TECHNICAL MANUAL

Glutamate-Glo™ Assay

Instructions for Use of Products
J7021 and J7022

3/17
TM495

Glutamate-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 Glutamate-Glo™ Assay is a bioluminescent assay for rapid, selective and sensitive detection of glutamate in biological samples. The assay couples glutamate oxidation and NADH production with a bioluminescent NADH detection system (1,2) (Figure 1). Glutamate dehydrogenase uses glutamate and NAD^+ to produce α-ketoglutarate and NADH. In the presence of NADH a pro-luciferin Reductase Substrate is converted by Reductase to luciferin that is then used by Ultra-Glo™ Recombinant Luciferase to produce light.

When Glutamate Detection Reagent, containing glutamate dehydrogenase, NAD^+, Reductase, Reductase Substrate and Luciferase, is added to a sample containing glutamate at a 1:1 ratio, the enzyme-coupled reactions start and run simultaneously (Figure 2). The luminescent signal is proportional to the amount of glutamate in the sample and increases until all glutamate is consumed, at which point a stable luminescent signal is achieved (Figure 3, Table 1).

The Glutamate-Glo™ Assay is a versatile system that is amenable to higher-throughput formats (3) and compatible with many sample types (Figure 2). Samples may require upfront sample processing, including dilutions, to fit into the linear range of the assay. They may also require inactivation of endogenous enzyme activity/deproteinization and NAD(P)H degradation (Section 3.C, Table 2). To simplify sample processing, methods for rapid enzyme inactivation and NAD(P)H degradation are provided that are compatible with 96- and 384-well plates and do not require sample centrifugation or spin columns.

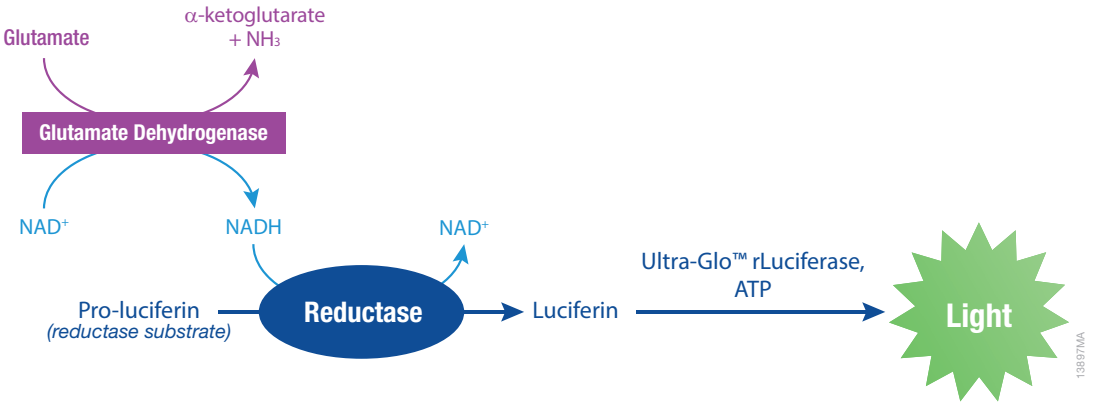

Figure 1. Schematic diagram of the Glutamate-Glo™ Assay principle. Glutamate dehydrogenase catalyzes the oxidation of glutamate with concomitant reduction of NAD^+ to NADH. In the presence of NADH, Reductase enzymatically reduces a pro-luciferin Reductase Substrate to luciferin. Luciferin is detected in a luciferase reaction using Ultra-Glo™ rLuciferase and ATP, and the amount of light produced is proportional to the amount of glutamate in the sample.

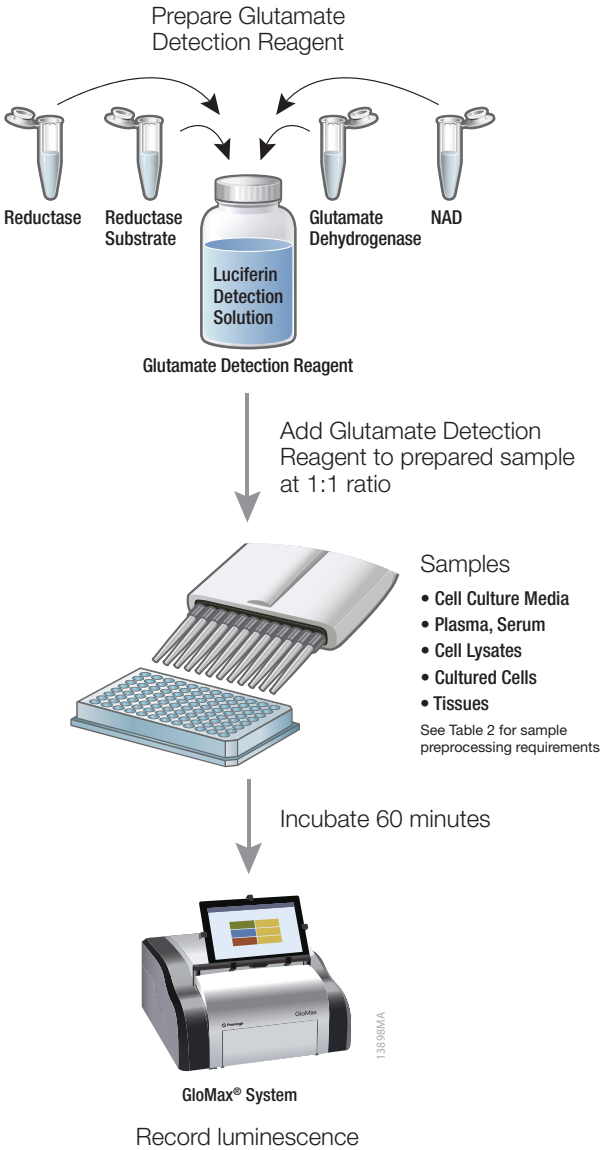

Figure 2. Glutamate-Glo™ Assay reagent preparation and protocol.

