Promega

TECHNICAL MANUAL

Malate-Glo™ Assay

Instructions for Use of Products
JE9100 and JE9200

11/23
TM696

Malate-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

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1. Description

The Malate-Glo™ Assay[a] is a bioluminescent assay for rapid, selective and sensitive detection of L-malate in biological samples. L-malate is a tricarboxylic acid cycle (TCA) intermediate and a critical component of the malate-aspartate shuttle. In plants, malate plays an important role in the Calvin cycle.

The Malate-Glo™ Assay couples malate oxidation and NADH production with a bioluminescent NADH detection system (Figure 1; 1–3). When malate detection reagent is added to a sample at a 1:1 ratio, the coupled-enzyme reactions start and run simultaneously (Figure 2). The luminescent signal is proportional to the amount of malate in the sample and increases until all malate is consumed, at which point a stable luminescent signal is achieved (Figure 3 and Table 1).

The assay sensitivity is <40nM (20pmol of malate in a 50µl sample) with linearity up to 25µM. The Malate-Glo™ Assay is versatile and compatible with many sample types. However, as with other enzyme-coupled malate detection methods, enzymes and reduced dinucleotides NAD(P)H in the samples may 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 for sample lysis, enzyme inactivation and NAD(P)H degradation. The workflow is compatible with 96- and 384-well plate formats, does not require sample centrifugation or spin columns and is well-suited for rapid analysis of multiple samples.

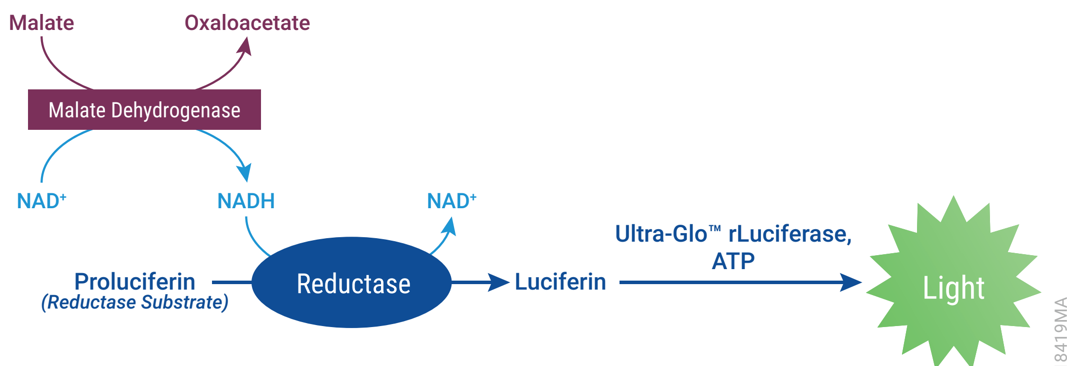

Figure 1. Schematic diagram of the Malate-Glo™ Assay principle. Malate dehydrogenase catalyzes the oxidation of malate with concomitant reduction of NAD+ to NADH. In the presence of NADH, reductase enzymatically reduces a proluciferin Reductase Substrate to luciferin. Luciferin is detected using Ultra-Glo™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

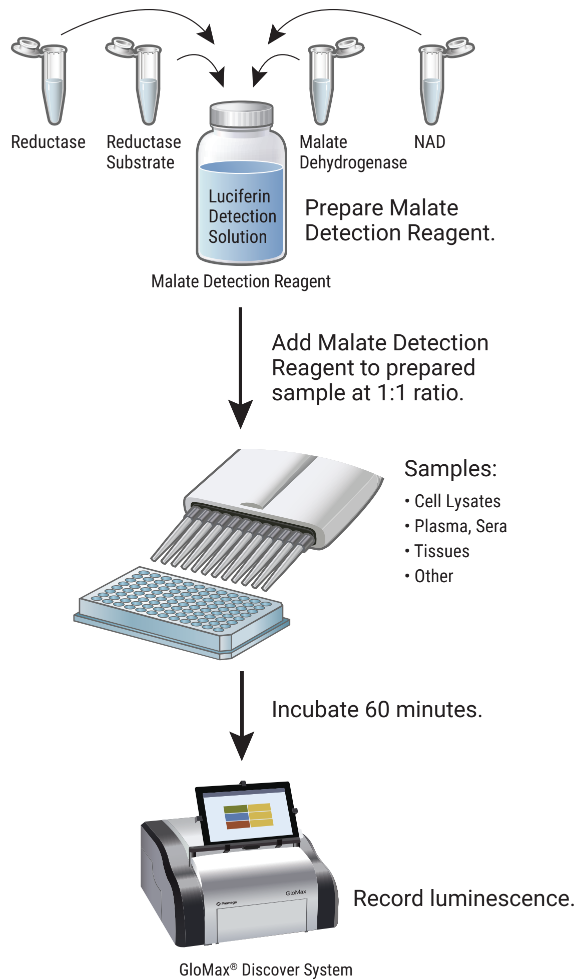

Figure 2. Malate-Glo™ Assay reagent preparation and protocol.

