TECHNICAL MANUAL

Glutamine/Glutamate-Glo™ Assay

Instructions for Use of Products
J8021 and J8022

Revised 8/19
TM496

Glutamine/Glutamate-Glo™ Assay

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The Glutamine/Glutamate-Glo™ Assay[a,b] is a bioluminescent assay for rapid, selective and sensitive detection of glutamine and glutamate in biological samples.

The assay is based on the conversion of glutamine to glutamate by Glutaminase enzyme. Next, glutamate oxidation and NADH production are coupled 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.

This assay uses two steps: 1) glutamine conversion to glutamate by Glutaminase; and 2) glutamate detection with the Glutamate Detection Reagent. 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 and increases until all glutamate is consumed, at which point a stable luminescent signal is achieved.

When using this assay, both glutamine and glutamate will be measured. For samples that contain both glutamate and glutamine, the light signal will be proportional to the starting concentration of total glutamine plus glutamate. Therefore, a second reaction without the Glutaminase enzyme is needed to measure the glutamate-only concentration. Measurement of total glutamine plus glutamate, and glutamate-only are performed in separate wells simultaneously. Glutamine levels are calculated by subtracting the glutamate-only signal from the total glutamine plus glutamate signal. The assay can also be used to measure only glutamate from a sample by omitting the Glutaminase step.

The assay has a linear range up to 50µM (Figure 3 and Table 1). The limit of detection is ~25–50nM (defined as the concentration at which the signal-to-noise ratio is 3; Table 1) and the maximum signal-to-background ratio is ~400. Glutaminase efficiently converts glutamine to glutamate; the result is that similar RLUs will be generated from equal concentrations of glutamine and glutamate (Figure 3 and Table 1).

The Glutamine/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.D, 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.

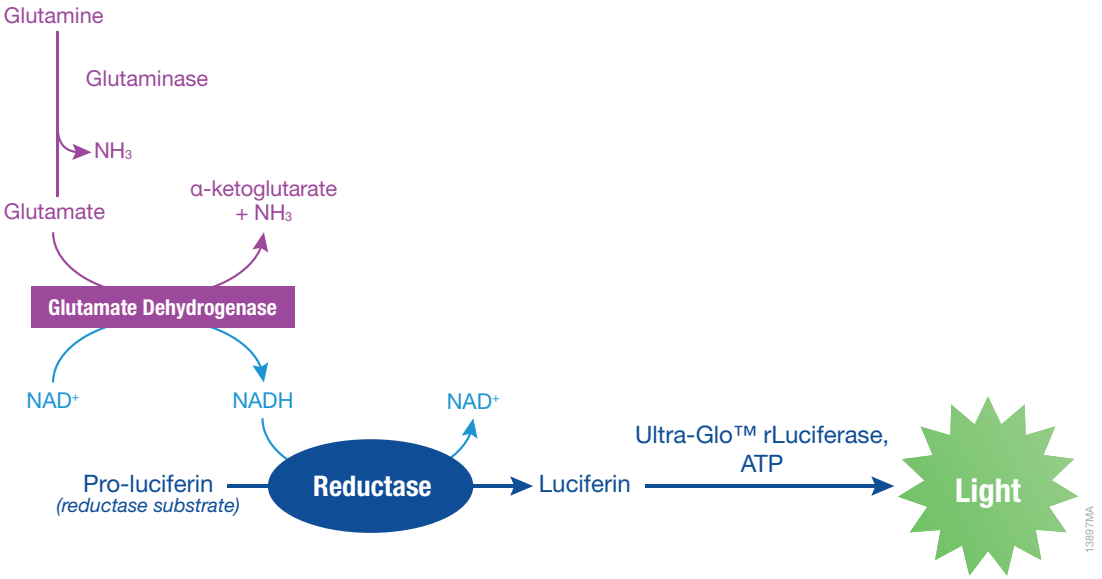

Figure 1. Schematic diagram of the Glutamine/Glutamate-Glo™ Assay principle. Glutaminase catalyzes the conversion of glutamine to glutamate. Glutamate Dehydrogenase catalyzes the oxidation of glutamate with concomitant reduction of NAD^+ to NADH. In the presence of NADH, Reductase enzymatically reduces 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.