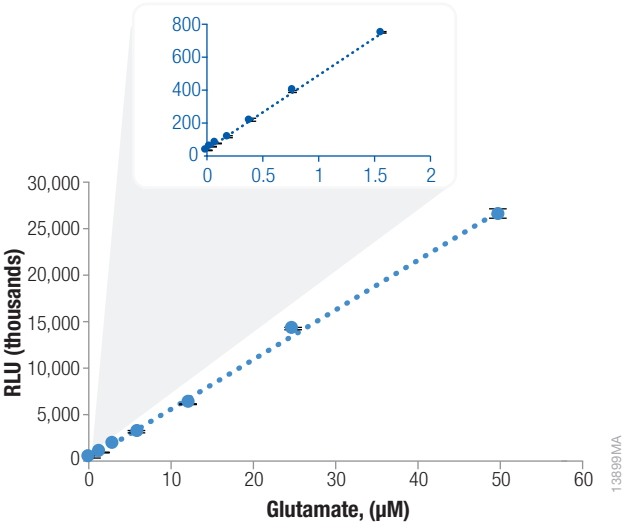

Figure 3. Glutamate titration curve. Twofold serial dilutions of glutamate were prepared in PBS, starting from 50µM. Aliquots of the prepared standards (50µl) were transferred to a 96-well plate and the assay was performed following the protocol in Section 3.B. Data represent the average of four replicates from readings using a GloMax® Luminometer.

Table 1. Glutamate Titration Data

Glutamate, µM	0	0.049	0.195	0.781	3.13	12.5	50
Ave. RLU (thousands)	24	44	109	385	1,482	5,878	26,572
St. Dev. (thousands)	0.45	0.33	0.95	1.63	5.71	13.5	450
CV	2%	1%	1%	0.4%	0.4%	0.2%	2%
S/B	–	1.9	4.6	16.2	62.3	247	1,117
S/N	–	46	189	800	3,226	12,592	58,732

Note: Average relative light unit (RLU) and standard deviation values are in thousands. Signal-to-background (S/B) was calculated by dividing the mean signal from samples by the mean signal from negative controls. Signal-to-noise (S/N) was calculated by dividing the net signal (mean signal minus mean negative control) by the standard deviation of the negative control. The table represents data from four-fold serial dilutions of glutamate.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Glutamate-Glo™ Assay	**5ml**	**J7021**

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

- 5ml Luciferin Detection Solution
- 55µl Reductase
- 55µl Reductase Substrate
- 100µl Glutamate Dehydrogenase
- 275µl NAD
- 50µl Glutamate (10mM)

PRODUCT	SIZE	CAT.#
Glutamate-Glo™ Assay	**50ml**	**J7022**

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

- 50ml Luciferin Detection Solution
- 275µl Reductase
- 275µl Reductase Substrate
- 1ml Glutamate Dehydrogenase
- 1ml NAD
- 50µl Glutamate (10mM)

Storage Conditions: Store complete kits 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. Do not freeze-thaw the kit components more than three times.

3. Measuring Glutamate

Materials to be Supplied by the User

- phosphate-buffered saline (PBS, e.g., Sigma Cat.# D8537 or Gibco Cat.# 14190) or other compatible buffer
- 96-well assay plates (white or clear bottom, e.g., Corning Cat.# 3903 or 3912)
- luminometer (e.g., GloMax® Discover Cat.# GM3000)

3.A. Reagent Preparation

This protocol is for a reaction with 50µl of sample and 50µl of Glutamate Detection Reagent in a 96-well plate. The assay can be adapted to other volumes provided the 1:1 ratio of Glutamate Detection Reagent volume to sample volume is maintained (e.g., 12.5µl of sample and 12.5µl Glutamate Detection Reagent in a 384-well format).

1. Thaw all components. Once thawed, equilibrate the Luciferin Detection Solution to room temperature; all other components should be placed on ice. Be sure to mix thawed components to ensure homogeneous solutions prior to use.
2. Prepare Glutamate Detection Reagent as shown in the table below. The amount of Glutamate Detection Reagent to prepare per reaction is for a 96-well plate format using 50µl of prepared sample. Prepare the amount of reagent needed for your experiment, factoring in that some volume may be lost during pipetting.

Component	Per Reaction	Per 5ml
Luciferin Detection Solution	50µl	5ml
Reductase	0.25µl	25µl
Reductase Substrate	0.25µl	25µl
Glutamate Dehydrogenase	1.0µl	100µl
NAD	1.0µl	100µl

3. Mix by gently inverting five times.

   Note: Return unused Luciferin Detection Solution, Reductase, NAD, and Glutamate Dehydrogenase to storage at less than –65°C or –30°C to –10°C. Return unused Reductase Substrate to storage at less than –65°C protected from light. Do not store unused Glutamate Detection Reagent.

3.B. Protocol

Upfront sample processing may be required. See Section 3.C for guidelines on preparing your specific sample type, including cell culture media, cell lysates, tissues, or plasma and sera.

