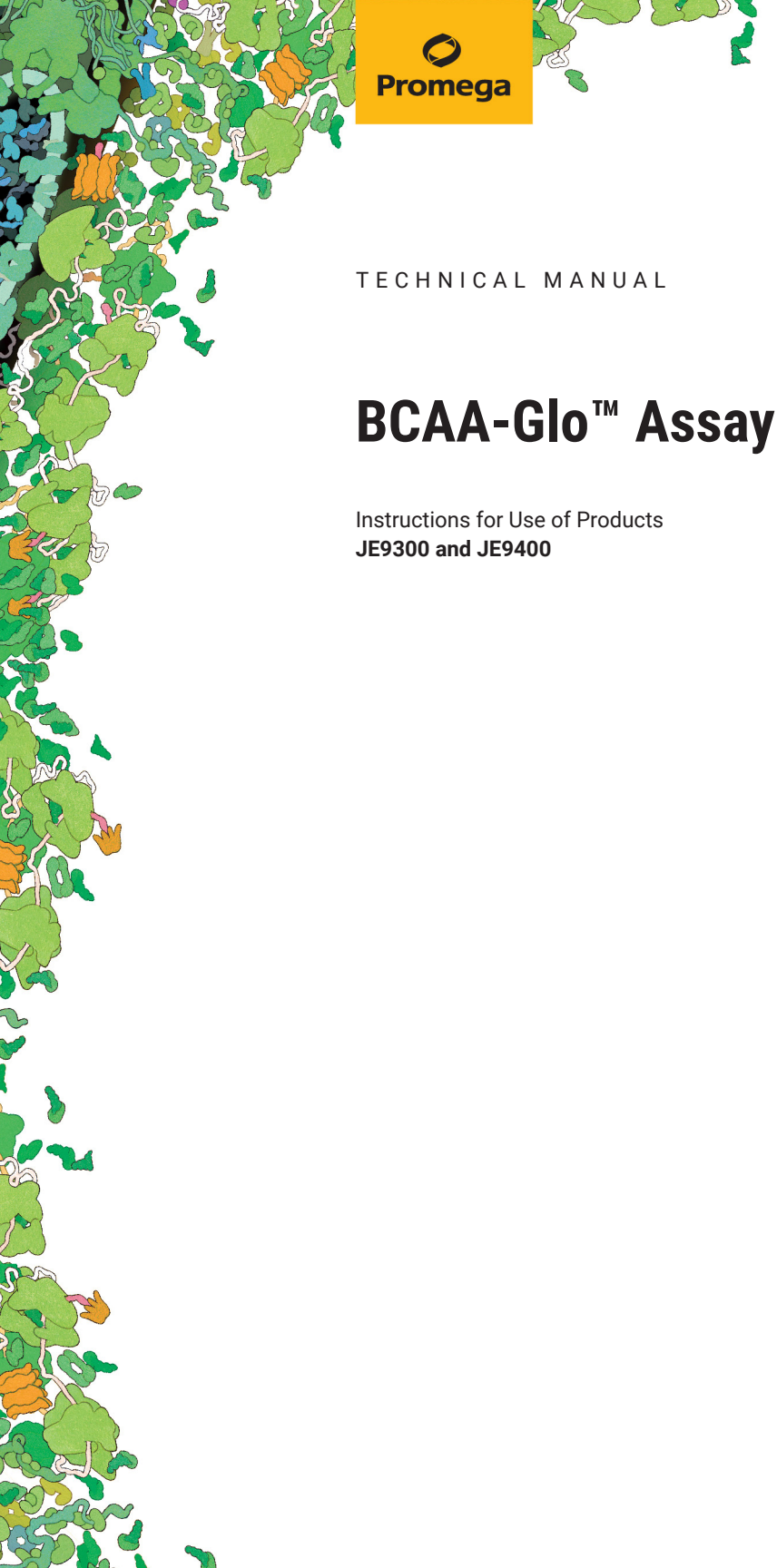

TECHNICAL MANUAL

BCAA-Glo™ Assay

Instructions for Use of Products
JE9300 and JE9400

Revised 7/24
TM712

BCAA-Glo™ Assay

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

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1. Description

The BCAA-Glo™ Assay[(a)] is a bioluminescent assay for rapid, selective and sensitive detection of branched-chain amino acids (BCAA) in biological samples. The term branched-chain amino acids refers to three amino acids: leucine, isoleucine and valine. BCAA participate in many central cell functions, including protein synthesis and metabolism, and elevated serum levels have been found to correlate with metabolic diseases such as diabetes.

The BCAA-Glo™ Assay couples BCAA oxidation and NADH production with a bioluminescent NADH detection system (Figure 1; 1–3). When BCAA detection reagent is added to a sample at a 1:1 ratio, the coupled-enzyme reactions are initiated and run simultaneously (Figure 2). The luminescent signal is proportional to the amount of BCAA in the sample, and increases until all BCAA is consumed, at which point a stable luminescent signal is achieved. The Leucine Dehydrogenase enzyme recognizes all three BCAA with similar efficiency (see Section 5.B). A single amino acid, Leucine, 10mM, is provided to serve as a positive control.

The BCAA-Glo™ Assay sensitivity is <50nM (25pmol of BCAA in a 50µl sample) with linearity up to 25µM (Figure 3 and Table 1). The BCAA-Glo™ Assay is versatile and compatible with many sample types, including mammalian cells, cell culture medium, tissues and serum. However, similar to other enzyme-coupled BCAA detection methods, enzymes and NAD(P)H reduced dinucleotides in samples can interfere with the assay. We recommend up-front sample preparation to inhibit endogenous enzyme activity and to degrade NAD(P)H. To simplify sample preparation, we provide a protocol that uses a strong acid (0.6N HCl) to lyse samples, inactivate enzymes and degrade NAD(P)H. 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.