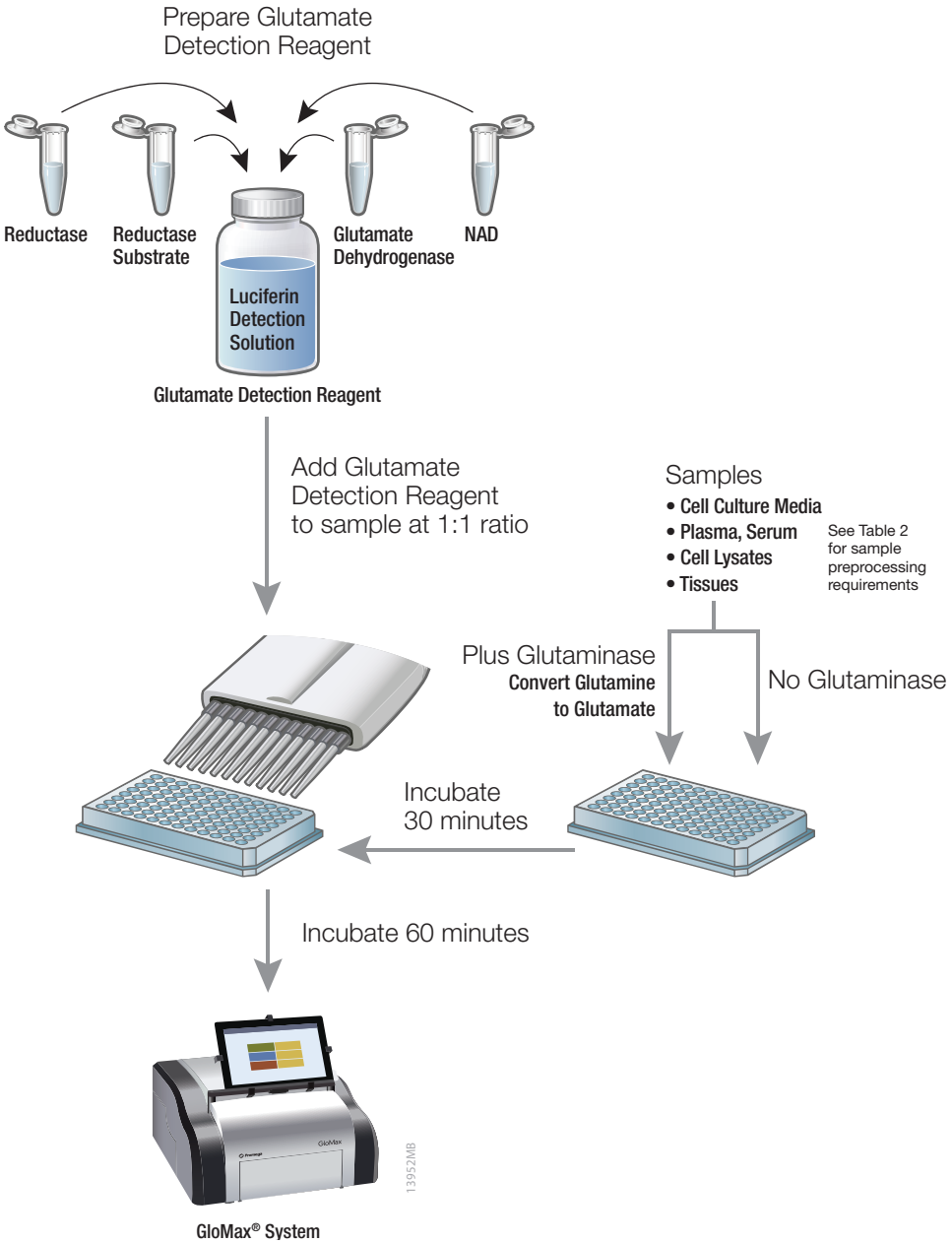

Figure 2. Glutamine/Glutamate-Glo™ Assay reagent preparation and protocol.

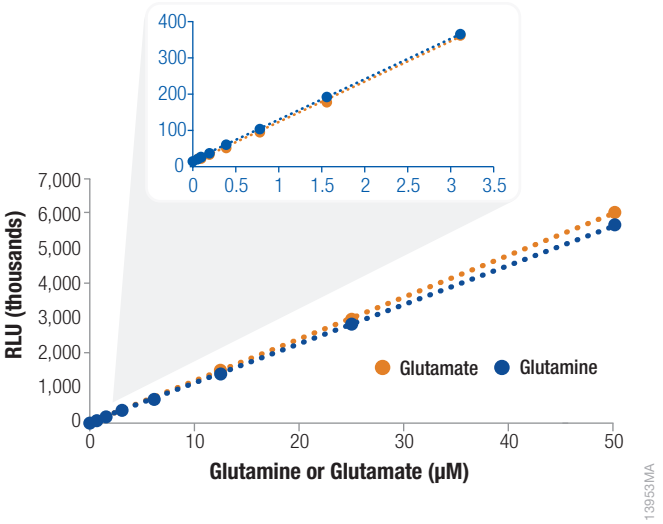

Figure 3. Glutamine and glutamate titration curves. Twofold serial dilutions of glutamine (blue circles) and glutamate (orange circles) controls were prepared in PBS, starting from 50µM. Aliquots of the prepared standards (25µl) were transferred to a 96-well plate and the assay was performed following the protocol in Section 3.C. All reactions were performed in quadruplicate and the data represent the average of the four replicates. Luminescence was recorded using a GloMax® Luminometer.

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Table 1. Glutamine and Glutamate Titration Data

Glutamine, µM	**0**	**0.049**	**0.195**	**0.781**	**3.13**	**12.5**	**50**
Ave. RLU (thousands)	15	22	37	103	366	1,415	5,665
St. Dev. (thousands)	0.7	1.2	0.3	1.2	7.0	39.6	97.4
CV	5%	5%	1%	1%	2%	3%	2%
S/B	1.0	1.4	2.5	7	24	94	376
S/N	–	9	32	128	514	2,049	8,273
Glutamate, µM	**0**	**0.049**	**0.195**	**0.781**	**3.13**	**13**	**50**
Ave. RLU (thousands)	15	22	33	96	364	1,481	6,061
St. Dev. (thousands)	1.1	1.4	0.7	1.7	9.3	49.5	166.5
CV	7%	6%	2%	2%	3%	3%	3%
S/B	1.0	1.4	2.2	6	24	96	392
S/N	–	6	16	71	308	1,295	5,341

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 glutamine or glutamate.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Glutamine/Glutamate-Glo™ Assay	**5ml**	**J8021**

The system contains sufficient reagents to perform 100 reactions in 96-well plates (25µl of sample + 25µl of Glutaminase reaction + 50µl of Glutamate Detection Reagent). This amount can be used to determine the glutamine and glutamate content of 50 samples. Includes:

- 5ml Luciferin Detection Solution
- 55µl Reductase
- 55µl Reductase Substrate
- 100µl Glutamate Dehydrogenase
- 275µl NAD
- 25µl Glutaminase
- 2.5ml Glutaminase Buffer
- 50µl Glutamate (10mM)

PRODUCT	SIZE	CAT.#
Glutamine/Glutamate-Glo™ Assay	**50ml**	**J8022**

The system contains sufficient reagents to perform 1,000 reactions in 96-well plates (25µl of sample + 25µl of Glutaminase reaction + 50µl of Glutamate Detection Reagent). This amount can be used to determine the glutamine and glutamate content of 500 samples. Includes:

- 50ml Luciferin Detection Solution
- 275µl Reductase
- 275µl Reductase Substrate
- 1ml Glutamate Dehydrogenase
- 1ml NAD
- 125µl Glutaminase
- 25ml Glutaminase Buffer
- 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 Glutamine and 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 (see Section 3.D)
- 96-well assay plates (white or clear bottom, e.g., Corning Cat.# 3903 or 3912)
- luminometer (e.g., GloMax® Discover Cat.# GM3000)
- L-glutamine (optional glutamine positive control; e.g., Sigma Cat.# G8540)

3.A. Reagent Preparation

This protocol is for a reaction with 25µl of sample and 25µl of Glutaminase, followed by the addition of 50µl of Glutamate Detection Reagent in a 96-well plate. The assay can be adapted to other volumes provided the 1:1:2 ratio of sample volume:Glutaminase Enzyme Solution:Glutamate Detection Reagent is maintained (e.g., 12.5µl of sample and 12.5µl of Glutaminase plus 25µl of Glutamate Detection Reagent in a 384-well format).

1. Once thawed, equilibrate the Luciferin Detection Solution to room temperature; all other components should be placed on ice.

   Note: Be sure to mix thawed components to ensure homogeneous solutions prior to use.

2. Prepare Glutaminase Enzyme Solution (Glutaminase Buffer + Glutaminase) as shown in the table below. In this example, the amount of Glutaminase per reaction is for a 96-well plate format using 25µl of prepared sample. Prepare the amount of Glutaminase Enzyme Solution needed for your experiment. To compensate for pipetting error, prepare material for one or two reactions more than you need.

Component	Per Reaction	Per 50 Reactions
Glutaminase Buffer	25µl	1.25ml
Glutaminase	0.125µl	6.25µl

3. Gently mix the diluted Glutaminase Enzyme Solution.

4. Prepare the same amount of Glutaminase Buffer as in step 2, without any added Glutaminase enzyme. This buffer will be used to generate the glutamate-only values.

5. Prepare Glutamate Detection Reagent as shown in the table below. The Glutamate Detection Reagent can be prepared during the Glutaminase reaction step (see Section 3.C). The amount of Glutamate Detection Reagent to prepare per reaction is for a 96-well plate format using 50µl of Glutaminase reaction. To compensate for pipetting error, prepare material for one or two reactions more than you need.

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

6. 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. Plate Layout

Use this layout as a guideline when plating samples and standards in Section 3.C.

	Add Glutaminase Enzyme Solution			Add Glutaminase Buffer Only			Add Glutaminase Enzyme Solution			Add Glutaminase Buffer Only		
	Samples						Glutamine Standard Curve (Optional)			Glutamate Standard Curve		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1			Sample 1			50µM			50µM		
B	Sample 2			Sample 2			25µM			25µM		
C	Sample 3			Sample 3			12.5µM			12.5µM		
D	Sample 4			Sample 4			6.25µM			6.25µM		
E							3.125µM			3.125µM		
F							1.56µM			1.56µM		
G							0.78µM			0.78µM		
H							0µM			0µM		

Notes:

1. Samples may also be assayed at multiple dilutions.
2. Prepare the standards in the same buffers as the samples.
3. The 0µM wells of the standard curve can serve as the negative control (buffer only) to measure assay background.

3.C. Protocol

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

Samples that contain both glutamine and glutamate require two reactions to determine the glutamine concentration. One reaction measures the starting glutamate concentration and the other measures the concentration of starting glutamate plus the glutamate that is the product of the glutamine/glutaminase reaction. The difference in signal represents the contribution from glutamine in the sample.

You can also assay positive controls. Using the 10mM Glutamate control stock solution that is included in the kit, you can generate a standard curve to confirm that samples are within the linear range of the assay and to calculate glutamine and glutamate concentrations. You can prepare dilutions in the same buffer as the samples (e.g., PBS) to encompass the linear range of the assay (50nM to 50µM). It is important to prepare controls in the same buffers used for preparing samples and to follow the same assay protocol. A glutamine positive control is not included in the kit. If you require a glutamine positive control, you can prepare a 10mM stock solution in water (e.g., using Sigma G8540).

1. For each prepared sample, transfer two 25µl aliquots into two wells of a white, 96-well luminometer plate that will be used for the assay. These two wells will be used to determine: i) total glutamine plus glutamate; and ii) glutamate only.

   Note: To minimize assay variability, it is preferable to assay total and glutamate-only wells in the same plate. (See Section 3.B for plate layout guidelines.)
2. Transfer 25µl of glutamate controls into a 96-well plate. Include a negative control (buffer only) for determining assay background.
3. Add 25µl of Glutaminase Enzyme Solution, prepared as described in Section 3.A, to the first set of wells. Add 25µl of Glutaminase Buffer (no Glutaminase) to the second set of wells and to the glutamate controls.
4. Shake the plate for 30–60 seconds.
5. Incubate for 30–40 minutes at room temperature.
6. Add 50µl of Glutamate Detection Reagent, prepared as described in Section 3.A, to all wells.
7. Shake the plate for 30–60 seconds.
8. 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.
9. Record luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.

3.D. Sample Processing

The glutamine and glutamate concentration in samples can vary significantly. For example, the glutamine concentration in medium can range from 2–4mM and decrease to micromolar levels as it is consumed by growing cells. The presence of dehydrogenases, reduced NAD(P)H dinucleotides and other factors in the samples can affect the Glutamine/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. Acidic Inactivation Solutions can be used for enzyme inactivation and NAD(P)H degradation. More information on the use of Inactivation Solutions is provided in Section 4.

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.