1. Transfer 50µl of sample or glutamate control into a 96-well plate. Include a negative control (buffer only) for determining assay background.
2. Add 50µl of Glutamate Detection Reagent prepared as described in Section 3.A.
3. Shake the plate for 30–60 seconds.
4. Incubate for 60 minutes at room temperature.

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

3.C. Sample Processing

The glutamate concentration in samples can vary significantly. For example, with cultured mammalian cells, the glutamate concentration can range from low micromolar values in freshly added medium to millimolar levels as cells grow and secrete glutamate. The presence of dehydrogenases, reduced NAD(P)H dinucleotides and other factors in the samples can affect the Glutamate-Glo™ Assay signal and background. Upfront sample processing, such as dilution to fit into the linear range and/or enzyme inactivation (deproteinization), may be required to avoid these issues.

Table 2 provides examples of glutamate concentration ranges in samples and suggestions for sample preparation. Section 4 provides example protocols for processing various sample types.

Promega

Table 2. Recommendations for Sample Processing

Recipes for Inactivation and Neutralization Solutions are provided in Table 3, Section 4.B.

Sample Type	Glutamate Concentration in Sample	Processing Recommendations
Cell culture medium (extracellular)	50µM to 2mM	• Dilute medium sample 40- to 100-fold in PBS
Cell lysates (intracellular)	10–30µM for 20,000 cells lysed in 50µl	• Remove media from cells, wash with PBS and add fresh PBS • Add Inactivation Solution (half of sample volume) • Add Neutralization Solution (same volume as Inactivation Solution)
Cells in culture (extracellular + intracellular)	Up to 50µM[1]	• Cells in medium or PBS • Add Inactivation Solution (1/8 of sample volume) • Add Neutralization Solution (the same volume as Inactivation Solution)
Tissues	25µM if 3mg of liver tissue is homogenized in 1ml	• Tissues in homogenization buffer • Add Inactivation Solution (1/8 of sample volume)[2] • Add Neutralization Solution (the same volume as Inactivation Solution)
Plasma and serum	50–100µM	• Dilute 10 to 50-fold in PBS

[1] Applicable for short incubation times (1–2 hours) when total glutamate production is within the linear range of the assay.

[2] Homogenizing tissue in buffer containing Inactivation Solution is the preferred method. If needed, Inactivation Solution can be added immediately after homogenization.

4. Example Protocols and Data for Various Sample Types

4.A. Cell Culture Medium

The Glutamate-Glo™Assay can be used to measure changes in glutamate concentration in mammalian cell culture medium. As growing cells continuously secrete glutamate, the concentration of glutamate in the medium will increase depending on cell type, cell density and time. Therefore, samples of the medium typically require dilution into the linear range of the Glutamate-Glo™ Assay. As a starting point, if the cells are plated in fresh medium and the secretion of glutamate is measured within 1–2 hours of incubation, no dilution is required. For longer incubation times, we recommend a 40- to 100-fold dilution in PBS.

An example showing measurement of glutamate secretion by ovarian carcinoma SKOV-3 cells is shown in Figure 4. Suspension cells can also be assayed; Figure 5 shows data with bone marrow leukemia K562 cells.

1. Plate 1,000–20,000 cells per well in a 96-well plate. Include control wells consisting of medium only.
2. Collect a sample of the medium at experimental time points by removing 2–5µl into 98–95µl PBS.
3. Proceed to Step 4 or freeze collected samples at –20°C until you are ready to perform the assay. Make sure the samples are well sealed. For example, collect the samples into a 96-well plate that is sealed with an adhesive plate sealer and a plastic plate lid.
4. On the day of the assay, thaw the samples and transfer 50µl to a 96-well assay plate.
5. Add 50µl of Glutamate Detection Reagent prepared as described in Section 3.A.
6. Shake the plate for 30–60 seconds to mix.
7. Incubate for 60 minutes at room temperature.
8. Record luminescence.

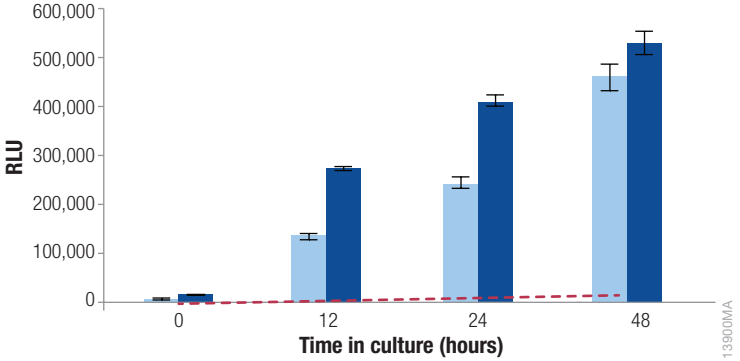

Figure 4. Glutamate secretion by SKOV-3 cells. SKOV-3 cells were plated at 5,000 (light blue bars) and 20,000 (dark blue bars) cells/well in DMEM medium (Gibco A14430) containing 5mM glucose, 2mM glutamine and 10% dialyzed FBS (Gibco 26400036). Wells with medium only were included as controls. At indicated time points, 2µl of medium was removed and diluted in 98µl PBS. The samples were frozen and stored at –20°C. On the day of the assay, the samples were thawed. A portion of the sample (25µl) was transferred to a 384-well assay plate and 25µl of Glutamate Detection Reagent was added. After 60 minutes at room temperature, luminescence was read using a Tecan instrument. The data represent the average of four replicates. The red line represents the luminescence values of the medium controls. A 25µM glutamate control corresponded to 906,931 RLUs and is not shown on the graph. All measured samples were within the linear range of the assay, and the calculated glutamate concentration in the medium increased from 3µM to 650µM for 5,000 cells/well and from 18µM to 750µM for 20,000 cells/well.