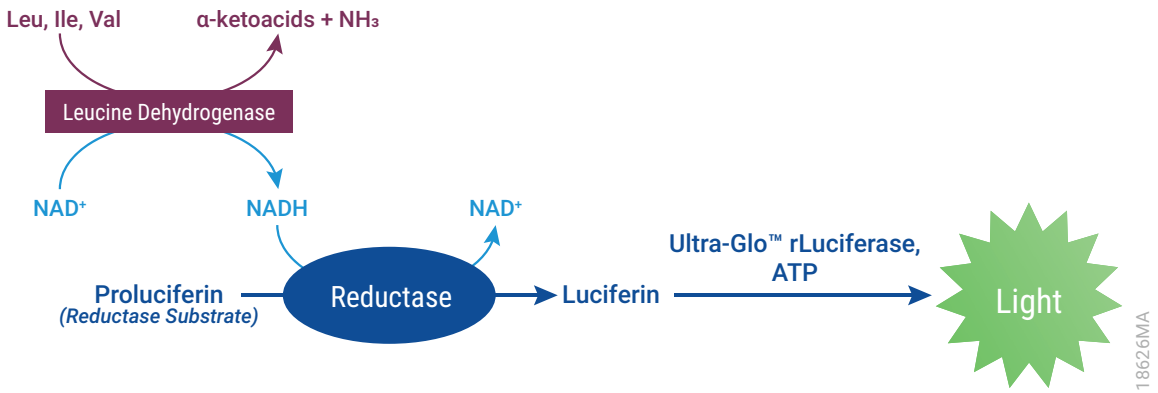

Figure 1. Schematic diagram of the BCAA-Glo™ Assay principle. Leucine Dehydrogenase catalyzes the oxidation of BCAA with concomitant reduction of NAD^+ to NADH. In the presence of NADH, Reductase enzymatically reduces a proluciferin Reductase Substrate to luciferin. Luciferin is detected using Ultra-Glo™ Recombinant Luciferase, and the amount of light produced is proportional to the amount of BCAA in the sample.

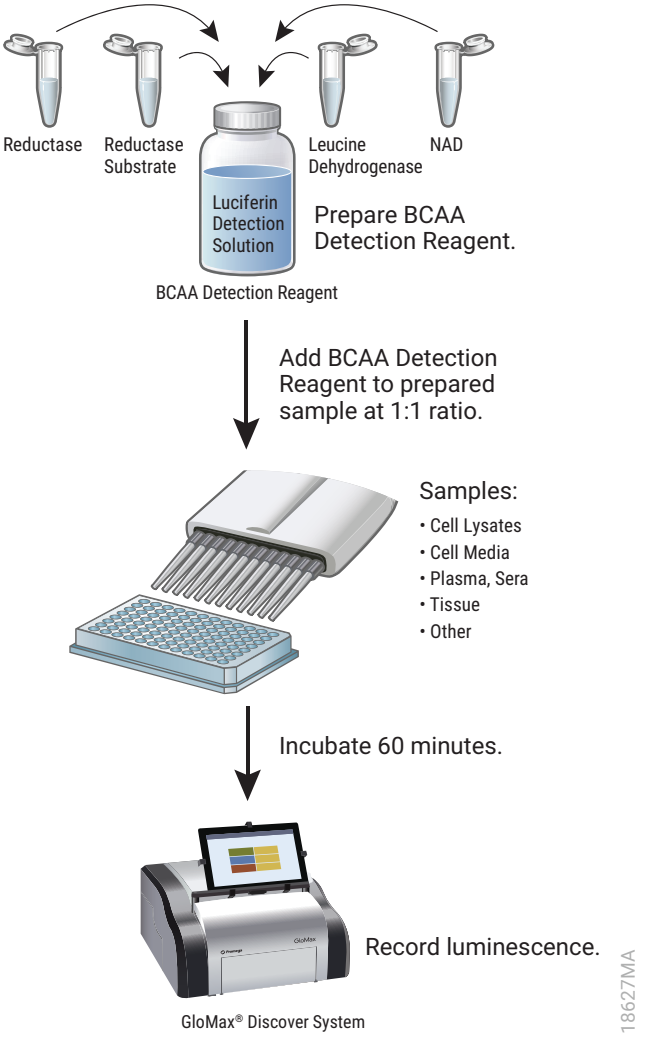

Figure 2. BCAA-Glo™ Assay reagent preparation and protocol.

1. Description (continued)

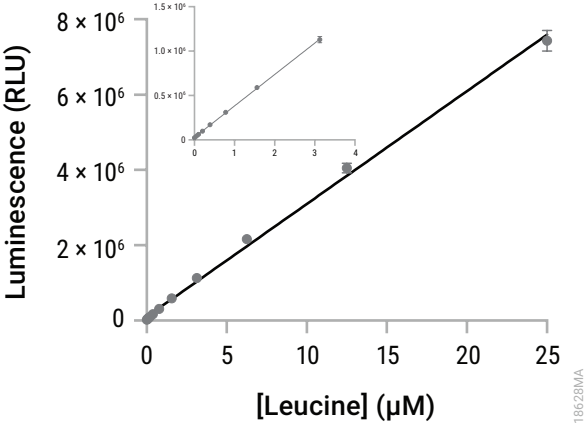

Figure 3. BCAA titration curve. Twofold serial dilutions of leucine in the range of 25µM to 50nM were prepared in phosphate-buffered saline (PBS). The negative control was PBS containing no leucine. Aliquots of the prepared dilutions (50µl) were transferred to a 96-well plate and the BCAA-Glo™Assay was performed following the protocol in Section 3.B. 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. BCAA Titration Data. Signal-to-background ratio (S/B) was calculated by dividing mean luminescence for samples by the mean luminescence for the negative control (no leucine). Signal-to-noise ratio (S/N) was calculated by dividing net luminescence (mean luminescence for the sample minus mean luminescence for the negative control) by the standard deviation of the negative control.