Table 2. Recommendations for Sample Processing

Sample Type	Glutamine and Glutamate Concentrations in Sample	Processing Recommendations
Cell culture medium (extracellular)	20µM to 2mM	• Dilute medium sample 40- to 100-fold in PBS
Cell lysates (intracellular)	4–40µM for 20,000 cells lysed in 50µl	• Remove media from cells, wash with PBS and add fresh PBS • Add Inactivation Solution I (half of sample volume)[1] • Add Tris Solution I (same volume as Inactivation Solution)[1]
Tissues	5–20µM for 3mg of tissue homogenized in 1ml	• Tissues in homogenization buffer • Add Inactivation Solution II (1/8 of sample volume)[2,3] • Add Tris Solution II (same volume as Inactivation Solution)[2]
Plasma and serum	400–600µM	• Dilute 10 to 100-fold in PBS

[1] See Table 3 in Section 4.B for solution compositions.

[2] See Table 4 in Section 4.D for solution compositions.

[3] 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 Glutamine/Glutamate-Glo™Assay can be used to measure changes in glutamine and glutamate concentrations in mammalian cell culture medium. As cells grow, they continuously consume glutamine and secrete glutamate. This process decreases the concentration of glutamine in the medium and increases the concentration of glutamate. The extent of the changes depends on cell type, cell density and time. Therefore, samples of the medium typically require dilution into the linear range of the Glutamine/Glutamate-Glo™ Assay. As a starting point, we recommend a 50-fold dilution in PBS.

An example showing measurement of glutamine consumption and 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 of 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.
5. For each prepared sample, transfer two 25µl aliquots into two wells of a white luminometer 96-well plate that will be used for the assay. These two wells will be used to determine: i) total glutamine plus glutamate; and ii) glutamate only.

   Note: To minimize assay variability, it is preferable to assay total and glutamate-only wells in the same plate. (See Section 3.B for plate layout guidelines.)
6. Transfer 25µl of glutamate controls into a 96-well plate. Include a negative control (PBS only) for determining assay background.
7. Add 25µl of Glutaminase in Glutaminase Buffer, prepared as described in Section 3.A, to the first set of wells. Add 25µl of Glutaminase Buffer (no Glutaminase) to the second set of wells and to the glutamate controls.
8. Shake the plate for 30–60 seconds.
9. Incubate for 30–40 minutes at room temperature.
10. Add 50µl of Glutamate Detection Reagent, prepared as described in Section 3.A, to all wells.
11. Shake the plate for 30–60 seconds.
12. 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.
13. Record luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.

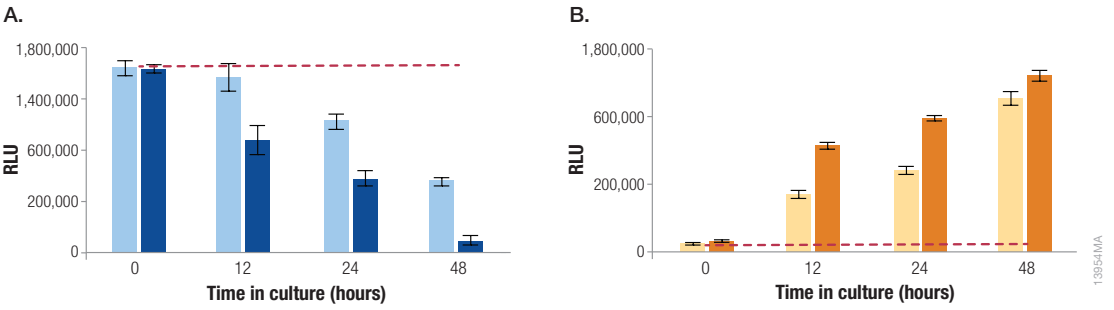

Figure 4. Glutamine consumption and glutamate secretion by SKOV-3 cells. Glutamine consumption (Panel A) and glutamate secretion (Panel B) by SKOV-3 cells were monitored over time. SKOV-3 cells were plated at 5,000 (light bars) and 20,000 (dark bars) cells/well in DMEM medium (Gibco A14430) containing 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 immediately stored at –20°C. On the day of the assay, the samples were thawed. Portions of the sample (2 × 12.5µl) were transferred to a 384-well assay plate and 12.5µl of Glutaminase Buffer or Glutaminase Enzyme Solution was added. The reactions were incubated for 30 minutes at room temperature. Twenty-five microliters of Glutamate Detection Reagent was added and, 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. Fifty micromolar glutamine and glutamate controls corresponded to 1.1×10^6 and 1.0×10^6 RLUs, respectively, and are not shown on the graph. All measured samples were within the linear range of the assay, and the calculated glutamine concentration in the medium decreased from 1.8mM to 0.70mM for 5,000 cells/well and 1.8mM to 0.13mM for 20,000 cells/well (determined by comparison to a standard curve). The glutamate concentration in the medium increased from 35µM to 570µM for 5,000 cells/well and 47µM to 650µM for 20,000 cells/well.

