing several dilutions. Dilute the tissue homogenate diluted in the homogenate solution (PBS + 0.3N HCl + Tris Buffer premixed in a 2:1:1 ratio). For quantitation, also use the homogenate solution to prepare positive (glycogen standards) and negative (buffer only) controls.

Example protocol for measuring glycogen in tissues.

1. Slice and weigh 5–15mg of tissue. Add 500µl of PBS.
2. Immediately add 1/2 volume (250µl) of 0.3N HCl.

   Note: The PBS (Step 1) and 0.3N HCl (Step 2) can be premixed before adding to tissue slice.
3. Homogenize for 20–30 seconds using a mechanical tissue homogenizer (e.g., Tissue-Tearor™, BioSpec Cat.# 985370-07).
4. Add 250µl of Tris Buffer to the homogenate (same volume as 0.3N HCl in Step 2).

   Note: To determine protein concentration in tissue lysate, analyze an aliquot of the sample; see Section 5.A.
5. Transfer 25µl of each homogenate into two separate wells of a 96-well assay plate. Include positive (glycogen standard) and negative (buffer only) controls. Controls should be prepared using the solution used to prepare the homogenates (PBS + 0.3N HCl + Tris Buffer premixed in a 2:1:1 ratio).

   Note: If dilutions are required to fit into the linear range of the assay, also use the premixed homogenate solution as the diluent.
6. Add 25µl of glucoamylase digestion solution, prepared as described Section 3.C, to one well and 25µl of Glucoamylase Buffer only to the second well. Add glucoamylase digestion solution to all control wells.
7. Mix gently on a plate shaker for 30–60 seconds.
8. Incubate at room temperature for 60 minutes.
9. Add 50µl of glucose detection reagent prepared as described in Section 3.C.
10. Mix gently on a plate shaker for 30–60 seconds.
11. Incubate at room temperature for 60–90 minutes.
12. Record luminescence.

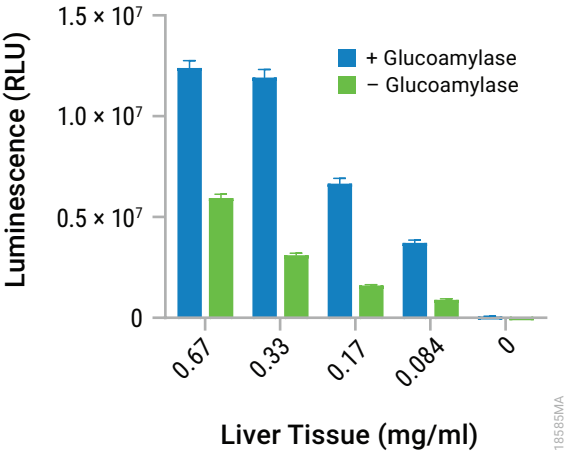

Figure 8. Measuring glycogen in tissues. A 10.7mg slice of frozen mouse liver tissue (BioIVT) was homogenized in PBS with 0.3N HCl, as described in the protocol (Section 4.B), for a final tissue concentration of 10.7mg/ml (1mg/ml protein). For glycogen detection, samples were serially diluted twofold in homogenate solution and 25µl of each dilution was transferred to two wells of an assay plate. Wells containing 25µl of homogenate solution were included as negative controls. Glycogen was measured following the example protocol (Section 4.B). Using a glycogen standard curve, the concentration of glycogen was determined to be 60µg glycogen/mg liver tissue. A ≥32-fold dilution to ≤0.33mg/ml, was necessary to fit into the linear range of the assay. There was also measurable glucose in the wells incubated with Glucoamylase Buffer only, which contributed to about 24% of the total signal.