Leucine (µM)	0	0.05	0.10	0.20	0.39	0.78	1.56	3.12	6.25	12.5	25
Average Luminescence (RLU × 10^3)	71	84	98	121	171	274	484	923	1,811	7,052	13,410
Standard Deviation (RLU × 10^3)	1.9	2.4	2.6	3.4	5.0	8.8	14.2	35.9	61.5	130	274
Coefficient of Variation (%)	8	6	4	3	3	3	2	3	3	3	4
S/B	1.0	1.7	2.5	4.0	6.9	12.5	23.8	45.6	87.5	164	300
S/N	—	9.7	19.6	38.9	76.5	150	297	581	1,128	2,120	3,898

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
BCAA-Glo™ Assay	**5ml**	**JE9300**

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

Metabolite-Glo™ Detection System, 5ml:
- 5ml Luciferin Detection Solution
- 55µl Reductase
- 55µl Reductase Substrate
- 275µl NAD
- 100µl NADP*
- 15ml 0.6N HCl
- 15ml Neutralization Buffer

BCAA-Glo™ Enzyme Pack, 5ml:
- 100µl Leucine Dehydrogenase
- 50µl Leucine, 10mM

PRODUCT	SIZE	CAT.#
BCAA-Glo™ Assay	**50ml**	**JE9400**

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

Metabolite-Glo™ Detection System, 50ml:
- 50ml Luciferin Detection Solution
- 275µl Reductase
- 275µl Reductase Substrate
- 1ml NAD
- 0.5ml NADP*
- 15ml 0.6N HCl
- 15ml Neutralization Buffer

BCAA-Glo™ Enzyme Pack, 50ml:
- 1ml Leucine Dehydrogenase
- 50µl Leucine, 10mM

Storage Conditions: Store the BCAA-Glo™ Assay (and all components contained therein) 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, except the 0.6N HCl and Neutralization Buffer, which 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.

*****NADP is a component of the Metabolite-Glo™ Detection System but is not used when performing the BCAA-Glo™ Assay.

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 BCAA

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 Cat.# 3912)
- luminometer (e.g., GloMax® Discover System Cat.# GM3000)

3.A. Sample Preparation

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

The BCAA-Glo™ Assay can be used to measure BCAA in samples such as mammalian cells, cell culture medium, tissues and serum. This requires preparing various sample types, including cell lysates and tissue homogenates, before assaying.

For sample preparation, we recommend using 0.6N HCl (acid) and Neutralization Buffer (1M Tris base) supplied with the kit. Acid treatment rapidly stops metabolism, inhibits endogenous protein activity and destroys reduced NAD(P)H dinucleotides. When dealing with difficult-to-lyse samples such as 3D cultures, Triton®X-100 can be added to a final concentration of 0.2%. Acid-treated and neutralized samples can be assayed immediately following the protocol provided in Section 3.C or stored at –20°C. If needed, an aliquot of the sample can be removed for protein measurement (see Section 5.A).

We do not recommend using detergent lysis without acid since many endogenous dehydrogenases remain active in detergent lysed samples, significantly increasing the BCAA-Glo™ Assay background. Endogenous dehydrogenase activity can be determined by using BCAA detection reagent prepared without Leucine Dehydrogenase.

Acid lysis is compatible with multiwell plate workflows and acid can be directly added to the cells plated in 96- or 384-well plates. Although cells lysed with acid might appear incompletely lysed when viewed under a microscope (Figure 4), metabolites are efficiently released.

Acid treatment can be used with different sample types and is highly recommended for unknown samples. Table 2 provides examples of BCAA concentration ranges in samples and suggestions for sample preparation. Section 4 provides example protocols for using the BCAA-Glo™ Assay with mammalian cells, cell culture media, tissues and serum.

Samples prepared using other methods, for example deproteinization using 10kDa filtration columns or heat inactivation, might be acceptable but must be tested for compatibility with the BCAA-Glo™ Assay using the provided Leucine, 10mM standard and controls described in Section 3.D.

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

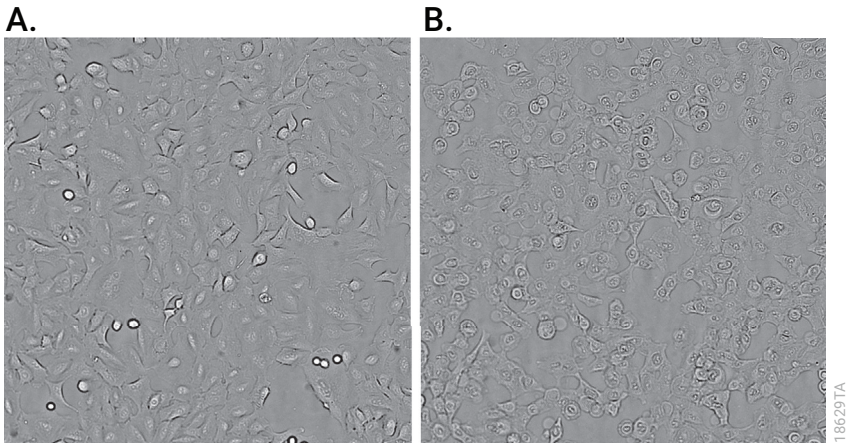

Figure 4. Cell imaging after adding acid. A549 cells were plated at 25,000 cells/well in DMEM (GIBCO® Cat.# A14430) and 10% dialyzed fetal bovine serum (FBS, GIBCO® Cat.# A3382001) in a 96-well plate (Greiner Cat.# 655094) overnight. Cells were washed three times with 100µl of PBS. PBS (**Panel A**) or PBS premixed with 0.6N HCl at 1:0.2 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. No significant difference in BCAA concentration was measured in samples treated with 0.6N HCl at 1/5 to 1/2 of the sample volume. Therefore, different ratios can be used to accommodate your experimental setup. Concentrations in this table represent experimental values shown in this technical manual. Optimization may be required for other sample types or sources.