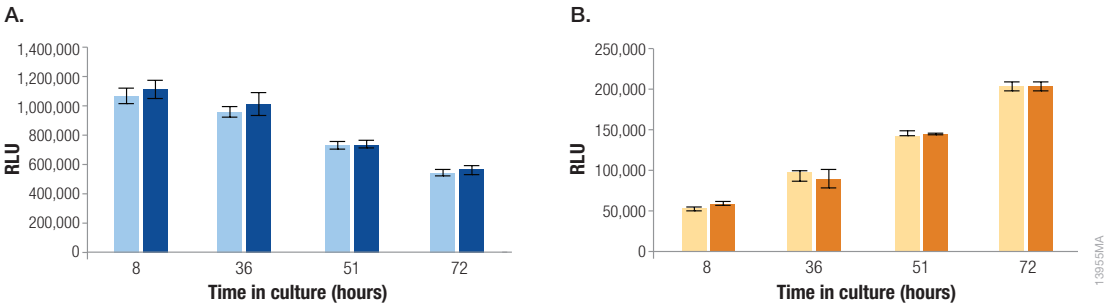

Figure 5. Glutamine consumption and glutamate secretion by K562 cells. Glutamine consumption (Panel A) and glutamate secretion (Panel B) by K562 cells were monitored over time. K562 cells were cultured in 75cm^2 tissue culture flasks in RPMI medium (Sigma R1383) supplemented with 5mM glucose and 10% 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 bars) or after removing the cells by centrifugation (dark bars). The diluted samples were frozen and stored at −20°C. The samples were thawed, diluted with PBS to a 40-fold final dilution and two 10µl aliquots were transferred into a 384-well assay plate. Ten microliters of Glutaminase Buffer or Glutaminase Enzyme Solution was added, and the reactions were incubated for 30 minutes at room temperature. Twenty microliters of Glutamate Detection Reagent was added to each sample. 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/Tris solutions showed no significant difference in glutamine and 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 Glutamine/Glutamate-Glo™Assay can be used for monitoring changes in intracellular glutamine and glutamate levels. When measuring intracellular concentrations, the cell culture medium must be removed and the cells washed with PBS to avoid contamination from glutamine and glutamate in the medium. Work quickly and use cold PBS to minimize changes in glutamine/glutamate metabolism.

After washing, the Inactivation Solution I 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 I rapidly stops metabolism, lyses the cells, inhibits activity of endogenous proteins and destroys reduced NAD(P)H dinucleotides. After the addition of Tris Solution I, the samples can be assayed immediately or stored at −20°C. Instructions for preparing Inactivation Solution I and Tris Solution I for cell lysates are provided in Table 3.

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

Table 3. Inactivation and Tris Solutions for Cell Lysates

Solution	Formulation	Preparation
Inactivation Solution I	0.3N HCl	Prepare 0.3N HCl from a concentrated stock solution, such as 1N HCl, by diluting with water. No pH adjustment is necessary.
Tris Solution I	450mM Tris (pH 8.0)	Prepare 450mM Tris (pH 8.0) in water.

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 homogenization efficiency, and glutamine and glutamate release. 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 glutamine and glutamate.

1. Plate 2,500–40,000 cells in 96-well plates. Add compounds to the cells if treatment is part of the experimental design.
2. After compound treatment, remove and discard the medium and wash cells twice with 200µl PBS per wash.
3. Add 30µl of PBS to the washed cells.
4. Add 15µl of Inactivation Solution I. Mix by shaking the plate for 5 minutes.

   Note: PBS can be combined with Inactivation Solution I and added together.
5. Add 15µl of Tris Solution I. Mix by shaking the plate for 30–60 seconds.
6. For each prepared sample, transfer two 25µl aliquots into two wells of a white luminometer 96-well plate that will be used for the assay. These two wells will be used to determine: i) total glutamine plus glutamate; and ii) glutamate only.

   Note: To minimize assay variability, assay total and glutamate-only wells in the same plate. (See Section 3.B for plate layout guidelines.)
7. Transfer 25µl of glutamate controls into a 96-well plate. Include a negative control (buffer only) for determining assay background.
8. Add 25µl of Glutaminase in Glutaminase Buffer, prepared as described in Section 3.A, to the first set of wells. Add 25µl of Glutaminase Buffer (no Glutaminase) to the second set of wells and to the glutamate controls.
9. Shake the plate for 30–60 seconds.
10. Incubate for 30–40 minutes at room temperature.
11. Add 50µl of Glutamate Detection Reagent, prepared as described in Section 3.A, to all wells.
12. Shake the plate for 30–60 seconds.
13. 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.