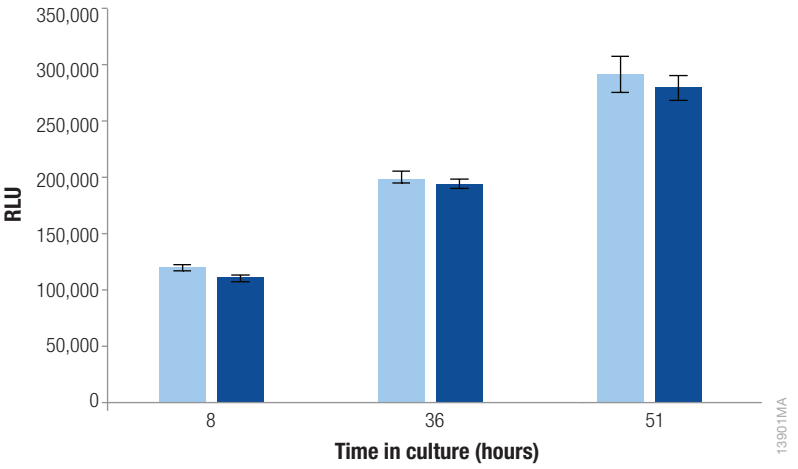

Figure 5. Glutamate secretion by K562 cells. K562 cells were cultured in 75 cm^2 tissue culture flasks in RPMI medium (Sigma R1383) supplemented with 5mM glucose and 10% dialyzed FBS. After 8, 36 and 51 hours, 1.5ml of cell culture was transferred into 1.5ml microcentrifuge tubes. A portion of each sample was diluted 10-fold in PBS directly (light blue bars) or after removing the cells by centrifugation (dark blue bars). The diluted samples were frozen and stored at −20°C. For glutamate detection, the samples were thawed, diluted with PBS to a 40-fold final dilution and 20µl was transferred into an assay plate. An equal volume of Glutamate Detection Reagent was added to the samples. After 60 minutes at room temperature, luminescence was read using a Tecan instrument. The data represent the average from four separate flasks. Samples treated and not treated with Inactivation/Neutralization solutions showed no significant difference in glutamate concentrations (data not shown). The data show that a small volume of suspension cell culture can be removed, diluted and assayed directly without pelleting the cells or treating the samples for protein inactivation and NAD(P)H degradation.

4.B. Cell Lysates

The Glutamate-Glo™Assay can be used for monitoring changes in intracellular glutamate levels. For measuring the intracellular glutamate concentration, the cell culture medium must be removed and the cells washed with PBS to avoid contamination from glutamate in the medium. Work quickly and use cold PBS to minimize changes in glutamate metabolism.

After washing, the Inactivation Solution can be added directly to the cells in a 96-well plate, eliminating the need for sample centrifugation or deproteinization using 10K spin columns required by other methods. The Inactivation Solution rapidly stops metabolism, lyses the cells, inhibits activity of endogenous proteins and destroys reduced NAD(P)H dinucleotides. After neutralization, the samples can be assayed immediately or stored at −20°C. Instructions for preparing Inactivation and Neutralization Solutions are provided in Table 3.

Samples deproteinized using other methods might be acceptable but have to be tested for compatibility with the Glutamate-Glo™ Assay. For example, perchloric acid/KOH treatment is not recommended for use with the Glutamate-Glo™ Assay.

Table 3. Inactivation and Neutralization Solutions

Solution	Formulation	Preparation
Inactivation Solution	0.6N HCl	Prepare 0.6N HCl from a concentrated stock solution, such as 1N HCl, by diluting with water. No pH adjustment is necessary.
Neutralization Solution	1M Tris Base	Dissolve 24.2g of Trizma® base powder (Sigma Cat. #T1503) in 200ml water. The final pH will be approximately 10.7. No pH adjustment is necessary.

Note: When homogenizing tissues or other hard-to-lyse samples, the addition of DTAB (dodecyltrimethylammonium bromide, Sigma Cat. #D8638) to the Inactivation Solution may increase the efficiency of homogenization and the release of glutamate**.** Prepare a 10% DTAB stock solution in water. If needed, warm the solution in a 37°C water bath to completely solubilize the DTAB. Add DTAB to the Inactivation Solution to a final concentration of 0.1–0.25% (v/v).

The following protocol measures changes in intracellular glutamate.

1. Plate 1,000–40,000 cells in 96-well plates. Add compounds to the cells if treatment is part of the experimental design.
2. After the compound treatment, remove and discard the medium and wash the cells twice with 200µl of PBS per wash.
3. Add 25µl of PBS to the washed cells.
4. Add 12.5µl of Inactivation Solution. Mix by shaking the plate for 5 minutes.

   Note: PBS can be combined with Inactivation Solution and added together.
5. Add 12.5µl of Neutralization Solution. Mix by shaking the plate for 30–60 seconds.
6. Add 50µl of Glutamate Detection Reagent prepared as described in Section 3.A.
7. Shake the plate for 30–60 seconds to mix.
8. Incubate for 60 minutes at room temperature.
9. Record luminescence.