Sample	BCAA Concentration in Sample	Recommendations
Cell medium (extracellular)	200µM–2.4mM in various media	• Cells in medium. • Add 0.6N HCl (1/5 to 1/2 of the sample volume). • Add Neutralization Buffer (the same volume as 0.6N HCl).
Cell lysates (intracellular)	0.1–15µM for 5,000 to 40,000 cells lysed in 50µl	• Cells in PBS. • Add 0.6N HCl (1/5 to 1/2 of the sample volume). • Add Neutralization Buffer (the same volume as 0.6N HCl).
Tissues	0.5–10µM for 10mg of wet tissue homogenized in 1ml	• Tissues in PBS. • Add 0.6N HCl (1/5 to 1/2 of the sample volume). • Add Neutralization Buffer (the same volume as 0.6N HCl).
Serum	200–800µM depending on human serum lot (varies)	• Serum samples diluted ≥40-fold in PBS. • Add 0.6N HCl (1/5 to 1/2 of the sample volume). • Add Neutralization Buffer (the same volume as 0.6N HCl).

3.B. Reagent Preparation

This protocol is for 50µl of sample and 50µl of BCAA detection reagent in a 96-well plate. The assay can be adapted to other volumes, provided the 1:1 ratio of BCAA detection reagent volume to sample volume is maintained (e.g., 12.5µl of sample and 12.5µl BCAA detection reagent in a 384-well plate format). To use a different assay format, scale the volumes of samples, controls, leucine standards and reagents accordingly.

1. Thaw all components. Once thawed, equilibrate the Luciferin Detection Solution to room temperature; place all other components on ice. Mix thawed components prior to use.
2. Calculate the volume of BCAA detection reagent needed. You will need 50µl of BCAA detection reagent for each assay in a 96-well plate with 50µl of sample. We recommend preparing additional reagent to compensate for pipetting error.
3. Prepare BCAA detection reagent by combining components as shown below.

   Note: Prepare only the volume of BCAA detection reagent calculated in Step 2. Unused BCAA detection reagent cannot be stored.

Component	Volume Per Reaction	Volume Per 100 Reactions
Luciferin Detection Solution	50µl	5ml
Reductase Substrate	0.25µl	25µl
Reductase	0.25µl	25µl
NAD	1µl	100µl
Leucine Dehydrogenase	1µl	100µl

4. Mix by gently inverting five times.

3.C. Protocol

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

Information on preparing and using appropriate positive and negative controls for the BCAA-Glo™ Assay can be found in Section 3.D.

1. Prepare samples using the appropriate method for your sample type. See Sections 3.A and 4 for more information.
2. Prepare the BCAA detection reagent as described in Section 3.B. Ensure that the reagent is at room temperature prior to use.
3. Transfer 50µl of each sample, positive controls (Leucine, 10mM standard diluted in the same buffer as the samples) and negative (buffer only) controls into a well of a 96-well plate.
4. Add 50µl of BCAA detection reagent to each well.
5. Mix the plate by shaking for 30–60 seconds.
6. Incubate for 60 minutes at room temperature.

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

7. Record luminescence using a plate-reading luminometer, following the instrument manufacturer's instructions.

3.D. Assay Controls and Data Analysis

There is a linear relationship between luminescence signal and BCAA 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 values 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 BCAA concentration and determine if your samples are within the linear range of the assay, a standard curve can be generated using a titration of Leucine, 10mM, included in the kit (see Table 1 for suggested concentrations). If the sample RLU values fall outside the linear range of the leucine standard curve, sample dilutions should be adjusted and reassayed.

Alternatively, in place of running a full standard curve, 2–4 concentrations of the Leucine, 10mM standard can be used and concentrations of BCAA in samples can be calculated based on RLU from those standards. We recommend including a high concentration of leucine (25µM), a low concentration (1µM) and concentrations that are near your sample concentration. These points can be adjusted based on concentrations expected in your sample. To determine assay background, a negative control (buffer only) should be included on the assay plate.

BCAA concentration in the sample can be calculated using the following formula if using one leucine standard concentration:

$$[\text{BCAA}] = \frac{[\text{Leucine standard, µM}] \times (\text{RLU}_{\text{sample}} - \text{RLU}_{\text{background}})}{(\text{RLU}_{\text{Leucine standard}} - \text{RLU}_{\text{background}})}$$

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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. 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, and should be considered when studying metabolism. Supplementing the culture medium with 5–10% fetal bovine serum (FBS) is a standard practice when culturing mammalian cells. However, FBS also contains variable amounts of metabolites. Using defined medium, for example DMEM (GIBCO® Cat.# A1443001) lacking major fuel sources such as glucose, glutamine and pyruvate, and adding those components at the desired concentrations, and then supplementing with dialyzed serum (e.g., GIBCO® Cat.# A3382001), provides better control for studying metabolic changes.

The BCAA-Glo™ Assay can be used to monitor changes in intracellular BCAA levels of cells plated in 96- or 384-well plates. Alternatively, cells can be collected and lysed, and samples transferred to 96- or 384-well plates for BCAA measurement.

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

1. Plate 1,000–50,000 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 three to five times with 200µl of cold PBS.

   Note: Work quickly to minimize changes in BCAA metabolism.
3. Add 25µl of PBS to the washed cells. Include a negative control (PBS, no cells) for determining assay background.
4. Add 12.5µl of 0.6N HCl. Mix by shaking the plate for 5 minutes.