5. Appendix

5.A. Multiplexing and Normalization for Mammalian Cells

The Glycogen-Glo™ Assay can be multiplexed with viability assays, including RealTime-Glo™ MT Cell Viability Assay (Cat.# G9711) and CellTiter-Fluor™ Cell Viability Assay (Cat.# G6080). Viability assays are useful tools for normalizing glycogen measurements to the number of viable cells and for separating immediate effects on glycogen metabolism from global effects on cell health. To multiplex the cell viability assays and glycogen detection using the same population of cells, perform the cell viability assay first as described in the appropriate technical manual. After viability measurements, remove the medium, wash cells with PBS and lyse as described in the example protocol for adherent cells (Section 4.A). After acid lysis and Tris Buffer addition, remove an aliquot of each sample for protein measurement and ATP detection using CellTiter-Glo® Viability Assay (Cat.# G9241). Use the remaining sample for the glycogen measurement, following the protocol described in Section 3.D.

5.A. Multiplexing and Normalization for Mammalian Cells (continued)

To measure protein concentration, we recommend the Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific Cat.# A53225), which is compatible with acid/base lysis and is sensitive enough to measure protein levels in 5,000–50,000 cells lysed in 100µl in a 96-well plate. At lower cell densities, we recommend using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific Cat. #23235), which has higher sensitivity (<5,000 cells/100µl). However, it is important to remove aliquots for protein determination before adding Tris Buffer because Tris interferes with the Micro BCA Protein Assay.

Representative data for multiplexing viability assays, ATP detection and glycogen measurements in A549 cancer cells are shown in Figure 9. Linear increases in signal with increasing cell number were observed with all assays. The data can be normalized by directly comparing the luminescent signal generated by the Glycogen-Glo™ Assay to the fluorescence and/or luminescence signals of the viability and ATP assays. The glycogen concentration can also be calculated and normalized to protein amount. The amount of glycogen per microgram (µg) of protein was similar for all three cell densities.