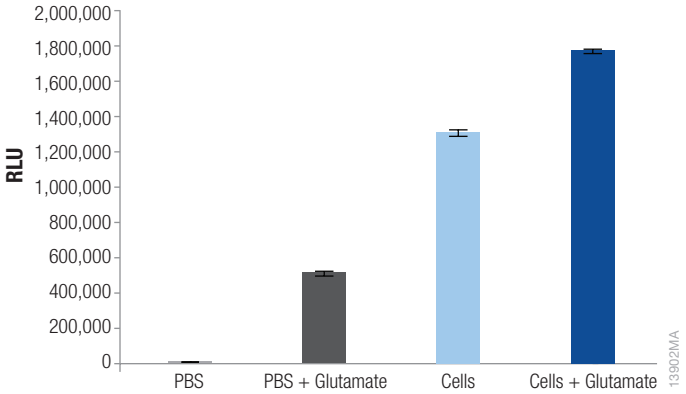

Figure 6. Intracellular glutamate in A549 cells. A549 cells in F12K medium (ATCC 30-2004) were plated at 10,000 cells/well in 96-well plates. After overnight incubation, the medium was removed, cells were washed twice with 200µl PBS, and then 25µl PBS or PBS containing 10µM glutamate was added to the cells. PBS and PBS containing 10µM glutamate (25µl) were included as controls. The cells with the glutamate spike were used to determine the efficiency of glutamate recovery and to calculate the glutamate concentration in the cell lysate. Samples were processed following the protocol in Section 4.B. The data represent the average of six replicates. The calculated glutamate concentration in the A549 cell lysate corresponds to 24µM (0.6nmol/25µl).

4.C. Cell Cultures Using a Homogeneous Assay Format

The homogeneous Glutamate-Glo™ Assay format was developed for measuring total glutamate directly in the well containing cells and is well suited for high-throughput applications. The protocol is applicable under experimental conditions when the total amount of glutamate (extracellular glutamate secreted into the medium plus the intracellular glutamate concentration) is within the linear range of the assay (0.05–50µM).

Because cells rapidly metabolize glutamine to glutamate, we recommend pre-incubating compounds with cells plated in glutamine-free medium. Glutamine can then be added to begin metabolism to glutamate.

1. Collect the cells, count, resuspend in glutamine-free medium at 10,000–200,000 cells/ml and plate 25µl in 96-well plates. When working with cells plated in growth medium overnight, remove the medium, wash the cells with PBS and add 25µl of glutamine-free medium.
2. Add 5µl of compounds diluted in the same glutamine-free medium and pre-incubate for 5–15 minutes.
3. Add 10µl of glutamine-free medium supplemented with 4X final concentration of glutamine. For example, add 8mM glutamine to give a final concentration of 2mM in the medium.
4. Incubate for the desired amount of time.

   Note: The optimal time has to be determined to make sure the glutamate concentration is within the linear range of the assay. Typically it is less than 2 hours after the addition of glutamine-containing medium.
5. Stop glutamate production by adding 5µl of Inactivation Solution. Shake the plate for 3–5 minutes to mix.
6. Add 5µl of Neutralization Solution. Shake the plate for 30–60 seconds to mix.

   Note: At this point the samples can be stored at room temperature for up to 2 hours or at –20°C for longer storage.
7. Add 50µl of Glutamate Detection Reagent prepared as described in Section 3.A.
8. Shake the plate for 30–60 seconds to mix.
9. Incubate for 60 minutes at room temperature.
10. Record luminescence.

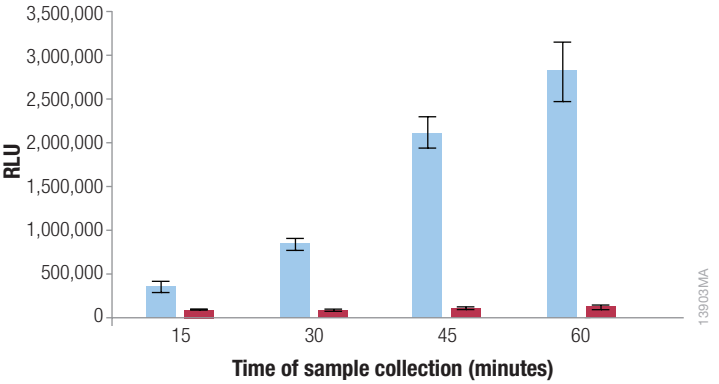

Figure 7. Homogeneous assay with glutaminase inhibitor BPTES. A549 cells were plated at 5,000 cells/well in 100µl culture medium. After an overnight incubation, the medium was removed and the cells were washed twice with PBS. Thirty microliters of DMEM (Gibco 14420) with 27µM BPTES (red bars) or without BPTES (blue bars) was added to the cells. After 10 minutes at room temperature, 10µl of DMEM with 4X glucose (20mM) and 4X glutamine (8mM) was added to the cells, resulting in a final BPTES concentration of 20µM. The plate was placed in the 37°C tissue culture incubator and, at indicated time points, 5µl of Inactivation Solution was added to stop cell metabolism. After incubation for 2–5 minutes, 5µl of Neutralization Solution was added and glutamate was measured by adding 50µl of Glutamate Detection Reagent. Luminescence was recorded using a GloMax® Luminometer and the data represent the average of quadruplicate wells. Cells without BPTES produced increasing amounts of glutamate with time. However, in cells exposed to BPTES, glutaminase was inhibited, preventing the conversion of glutamine to glutamate. Controls included glutamate without cells and the RealTime-Glo™ MT Cell Viability Assay, which indicated that BPTES had no effect on the assay, or on cell viability (data not shown).