   Note: Alternatively, PBS (Step 3) can be combined with 0.6N HCl (Step 4) and added simultaneously.
5. Add 12.5µl of Neutralization Buffer. Mix by shaking the plate for 30–60 seconds.
6. Add 50µl of BCAA detection reagent, prepared as described in Section 3.B.
7. Mix by shaking the plate for 30–60 seconds.
8. Incubate for 60 minutes at room temperature.
9. Record luminescence.

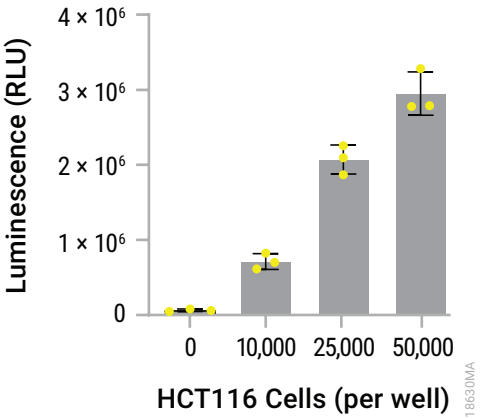

Figure 5. Intracellular BCAA. HCT116 cells in DMEM supplemented with 10% serum were plated at 10,000, 25,000 and 50,000 cells/well. After overnight incubation, the medium was removed, cells were washed with 200µl of cold PBS and BCAA was measured as described in the preceding protocol. The data show increased signal with increasing cell number. The intracellular BCAA was measured at > tenfold signal above background with 10,000 HCT116 cells plated per well. Data are the average of three replicates (yellow dots).

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

1. Collect cells, wash with cold PBS three to four times and resuspend at a concentration of 0.5×10^5–1.6×10^6 cells/ml.
2. Add 1/5 volume of 0.6N HCl (e.g., add 200µl of 0.6N HCl per 1ml of cells in PBS). Mix well and incubate for 5 minutes at room temperature.
3. Add the same volume of Neutralization Buffer as 0.6N HCl in Step 2. Mix well.

   Notes: Aliquots can be removed for protein measurements or samples can be stored below –10°C.
4. Premix PBS, 0.6N HCl and Neutralization Buffer at 5:1:1 ratio. Use this mixture as a negative control to determine assay background and to prepare 1µM leucine standard as a positive control.
5. Transfer 50µl of cell lysates, negative and positive controls to the assay plate.

   Note: If cell lysates need to be diluted, use buffer prepared in Step 4.
6. Add 50µl of BCAA detection reagent prepared as described in Section 3.B.
7. Mix by shaking the plate for 30–60 seconds.
8. Incubate for 60 minutes at room temperature.
9. Record luminescence.

4.A. Mammalian Cells (continued)

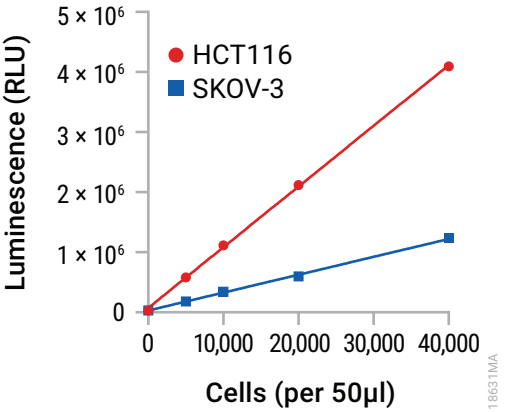

Figure 6. Measuring BCAA in cell lysates. HCT116 and SKOV-3 cells (1.6 × 10^6 cells/ml) were prepared as described in the protocol. To determine the linear range of BCAA in cell lysates, twofold serial dilutions were prepared in PBS premixed with 0.6N HCl and Neutralization Buffer, at 5:1:1 ratio. Each sample (50µl) was transferred to a 96-well assay plate. BCAA detection reagent (50µl) was added to the samples and after a 60-minute incubation at room temperature, luminescence was recorded using a GloMax® 96 Luminometer. Data represent the average of three replicates. The data show a linear relationship between light signal and cell density, indicating that BCAA measurements are within the linear range of the assay. The assay sensitivity and wide linearity allows for measurement of BCAA using 5,000–40,000 cells/50µl. The calculated BCAA concentration in the HCT116 and SKOV-3 cells corresponded to approximately 9.2 and 2.6 fmol/cell, respectively.

4.B. Cell Culture Media

The BCAA-Glo™ Assay can be used for measuring BCAA in cell culture media. Formulations of commonly used cell culture media include 200µM to 2.4mM BCAA and will require dilution (>8- to 100-fold) to fit into the linear range of the assay (e.g., to <25µM).Changes in BCAA levels can be monitored over time by removing small aliquots at different time points. The collected samples can be diluted directly into PBS and frozen until the completion of the time course.

1. Add 5,000–50,000 cells per well to a 96-well plate. Include control wells containing medium only.
2. Collect medium samples at experimental time points by removing 2–5µl and diluting into 98µl or 95µl of PBS.

   Note: Samples may be assayed immediately or frozen at –10°C or below.
3. On the day of the assay, thaw the samples and transfer 25µl to a 96-well plate. Include negative (buffer only) and positive (leucine standard) controls as described in Section 3.B.

   Note: The medium samples may need to be further diluted (e.g., in PBS) to fit into the linear range of the assay.
4. Add 12.5µl of 0.6N HCl and mix by shaking the plate for 5 minutes.

5. Add 12.5µl of Neutralization Buffer. Mix by shaking the plate for 30–60 seconds.
6. Add 50µl of BCAA detection reagent, prepared as described in Section 3.B.
7. Mix by shaking the plate for 30–60 seconds.
8. Incubate for 60 minutes at room temperature.
9. Record luminescence.