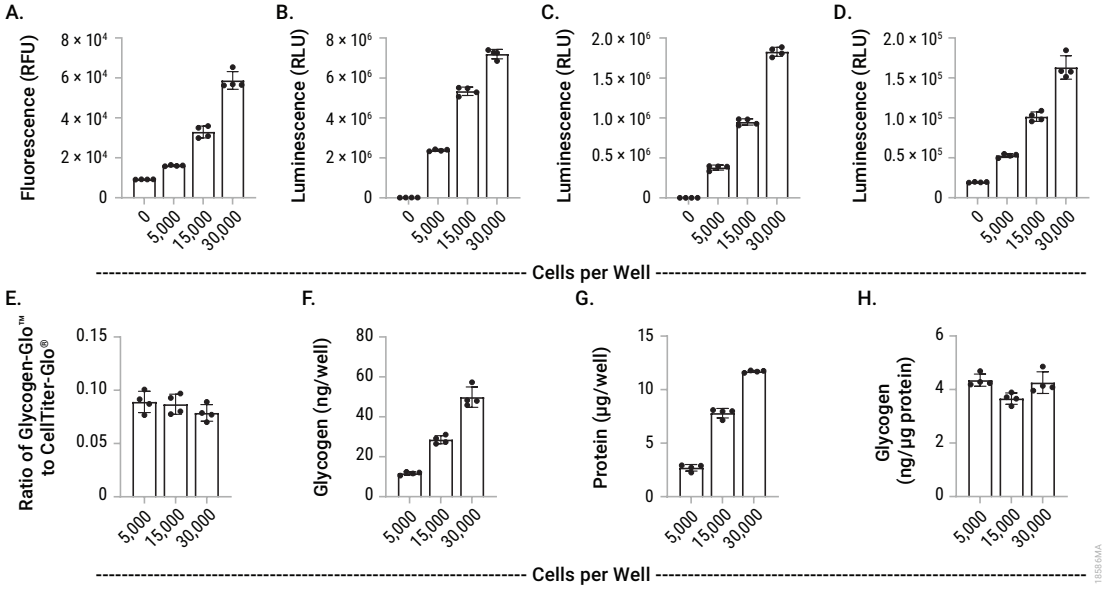

Figure 9. Multiplexing viability, ATP and protein measurements with glycogen detection. A549 cells in F-12 medium with 10% serum, were plated at 5,000, 15,000 and 30,000 cells per well. The Glycogen-Glo™ Assay was performed using the protocol in Section 3.D with the following modifications: On the day of the assay and before 0.3N HCl treatment, viability was assessed using (**Panel A**) the CellTiter-Fluor™ Assay (Cat.# G9260) and (**Panel B**) the RealTime-Glo™ MT Cell Viability Assay (Cat.# G9711). After 0.3N HCl and Tris Buffer treatment, an aliquot was removed to analyze ATP content (**Panel C**) using the CellTiter-Glo® Assay (Cat.# G7570). Glycogen (**Panel D**) was then measured using the described protocol and normalized to ATP content (**Panel E**). The amount of glycogen per well (**Panel F**) was calculated based on the glycogen standard. Prior to glycogen detection, an aliquot was removed for protein measurement (**Panel G**) using the Pierce™ Rapid Gold BCA Protein Assay Kit (Cat.# A53225), and the amount of glycogen was normalized to total protein (**Panel H**).

5.B. Temperature and Reagent Compatibility

The intensity and stability of the luminescent signal is temperature sensitive. For consistent results, equilibrate the reagent to room temperature before using.

Avoid the presence of DTT and other reducing agents in the samples to be tested. Reducing agents will react with the Reductase Substrate and increase background.

5.C. Plates and Equipment

Most standard plate readers are designed to measure luminescence and are suitable for this assay. Some instruments do not require gain adjustment while others might require optimizing the gain settings to achieve sensitivity and dynamic range. An integration time of 0.25–1 second per well should serve as a guidance. For exact instrument settings, consult the instrument manual.

Use opaque, white multiwell plates that are compatible with your luminometer (e.g., Corning® Costar® 96-well plates, Cat.# 3912, Costar® 384-well plates, Cat.# 3570). For cultured cells, white-walled clear bottom tissue culture plates (e.g., Corning® 96-well plates, Cat.# 3903) are acceptable. Luminescent metabolite assays are well suited for miniaturization. When samples are limited, consider using 96-half area (Corning® Cat. #3696), 384-well (Costar® Cat.# 3570) or 384-low volume (Corning® Cat.# 4512) plates. We do not recommend black or clear plates. Light 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 data. Although relative luminescence output will vary with different instruments, this variation does not affect assay performance.

5.D. References

1. Zhou, W. *et al.* (2014) Self-immolative bioluminogenic quinone luciferins for NAD(P)H assays and reducing capacity-based cell viability assays. *ChemBioChem.* **15**, 670–5.
2. 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.
3. Leippe, D. *et al.* (2017) Bioluminescent assays for glucose and glutamine metabolism: High-throughput screening for changes in extracellular and intracellular metabolites. *SLAS Discov.* **22**, 366–77.
4. Rousset, M., Zweibaum, A. and Fogh, J. (1981) Presence of glycogen and growth-related variations in 58 cultured human tumor cell lines of various tissue origins. *Cancer Res.* **41**, 1165–70.

Promega

5.E. Related Products

Energy Metabolism Assays

Product	Size	Cat.#
BCAA-Glo™ Assay	5ml	JE9300
BHB-Glo™ (Ketone Body) Assay	5ml	JE9500
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190
Dehydrogenase-Glo™ Detection System	5ml	J9010
Glucose-Glo™ Assay	5ml	J6021
Glucose Uptake-Glo™ Assay	5ml	J1341
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycogen-Glo™ Assay	5ml	J5051
Lactate-Glo™ Assay	5ml	J5021
Malate-Glo™ Assay	5ml	JE9100
Metabolite-Glo™ Detection System	5ml	J9030
Triglyceride-Glo™ Assay	5ml	J3160

Additional sizes available.

Oxidative Stress Assays

Product	Size	Cat.#
GSH/GSSG-Glo™ Assay	10ml	V6611
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
ROS-Glo™ H_2O_2 Assay	10ml	G8820

Additional sizes available.

Cell Viability, Cytotoxicity and Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay System	2.5ml	G8090
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011
RealTime-Glo™ MT Cell Viability Assay	100 assays	G9711

Additional sizes available.

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