4.D. Measuring Multiple Metabolites from One Sample

Four metabolites important to the energetic state of the cell—glucose, lactate, glutamate and glutamine—can be measured in parallel using the bioluminescent Glucose-Glo™ (Cat.# J6021), Lactate-Glo™ (Cat.# J5021), Glutamine/Glutamate-Glo™ (Cat.# J8021) and Glutamate-Glo™ (Cat.# J7021) Assays. Sample processing compatible with all of the bioluminescent metabolite assays allows the same sample to be used for detection of all four metabolites. This includes sample types such as culture media, sera, plasma and tissues.

When measuring metabolites in medium, only a small amount of sample is required for any assay. Therefore, cells can be grown in multiwell plates, and medium (2–5µl) can be collected at multiple time points from the same well. All four metabolites can then be assayed from the same collected medium samples (Figure 8).

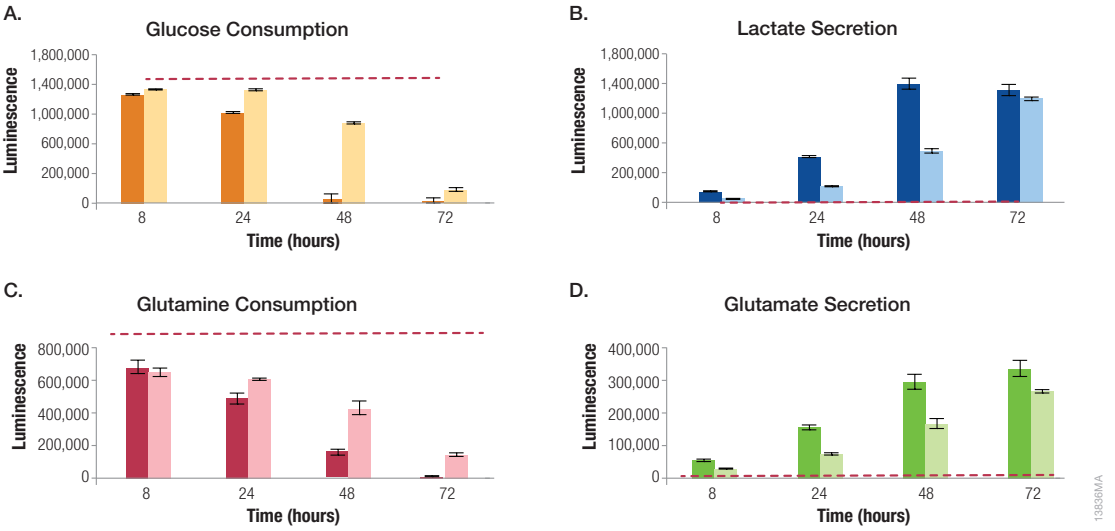

Figure 8. Measuring extracellular metabolites. A549 cells were plated at 15,000 (dark bars) or 5,000 (light bars) cells/well in 100µl DMEM with 5mM glucose, 2mM glutamine and 10% dialyzed serum. At the indicated time points, 2.5µl of medium was removed, diluted in 97.5µl PBS and stored frozen at –20°C. At the end of the experiment, samples were thawed and aliquots were transferred to a 384-well plate. Each sample was transferred into four wells, one for each metabolite. The following volumes were used from the thawed sample to detect each of the four metabolites: 25µl for lactate, 12.5µl plus an additional 12.5µl PBS for glucose, 12.5µl for glutamine and 12.5µl for glutamate. The metabolites were then detected using the Glucose-Glo™ (Panel A), Lactate-Glo™ (Panel B), and Glutamine/Glutamate-Glo™ Assays (Panels C and D), respectively. Luminescence was recorded using a Tecan instrument. The glutamate secretion data were obtained using the Glutamine/Glutamate-Glo™ Assay; similar results were obtained with the Glutamate-Glo™ Assay (data not shown). The red lines depict the signals from control wells containing medium but no cells.

4.E. Tissues

The Glutamate-Glo™ Assay can be used to measure the glutamate concentration in homogenized tissues (Table 4). We recommend homogenizing the tissues in 50mM Tris (pH 7.5) pre-mixed with Inactivation Solution (8:1 v/v) at 3–15 mg of tissue/ml. Other buffers, such as RIPA, can be used but should be tested for compatibility with the Glutamate-Glo™ Assay. If other buffers are used, the Inactivation Solution should be added immediately after tissue homogenization. After homogenization, treat the samples with Neutralization Solution (the same volume as the Inactivation Solution) and, if necessary, diluted to the linear range of the assay. As a starting point, we recommend using 0.05–0.3mg of tissue (0.005–0.03mg of protein) in a 50µl reaction volume.

See Table 3, Section 4.B for preparation of Inactivation and Neutralization Solutions.

1. Slice frozen tissue and place in a pre-weighed tube. Target 3–15mg tissue per slice. Weigh the sample and pre-weighed tube, then subtract the weight of the tube to get the tissue weight. Immediately place samples on dry ice.
2. Premix 50mM Tris (pH 7.5) buffer (Homogenization Buffer) with Inactivation Solution at a 8:1 ratio (e.g., 1ml buffer + 0.125ml of Inactivation Solution) and add 1.125ml for every 3–15mg of frozen tissue.
3. Homogenize for 20–30 seconds using a Tissue Tearor (BioSpec Cat.# 985370-07) or other mechanical homogenization.
4. Neutralize the tissue homogenate by adding 0.125ml of Neutralization Solution per 1.125ml of homogenate.