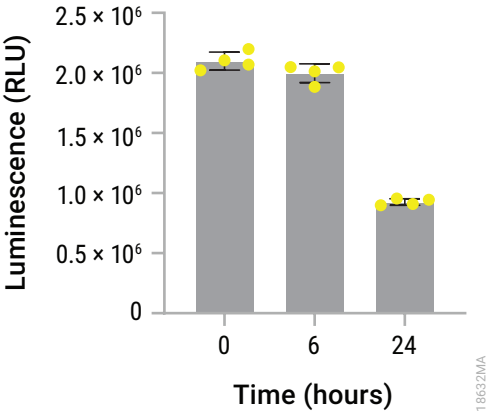

Figure 7. BCAA in cell culture medium. HCT116 cells were plated at 50,000 cells/well in a 96-well plate overnight in 100µl of DMEM supplemented with 10% FBS. The medium was then removed and cells were washed three times to remove residual BCAA. Fresh DMEM with 10% FBS was added. Aliquots of medium were removed after 6 and 24 hours and frozen below –10°C until assayed. Samples were thawed and diluted 200-fold with PBS before measuring BCAA as described in Section 4.B. BCAA levels in the medium decreased to approximately 50% of the starting concentration in 24 hours due to cell consumption. Data are the average of four replicates (shown as dots on the graph).

4.C. Tissues

The BCAA-Glo™ Assay can be used to measure the BCAA concentration in homogenized tissues. To fit into the BCAA-Glo™ Assay linear range and avoid assay interference, we recommend homogenizing the tissues at 5–15mg/ml of tissue in PBS with 0.6N HCl. Homogenized and neutralized tissues can be assayed immediately or stored below –10°C.

1. Weigh 5–15mg of tissue and add 1ml of PBS.
2. Immediately add 200µl (1/5 sample volume) of 0.6N HCl and homogenize for 20–30 seconds using a mechanical homogenizer (e.g., Tissue-Tearor™, BioSpec Cat. #985370-07).

   Note: PBS and 0.6N HCl can be premixed before adding to the tissue.
3. Add 200µl (same volume as 0.6N HCl) of Neutralization Buffer to the homogenate.

   Note: An aliquot of tissue homogenate can be removed at this point to determine protein concentration; see Section 5.A.

4.C. Tissues (continued)

4. Transfer 50µl of each prepared sample to a 96-well assay plate. Include negative controls (buffer only) and positive controls (leucine standard). Prepare controls in the same buffer as samples (e.g., PBS + 0.6N HCl + Neutralization Buffer at a 5:1:1 ratio).
5. Add 50µl of BCAA detection reagent, prepared as described Section 3.B.
6. Mix by shaking the plate for 30–60 seconds.
7. Incubate at room temperature for 60 minutes.
8. Record luminescence.

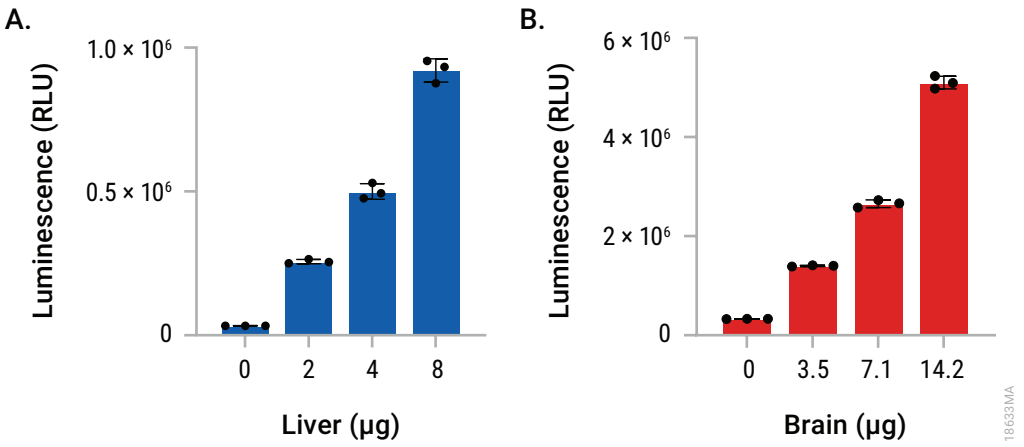

Figure 8. Measuring BCAA in tissues. Samples (11mg/ml) of frozen mouse liver (**Panel A**) and brain (**Panel B**) tissue were homogenized as described in the protocol. An aliquot of each sample was removed for protein measurement. The protein concentration was 1.28mg/ml for liver (**Panel A**) and 1.14mg/ml for brain (**Panel B**). For BCAA detection, samples were serially diluted twofold in dilution buffer (PBS + 0.6N HCl + Neutralization Buffer premixed in a 5:1:1 ratio) and 50µl was transferred to the assay plate. Wells containing 50µl of homogenization buffer with and without 1µM leucine were included as the positive and negative controls, respectively. BCAA detection reagent (50µl) was added, and after a 60-minute incubation at room temperature, luminescence was recorded. Dilutions in the 1:2 to 1:16 range were tested. BCAA concentration was approximately 9.1µM in the liver homogenate and approximately 2.8µM in the brain homogenate. Data are the average of three replicates (shown as dots on the graph).

4.D. Serum

The BCAA-Glo™ Assay can be used to measure BCAA in serum or plasma. Levels in serum and plasma can be high, 200–800µM, and therefore must be diluted at least 8- to 32-fold, to <25µM, to fit into the linear range of the assay. We recommend testing a series of serum dilutions to determine the optimal dilution range, which will depend on the specific serum sample.

In this protocol we also include optional steps for incorporating a leucine spike into the experiment. The spike can serve as a means of determining concentration and also demonstrate the lack of any interference by serum.

A 'spike' is a known concentration of Leucine, 10mM standard added to the sample. It is important when doing spike experiments to know the concentration of the spike you are adding to the sample, and to run the sample with and without spike, treating the wells the same. When choosing the concentration of spike, target 40–100% of the sample concentration. The sum of the sample and spike must also remain within the linear range of the assay. Interference can be measured based on the recovery of the added spike relative to a leucine-only control as shown in Figure 9, Panel B.