14. Record luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.

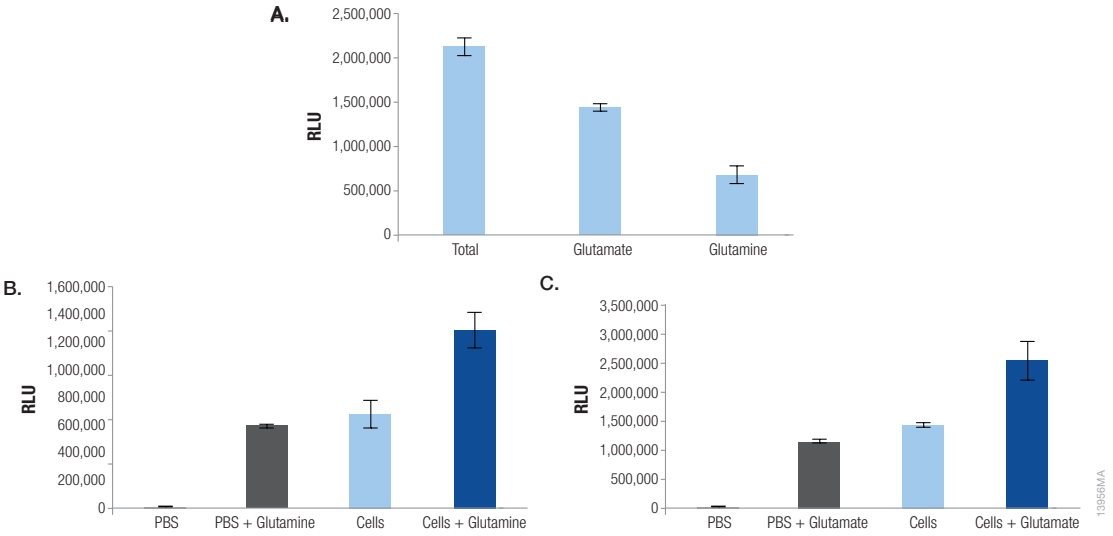

Figure 6. Intracellular glutamine and glutamate in A549 cells. A549 cells were plated at 20,000 cells/well in 96-well plates in DMEM (Gibco 14430) supplemented with 5mM glucose, 2mM glutamine and 10% dialyzed FBS. After overnight incubation, the medium was removed and cells were washed twice with PBS. A 30µl aliquot of PBS, PBS containing 10µM glutamine, or PBS containing 20µM glutamate was added to the cells. Controls without cells included PBS, PBS containing 10µM glutamine, and PBS containing 20µM glutamate. The cells (and PBS controls) were processed by adding 15µl of Inactivation Solution I followed by 15µl of Tris Solution I. Two 25µl aliquots were transferred to a 96-well plate for the assay. Samples were processed following the protocol in Section 4.B. The data represent the average of four replicates. To calculate glutamine signal (Panel A), the average signal from the glutamate-only wells was subtracted from the signal from the total glutamine plus glutamate wells. Cells with a glutamine or glutamate spike were used to determine the recovery efficiency of glutamine (Panel B) or glutamate (Panel C), respectively, and to calculate the glutamine and glutamate concentrations in the cell lysate. The calculated concentrations of glutamate and glutamine in the A549 cell lysate were 25µM and 11µM, respectively.

4.C. 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 7).

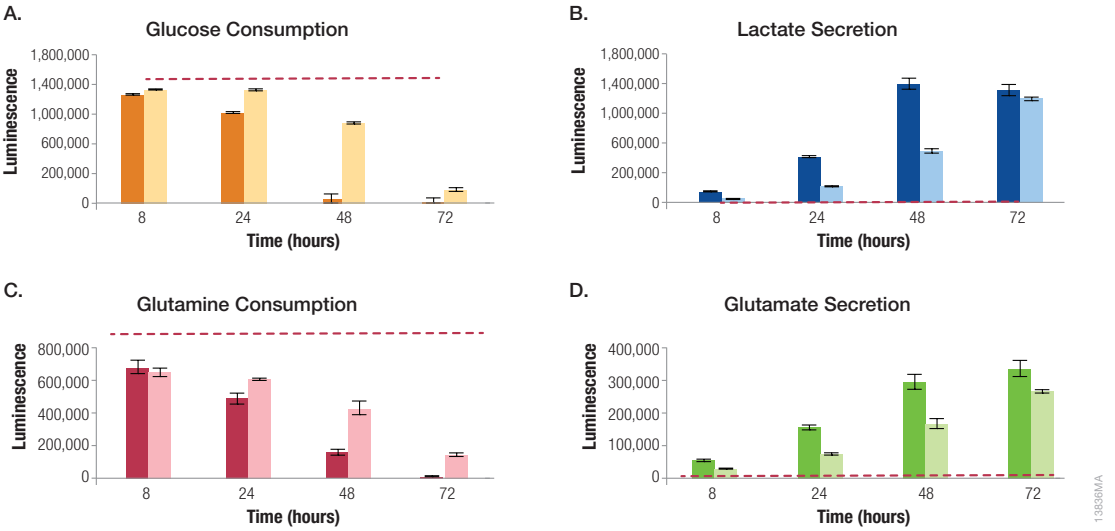

Figure 7. Measuring extracellular metabolites. A549 cells were plated at 15,000 (dark bars) or 5,000 (light bars) cells/well in 100µl DMEM with 5mM glucose, 2mM glutamine and 10% dialyzed serum. At the indicated time points, 2.5µl of medium was removed, diluted in 97.5µl PBS and stored frozen at –20°C. 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.D. Tissues

The Glutamine/Glutamate-Glo™ Assay can be used to measure the glutamine and glutamate concentrations in homogenized tissues. We recommend homogenizing the tissues in 50mM Tris (pH 7.5) pre-mixed with Inactivation Solution II (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 Glutamine/Glutamate-Glo™ Assay. If other buffers are used, the Inactivation Solution II should be added immediately after tissue homogenization. After homogenization, treat the samples with Tris Solution II (the same volume as the Inactivation Solution) and, if necessary, dilute them to fit the linear range of the assay. As a starting point, we recommend using 0.025–0.15mg of tissue (0.0025–0.015mg of protein) in a 25µl reaction volume. Instructions for preparing Inactivation Solution II and Tris Solution II are provided in Table 4. Representative results are shown in Table 5.

Table 4. Inactivation and Tris Solutions for Tissue Homegenates

Solution	Formulation	Preparation
Inactivation Solution II	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.
Tris Solution II	600mM Tris (pH 8.5)	Prepare 600mM Tris (pH 8.5) in water.

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 glutamine and 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).

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 II at an 8:1 ratio (e.g., 1ml buffer + 0.125ml of Inactivation Solution II) 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. Add 0.125ml of Tris Solution II 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 II and Tris Solution II at a 8:1:1 ratio (e.g., 1ml + 0.125ml + 0.125ml).
6. Dilute the tissue homogenate to fit the linear range of the assay.

   Note: The samples can be transferred directly. The centrifugation step commonly used by other methods is not required.

7. For each prepared sample, transfer two 25µl aliquots into two wells of a white luminometer 96-well plate that will be used for the assay. These two wells will be used to determine: i) total glutamine plus glutamate; and ii) glutamate only.

   Note: To minimize assay variability, it is preferable to assay total and glutamate-only wells in the same plate. (See Section 3.B for plate layout guidelines.)

8. Transfer 25µl of glutamate controls into a 96-well plate. Include a negative control (buffer only) for determining assay background.
9. Add 25µl of Glutaminase in Glutaminase Buffer, prepared as described in Section 3.A, to the first set of wells. Add 25µl of Glutaminase Buffer (no Glutaminase) to the second set of wells and to the glutamate controls.
10. Shake the plate for 30–60 seconds.
11. Incubate for 30–40 minutes at room temperature.
12. Add 50µl of Glutamate Detection Reagent, prepared as described in Section 3.A, to all wells.
13. Shake the plate for 30–60 seconds.
14. 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.