   Note: Samples of tissue homogenate can be used for protein determination. If detergents such as DTAB are included in the homogenization protocol, we recommend using the Pierce 660nm Protein Assay with Ionic Detergent Compatibility Reagent.
5. Make a sample dilution buffer by premixing Homogenization Buffer with Inactivation Solution and Neutralization Solution at an 8:1:1 ratio (1ml + 0.125ml + 0.125ml).
6. Dilute the tissue homogenate to fit the linear range of the assay.
7. Transfer 50µl of prepared samples into a 96-well assay plate.

   Note: The samples can be transferred directly. The centrifugation step commonly used by other methods is not required.
8. Add 50µl of Glutamate Detection Reagent prepared as described in Section 3.A.
9. Shake the plate for 30–60 seconds to mix.
10. Incubate for 60 minutes at room temperature.
11. Record luminescence.

Table 4. Glutamate in Tissues

Sample	RLUs
Assay background	2,004 ± 112
Control (25µM glutamate)	1,090,199 ± 11,970
Glutamate in brain tissue	898,061 ± 13,651

A sample of frozen mouse liver tissue (BioreclamationIVT) was homogenized, neutralized, and diluted to yield 3mg of tissue/ml (0.3mg/ml protein). Dilutions were prepared and aliquots (50µl) were transferred to a 96-well assay plate. Wells containing 50µl of Dilution Buffer with or without 25µM glutamate were included in the same plate as controls. The measurements were done in triplicate. The glutamate concentration in the sample was 24µM and the luminescent signal was 448-fold above background.

4.F. Plasma and Serum

Plasma and serum samples must be diluted to fit the linear range of the Glutamate-Glo™ Assay (0.05–50µM). The sensitivity of the assay requires that only a small amount of plasma or serum be used, e.g., 10µl diluted 10-fold or more.

1. Dilute the plasma or serum sample 10- to 50-fold in PBS. Multiple dilutions can be tested.
2. Transfer 50µl of diluted plasma or serum into the wells of a white 96-well assay plate.
3. Add 50µl of Glutamate Detection Reagent prepared as described in Section 3.A.
4. Shake the plate for 30–60 seconds to mix.
5. Incubate for 60 minutes at room temperature.
6. Record luminescence.

Note: If plasma or serum is diluted less than 10-fold, the Inactivation and Neutralization Solutions may be required to inactivate endogenous enzymes. Add 25µl of the diluted sample to a well, followed by 12.5µl of Inactivation Solution. Mix and incubate for 5–10 minutes at room temperature. Then add 12.5µl of Neutralization Solution and proceed with Step 3 above.

5. Appendix

5.A. Assay Controls and Data Analysis

There is a linear relationship between luminescence signal and glutamate concentration, and many luminescence measurements can be described simply in terms of RLUs. The most important information is the change in RLUs that occurs between the experimental controls and test conditions. To calculate glutamate concentration, a glutamate titration curve or a "spike" of glutamate at a known concentration has to be included in the experimental set up and assayed under the same experimental conditions, preferably in the same assay plate.

Different buffers can affect light output; therefore, controls should be prepared using the same buffers as the samples. The Glutamate-Glo™ Assay includes 10mM Glutamate as a **positive control**. Wells containing buffer only should be included as **negative controls**. These wells can be used to measure the background signal and calculate signal-to-background ratios.

5.B. Use of Medium and Serum

The formulations of commonly used cell culture media, such as DMEM and RPMI-1640, contain glucose, glutamine, amino acids and other components that may influence the metabolic rate of cells. Therefore, it is important to carefully define the culture medium used in assays measuring metabolic pathways. We recommend using medium lacking glucose, glutamine and pyruvate, and adding those components at the desired final concentration on the day of the experiment. We use DMEM (Gibco #14430) and add 5mM glucose and 2mM glutamine.

Supplementing the culture medium with 5–10% of fetal bovine serum (FBS) is a standard practice when culturing mammalian cells. We have found FBS may contain significant levels of metabolites that should be taken into account when planning experiments. For short-term experiments, media without serum can be used. Otherwise, we recommend using dialyzed serum (e.g., Gibco 26400036).

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 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.D. Multiplexing and Normalization

The Glutamate-Glo™ Assay can be multiplexed to normalize for changes in viability and to account for well-to-well variation. Changes in the glutamate concentration in the medium can be measured by removing a small amount of medium (2–5µl) for glutamate detection and using the remainder of the sample for RealTime-Glo™, CellTiter-Fluor™ and CellTiter-Glo® viability measurements, following the protocols provided with the respective assays.

Multiplexing intracellular glutamate detection with viability assays starts by adding RealTime-Glo™ and CellTiter-Fluor™ reagents to the medium and measuring viability after incubation, followed by removal of the medium and lysing the cells (Section 4.B). An aliquot of the cell lysate can be removed for quantitation measurements using the CellTiter-Glo® Assay. An equal volume of Glutamate Detection Reagent is added to the remainder of the cell lysate for measurement of intracellular glutamate concentration.