1. Make serum serial dilutions in PBS. Include a negative control (buffer only).
2. Prepare a 4µM leucine spike by diluting the Leucine, 10mM standard in PBS.
3. Combine diluted serum samples and negative control with either PBS (no spike control) or 4µM leucine spike at a 1:1 ratio (final concentration 2µM leucine spike).
4. Transfer 30µl of each prepared sample into a 96-well assay plate.
5. Add 10µl of 0.6N HCl. Mix by shaking the plate for 5 minutes.
6. Add 10µl of Neutralization Buffer. Mix by shaking the plate for 30–60 seconds.
7. Add 50µl of BCAA detection reagent prepared as described Section 3.B.
8. Mix on a plate shaker for 30–60 seconds.
9. Incubate at room temperature for 60 minutes.
10. Record luminescence.
11. Calculate percent recovery by subtracting the negative control (assay background) values as shown in this formula:

$$\text{Percent Recovery} = \frac{(RLU_{\text{serum + leucine spike}} - RLU_{\text{serum}})}{(RLU_{\text{leucine standard}})} \times 100$$

Note: After assay conditions are optimized to reduce interference, Steps 2, 3 and 11 can be omitted.

4.D. Serum (continued)

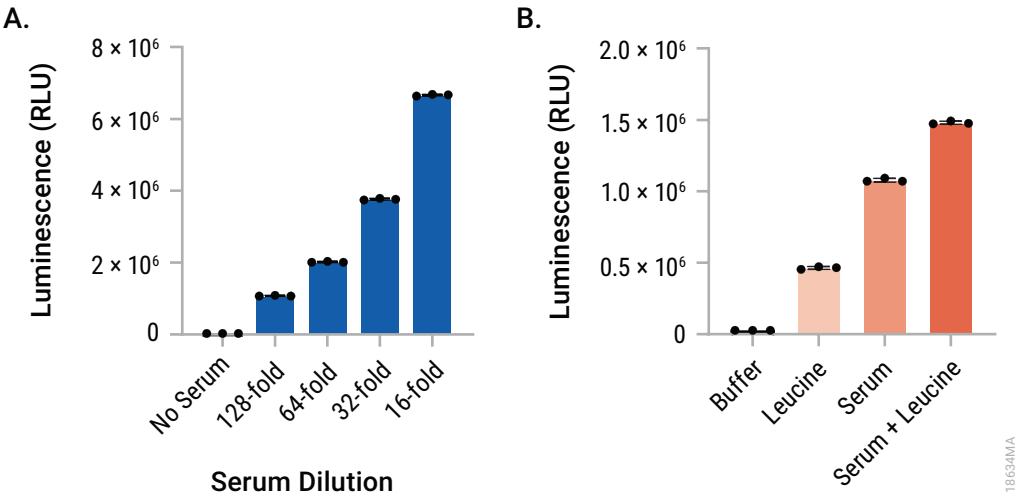

Figure 9. Optimization of BCAA detection in serum. Serial dilutions of human serum (BioIVT) were prepared in PBS and then mixed 1:1 with PBS (**Panel A**) or 1:1 with 4µM leucine standard (2µM final) for spike samples (**Panel B**). Dilutions were transferred to a white 96-well plate (30µl per well). Samples were acidified with 0.6N HCl (10µl per well) for 5 minutes, then Neutralization Buffer added (10µl per well) . Human serum had to be diluted at least 16-fold to fit into the linear range of the assay and was linear with samples diluted from 16- to 128-fold (**Panel A**). Serum diluted 128-fold was measured with 40-fold signal above background, indicating that BCAA concentration in serum can be measured with high sensitivity and wide assay window. The serum diluted 128-fold was used for the spike recovery test. Recovery of the 2µM spike was approximately 92% (**Panel B**). Based on the leucine spike, the concentration of BCAA in the human serum sample was calculated to be approximately 590µM. Luminescence was analyzed on a GloMax® Discover System. Data are the average of three replicates (shown as dots on the graph).

5. Appendix

5.A. Multiplexing and Normalizing for Mammalian Cells

The BCAA-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 can be used to normalize BCAA measurements to the number of viable cells and separate immediate effects on BCAA metabolism from global effects on cell health. To multiplex the cell viability assays and BCAA 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 neutralization, remove an aliquot of each sample for protein measurement and ATP detection using CellTiter-Glo® Cell Viability Assay (Cat.# G9241). Use the remaining sample for the BCAA measurement following the protocol described in Section 3.B.

To measure protein concentration, we recommend the PIERCE™ Dilution-Free™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific Cat.# A55862), which is compatible with acid/base lysis and sensitive enough to measure protein levels in 10,000–50,000 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, remove aliquots for protein determination before sample neutralization because Neutralization Buffer interferes with the Micro BCA Protein Assay.

Representative data for multiplexing viability assays, ATP detection and BCAA measurements in A549 cancer cells are shown in Figure 10. Linear increases in signal with increasing cell number was observed with all assays. The data can be normalized by directly comparing the luminescent signal generated by the BCAA-Glo™ Assay to the fluorescence and/or luminescence signals of the viability and ATP assays. The BCAA concentration can also be calculated and normalized to the protein amount.