15. Record luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.

Table 5. Glutamine and Glutamate in Liver Tissues

Sample	RLUs
Assay background	68,970 ± 1,580
Control (6.25µM glutamate)	1,549,699 ± 36,092
Total glutamine + glutamate in liver tissue	3,176,405 ± 69,228
Glutamate in liver tissue	1,451,041 ± 14,503
Glutamine in liver tissue (ΔRLU)	1,725,364 ± 70,731

A sample of frozen mouse liver tissue (BioreclamationIVT) was homogenized, in acid. Tris Solution II was added and the homogenate was diluted to 3mg of tissue/ml (0.3mg/ml protein). Additional dilutions were prepared in dilution buffer (see Step 5) and aliquots (25µl) were transferred to a 96-well assay plate. Wells containing 25µl of Dilution Buffer (prepared in step 5) with or without 6.25µM glutamate were included in the same plate as controls. The measurements were done in triplicate. The RLUs from the glutamate reaction were subtracted from the total glutamine plus glutamate reaction to get ΔRLUs for glutamine. The glutamine and glutamate concentrations in the liver tissue were found to be 7.0 and 5.9µM, respectively (by comparison to a standard curve), and the luminescent signals were 20-fold above background.

4.E. Plasma and Serum

Plasma and serum samples must be diluted to fit the linear range of the Glutamine/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 100-fold in PBS. Multiple dilutions can be tested.
2. For each prepared sample, transfer two 25µl aliquots into two wells of a white luminometer 96-well plate that will be used for the assay. These two wells will be used to determine: i) total glutamine plus glutamate; and ii) glutamate only.

   Note: To minimize assay variability, it is preferable to assay total and glutamate-only wells in the same plate. (See Section 3.B for plate layout guidelines.)
3. Transfer 25µl of glutamate controls into a 96-well plate. Include a negative control (PBS only) for determining assay background.
4. Add 25µl of Glutaminase in Glutaminase Buffer, prepared as described in Section 3.A, to the first set of wells. Add 25µl of Glutaminase Buffer (no Glutaminase) to the second set of wells and to the glutamate controls.
5. Shake the plate for 30–60 seconds.
6. Incubate for 30–40 minutes at room temperature.
7. Add 50µl of Glutamate Detection Reagent, prepared as described in Section 3.A, to all wells.
8. Shake the plate for 30–60 seconds.
9. 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.
10. Record luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.

Note: If plasma or serum is diluted less than 10-fold, the Inactivation and Tris Solutions may be required to inactivate endogenous enzymes. Add 30µl of the diluted sample to a well, followed by 15µl of Inactivation Solution I (see Table 3). Mix and incubate for 5–10 minutes at room temperature. Then add 15µl of Tris Solution I (see Table 3) and proceed with Step 2 above.

5. Appendix

5.A. Assay Controls and Data Analysis

There is a linear relationship between luminescence signal and the concentrations of glutamine and glutamate. Many luminescence measurements can be described simply in terms of RLUs. To calculate glutamine and glutamate concentrations, a standard curve using titrations of glutamate or glutamine can be used. Alternatively, a "spike" of glutamine or glutamate at a known concentration can be included in the experimental set up and assayed under the same experimental conditions, preferably on the same assay plate.

Different buffers can affect light output; therefore, controls should be prepared using the same buffers as the samples. The Glutamine/Glutamate-Glo™ Assay includes 10mM Glutamate as a **positive control**. Wells containing buffer only should be included as **negative controls** (See Section 3B). These wells can be used to measure the background signal and calculate signal-to-background ratios.

RLUs can be converted into glutamate concentration using a glutamate standard curve. Since a given concentration of glutamine is expected to generate a light signal similar in intensity to an equivalent concentration of glutamate (Figure 3), the glutamate standard curve can be used to calculate glutamine concentration. If a glutamine positive control is also needed or preferred, a stock solution of glutamine can be prepared in water (e.g., using L-Glutamine; Sigma Cat.# G8540).

Samples that contain both glutamine and glutamate require two reactions to determine the glutamine concentration. One reaction measures the starting glutamate concentration and the other measures the concentration of starting glutamate plus the glutamate that is the product of the glutaminase reaction. The difference in signal is contributed by the glutamine in the sample.

Representative results from studying a mixture of glutamine and glutamate are shown in Table 6 and serve to illustrate the principles of the assay. Glutamine and glutamate controls were assayed individually or as a mixture, with and without Glutaminase enzyme. Glutamine produced a signal above background (Bkgd) only when Glutaminase was present (A and B), whereas glutamate resulted in a signal in the presence or absence of Glutaminase (C and D). Signals from equal concentrations of glutaminase and glutamate produced similar results (A and B compared to C and D). Mixtures of glutamine and glutamate are additive. When a mixture of 10µM glutamine and 10µM glutamate (20 µM total) is assayed in the presence of Glutaminase, the light signal is similar to 20 µM glutamine or 20 µM glutamate in the presence of Glutaminase (E vs A or C). However, in the absence of Glutaminase, only the glutamate portion is detected and the signal is equivalent to 10µM glutamate (E vs D).

Table 6. Assaying Mixtures of Glutamine and Glutamate

		RLU	
Mixture	**Formulation**	**With Glutaminase**	**Without Glutaminase**
A	Glutamine, 20µM	2,449,189	17,304
B	Glutamine, 10µM	1,241,521	17,327
C	Glutamate, 20µM	2,494,288	2,660,110
D	Glutamate, 10µM	1,174,706	1,366,632
E	Glutamine 10µM + Glutamate 10µM	2,543,057	1,306,659
F	Glutamine 5µM + Glutamate, 5µM	1,283,518	677,530
Bkgd	Background	17,856	18,036

Mixtures E and F from Table 6 can be used to calculate glutamine concentration. The net RLUs of the reactions with and without Glutaminase and the difference between the two reactions were calculated (Table 7). The difference reflects the light signal contribution from glutamine. Titrations of glutamate control were also included in this experiment and a standard curve was generated using the net RLU values (calculated by subtracting the average background signal from the average signals). The net RLU values in Table 7 and the equation of the trend line (y = 120,263x) were used to calculate the concentrations of the metabolites in the mixtures.