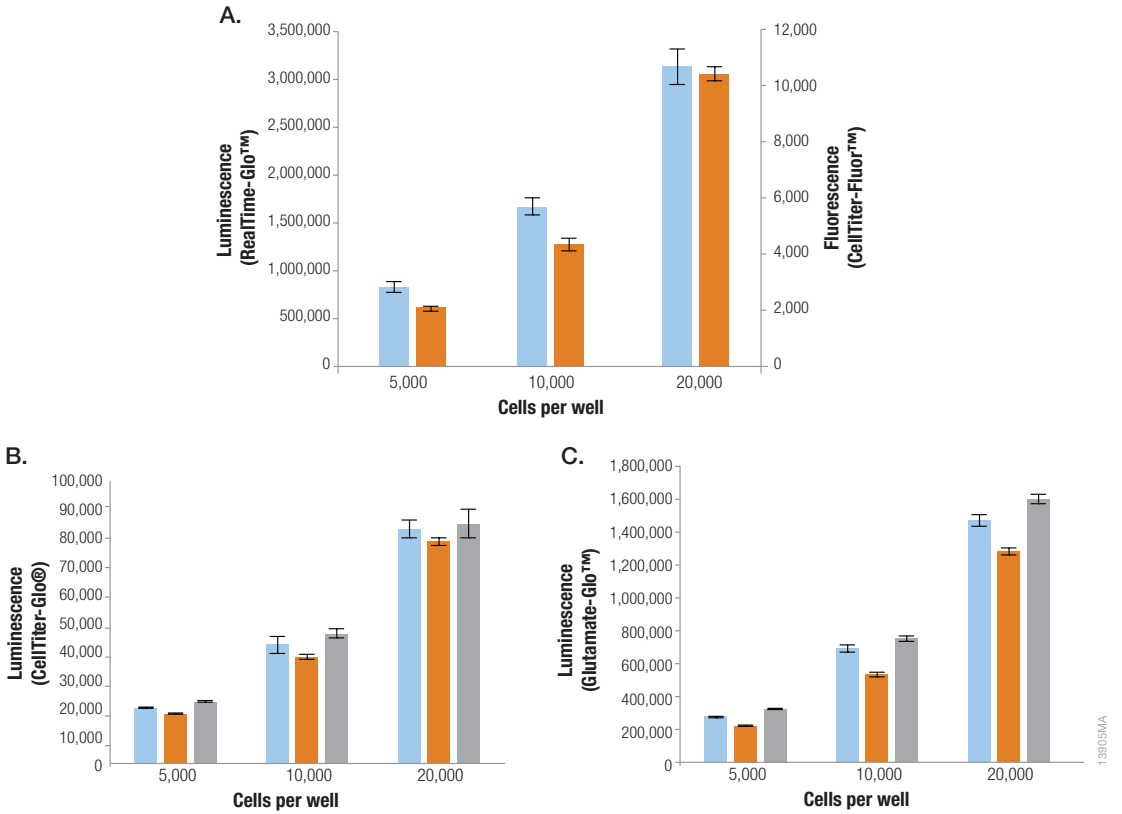

Figure 9. Multiplexing with viability assays. A549 cells were diluted in DMEM with 5mM glucose, 2mM glutamine and 10% dialyzed serum at three cell densities (100µl per well). After overnight incubation, 25µl of 5X RealTime-Glo™ (blue bars) or 5X CellTiter-Fluor™ (orange bars) reagents was added to the wells. Medium without viability reagent was added to a third set of wells (grey bars). After 30 minutes at 37°C, luminescence and fluorescence were recorded. All medium was removed, and the cells were washed twice with cold PBS. After the second wash, 25µl of PBS was added to each well of cells, followed by 12.5µl of Inactivation Solution. After mixing for 5 minutes on a plate shaker, 12.5µl of Neutralization Solution was added to the wells. An aliquot (2µl) of the cell lysate was removed to a 384-well luminometer plate and mixed with an equal volume of CellTiter-Glo® reagent. After 10 minutes at room temperature, luminescence was recorded . The remaining cell lysate was combined with an equal volume of Glutamate Detection Reagent. After 60 minutes at room temperature, the luminescence was recorded using a GloMax® luminometer. A linear increase in signal with increasing amounts of cells was detected with the viability assays (Panels A and B). The addition of viability reagents to the medium had no effect on glutamate measurements (Panel C).

5.E. Assay Plates and Equipment

Most standard plate readers are designed for measuring 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® #3917 96-well or Costar® #3570 384-well plates). For cultured cell samples, white-walled, clear-bottom tissue culture plates (e.g., Corning Costar #3903 96-well plates) are acceptable. Light signal is diminished in black plates, and increased well-to-well cross-talk 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.

6. 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**, 670-675.
2. Vidugiriene, J. *et al.* (2014) Bioluminescent Cell-based NAD(P)/NAD(P)H Assays for Rapid Dinucleotide Measurement and Inhibitor Screening. ASSAY and Drug Development Technologies, **12**, 514-526.
3. Leippe, D. *et al.* (2016) Bioluminescent Assays for Glucose and Glutamine Metabolism: High-Throughput Screening for Changes in Extracellular and Intracellular Metabolites. *SLAS Discovery* **22(4)**, 366–377.

7. Related Products

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

Cytotoxicity Assays

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

Promega

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 for viability, cytotoxicity and multiplex assays.

Energy Metabolism Assays

Product	Size	Cat.#
Glucose Uptake-Glo™ Assay	5ml	J1341
Glucose-Glo™ Assay	5ml	J6021
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
	100ml	G8001

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