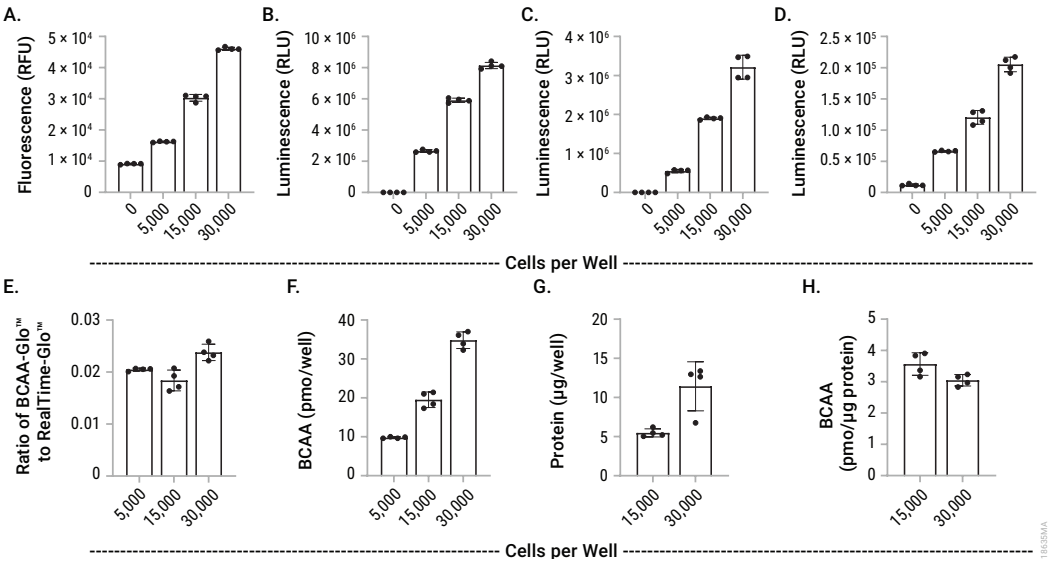

Figure 10. Multiplexing viability, ATP and protein measurements with BCAA detection. A549 cells in 100µl of F-12 medium supplemented with 10% serum were plated at 5,000, 15,000 and 30,000 cells/well. The BCAA-Glo™ Assay was performed using the protocol in Section 4.A with the following modifications: On the day of the assay and before 0.6N HCl treatment, viability was assessed (**Panel A**) using the CellTiter-Fluor™ Assay (Cat.# G9260) and (**Panel B**) the RealTime-Glo™ MT Cell Viability Assay (Cat.# G9711). After 0.6N HCl and Neutralization Buffer treatment, an aliquot was removed to analyze ATP content (**Panel C**) using CellTiter-Glo® Assay (Cat.# G7570). BCAA (**Panel D**) was then measured using the protocol described in this technical manual, and normalized to the RealTime-Glo™ MT Assay (**Panel E**). The amount of BCAA per well (**Panel F**) was calculated based on the Leucine, 10mM standard. Prior to BCAA detection, an aliquot was removed for protein measurement (**Panel G**) using the PIERCE™ Dilution-Free™ Rapid Gold BCA Protein Assay Kit (Cat.# A55862), and the amount of BCAA was normalized to total protein (**Panel H**).

5.B. Leucine Dehydrogenase Activity with BCAA

The Leucine Dehydrogenase enzyme recognizes all three BCAA with similar efficiency (Figure 11). It is specific for BCAA L-isomers (data not shown).

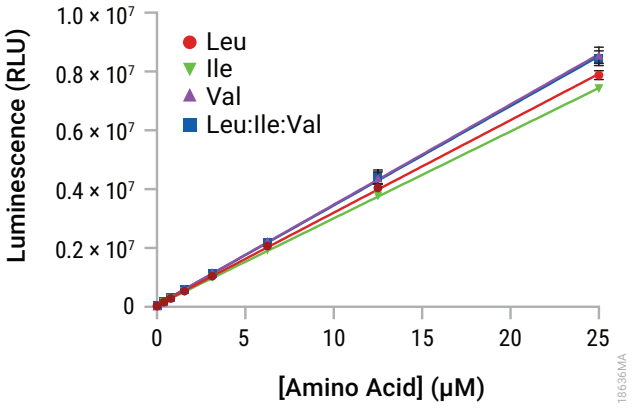

Figure 11. Activity of Leucine Dehydrogenase with BCAA. Stocks of valine and isoleucine (10mM, Sigma Cat.# 94619 and Sigma Cat.# I2752, respectively) were prepared in water and used with the Leucine, 10mM standard to test the activity of the Leucine Dehydrogenase. Twofold serial dilutions of leucine (Leu), valine (Val) and isoleucine (Ile) were prepared in PBS. A 1:1:1 mixture of the three amino acids was also prepared and serially diluted. Aliquots of the prepared dilutions (50µl) were transferred to a 96-well assay plate and the assay was performed following the protocol in Section 3.C. Each data point represents the average of four replicates.

5.C. Temperature and Reagent Compatibility

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

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. 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-well low volume (Corning® Cat.# 4512) plates. We do not recommend black or clear plates. The light signal is diminished in black plates and there is increased well-to-well crosstalk in clear plates.

Note: 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.E. 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.

Promega

5.F. Related Products

Energy Metabolism Assays

Product	Size	Cat.#
Glucose Uptake-Glo™ Assay	5ml	J1341
Lactate-Glo™ Assay	5ml	J5021
Pyruvate-Glo™ Assay	5ml	J4051
Glucose-Glo™ Assay	5ml	J6021
Glycogen-Glo™ Assay	5ml	J5051
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycerol-Glo™ Assay	5ml	J3150
Triglyceride-Glo™ Assay	5ml	J3160
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190
Malate-Glo™ Assay	5ml	JE9100
BHB-Glo™ (Ketone Body) Assay	5ml	JE9500
Metabolite-Glo™ Detection System	5ml	J9030
Dehydrogenase-Glo™ Detection System	5ml	J9010

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

Additional sizes available.

6. Summary of Changes

The following changes have been made to the 7/24 revision of this document:

1. In Section 5.A, two Thermo Fisher Scientific catalog numbers were updated.
2. Third party trademarks were added or updated.

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