Table 7. Calculated Glutamine Concentrations in Mixtures of Glutamine and Glutamate

		Net RLU and concentration (µM)		
Mixture	**Formulation**	**With Glutaminase**	**Without Glutaminase**	**Difference in RLU**
E	Glutamine 10µM+ Glutamate 10µM	2,525,202 (21.0)	1,288,624 (10.7)	1,236,578 (10.4)
F	Glutamine 5µM + Glutamate, 5µM	1,265,663 (10.5)	659,494 (5.5)	606,169 (5.0)
Bkgd	Background	0	0	0

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) containing no glucose or glutamine and add glucose and glutamine to the desired concentrations, typically 5mM and 2mM, respectively. GlutaMAX™ (Gibco), a dipeptide used as a glutamine substitute, is not used by the Glutamine/Glutamate-Glo Assay.

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. Assay Multiplexing and Normalization

The Glutamine/Glutamate-Glo™ Assay can be multiplexed with viability assays, including the RealTime-Glo™ MT, CellTiter-Glo® and CellTiter-Fluor™ Cell Viability Assays (Figure 8). Changes in the glutamine and glutamate concentrations in the medium can be measured by removing a small amount of medium (2–5µl) for glutamine and 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. The remainder of the cell lysate can be used for metabolite detection.

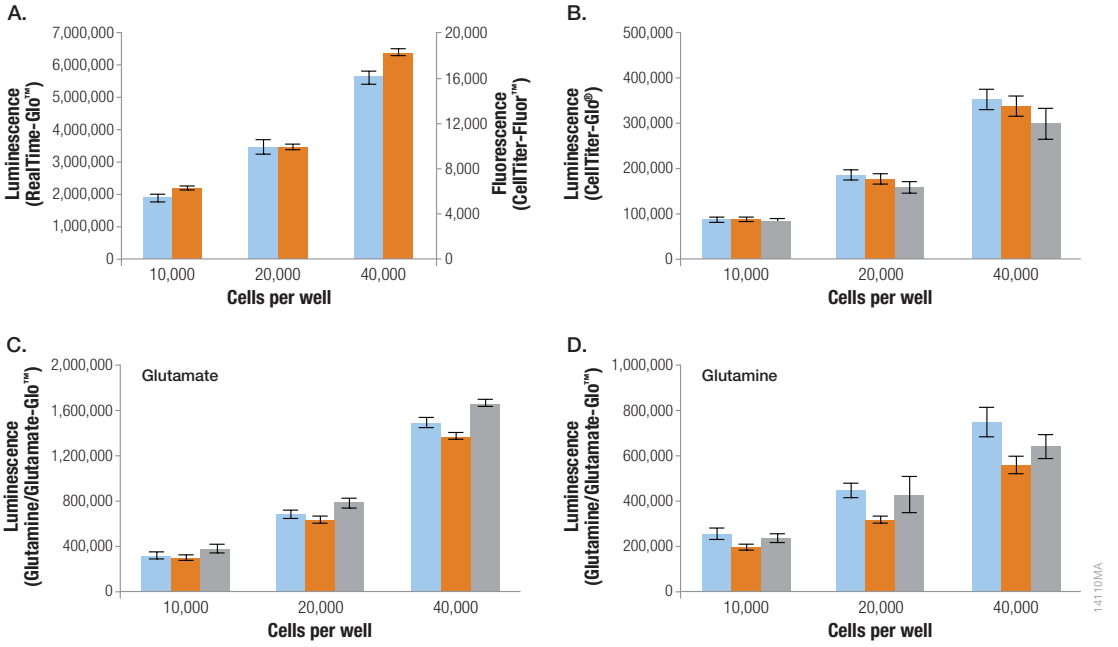

Figure 8. Multiplexing with viability assays. A549 cells were plated in DMEM with 5mM glucose, 2mM glutamine and 10% dialyzed serum at three cell densities (100µl per well). After overnight incubation, 25µl of 5X RealTime-Glo™ (blue bars) or 5X CellTiter-Fluor™ (orange bars) reagent was added to the 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 then removed, the cells were washed twice with cold PBS and 30µl of PBS followed by 15µl of Inactivation Solution I was added to each well. After mixing for 5 minutes on a plate shaker, 15µl of Tris Solution I was added to the wells. A 5µl aliquot 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 incubation at room temperature, luminescence was recorded. The remaining cell lysate was used to measure (1) total glutamine and glutamate and (2) glutamate only following the protocol in Section 4.B. Briefly, 25µl of lysate was transferred into each of two wells. 25µl Glutaminase Enzyme Solution was added to one well, and 25µl Glutaminase Buffer was added to the second well. After 30 minutes, 50µl Detection Reagent was added to each well and the reactions incubated for 60 minutes at room temperature. Luminescence was recorded using a GloMax® luminometer and the contribution from glutamine was calculated as the difference between signals from the two wells (as described in Section 5.A). 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 media did not affect the ability to measure glutamate (Panel C) and glutamine (Panel D).

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.

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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

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
Glutamate-Glo™ Assay	5ml	J7021
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

8. Summary of Change

The following change was made to the 8/19 revision of this document:

Updated data in Table 7.

[a]U.S. Pat. No. 9,273,343 and other patents pending.

[b]European Pat. No. 1131441 and Japanese Pat. No. 4520084.

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