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1. Description (continued)

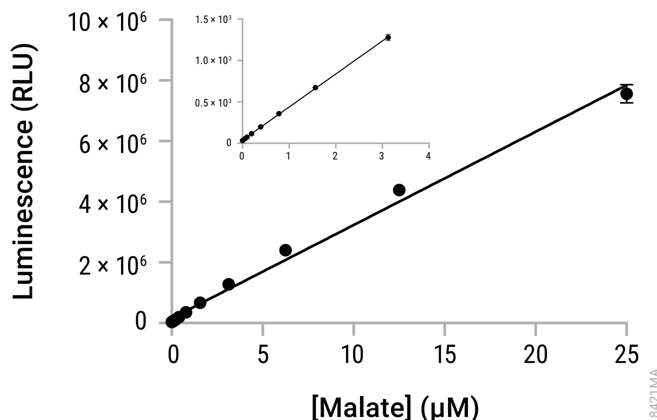


Figure 3. Malate titration curve. Twofold serial dilutions of malate in the range of 25µM to 40nM were prepared in phosphate-buffered saline (PBS). The negative control was PBS containing no malate. Aliquots of the prepared dilutions (50µl) were transferred to a 96-well plate and the assay was performed following the protocol in Section 3.B. Luminescence was measured in relative light units (RLU) using a GloMax® Discover luminometer. Each data point represents the average of four replicates.

Table 1. Malate 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 malate). Signal-to-noise ratio (S/N) was calculated by dividing net luminescence (mean luminescence for the sample minus mean luminescence for the negative controls) by the standard deviation of the negative control.

Malate (µM)	0	0.05	0.1	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25
Average Luminescence (RLU × 10^3)	33	54	73	116	198	357	671	1,277	2,403	4,383	7,559
Standard Deviation (RLU × 10^3)	3.0	3.4	3.3	4.1	6.0	10.7	17.7	38.7	69.8	151	300
Coefficient of Variation (%)	9	6	4	3	3	3	3	3	3	3	4
S/B	1.0	1.6	2.2	3.5	5.9	10.7	20.1	38.2	71.8	131	225
S/N		6.9	13.5	27.5	55	108	212	414	790	1,450	2,508

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Malate-Glo™ Assay	**5ml**	**JE9100**

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

Malate-Glo™ Enzyme Pack, 5ml:

- 25µl Malate Dehydrogenase
- 50µl Malate, 10mM

PRODUCT	SIZE	CAT.#
Malate-Glo™ Assay	**50ml**	**JE9200**

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

Metabolite-Glo™ Detection System, 50ml:

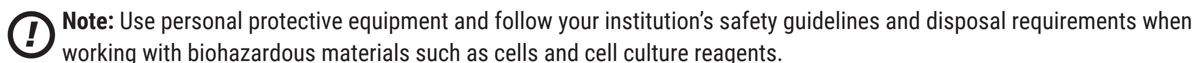
- 50ml Luciferin Detection Solution
- 275µl Reductase
- 275µl Reductase Substrate
- 1ml NAD
- 0.5ml NADP*
- 15ml 0.6N HCl
- 15ml Neutralization Buffer

Malate-Glo™ Enzyme Pack, 50ml:

- 250µl Malate Dehydrogenase
- 50µl Malate, 10mM

Storage Conditions: Store the Malate-Glo™ Assay at less than –65°C. Alternatively, store the Reductase Substrate at less than –65°C protected from light and all other components at –30°C to –10°C, 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 Malate-Glo™ Assay.



Note: Use personal protective equipment and follow your institution's safety guidelines and disposal requirements when working with biohazardous materials such as cells and cell culture reagents.

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3. Measuring Malate

Materials to Be Supplied By the User

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

3.A. Sample Preparation

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

The Malate-Glo™ Assay can be used to measure malate in samples such as cells, 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.B or stored at –20°C. If needed, an aliquot of the sample can be removed for protein measurement (see Section 5.A).

We also do not recommend using detergent lysis without acid since many dehydrogenases remain active in detergent lysed samples, significantly increasing the Malate-Glo™ Assay background. Endogenous dehydrogenase activity can be determined by using malate detection reagent prepared without Malate Dehydrogenase. If, in the absence of Malate Dehydrogenase, sample luminescence signal is higher compared to assay background (buffer only) control, sample deproteinization/enzyme inactivation is required.

Acid lysis is compatible with 96-well plate workflow and can be directly added to the cells plated in 96- or 384-well plates. Although cells treated with acid might appear incompletely lysed when viewed under a microscope (Figure 4, Panels A–C), metabolites are successfully released from cells. This is demonstrated in the comparison with conventional cell lysis, using Triton®X-100, where there is no difference in light output between acid only lysis and acid with Triton®X-100 lysis (Figure 4, Panel D).

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

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

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

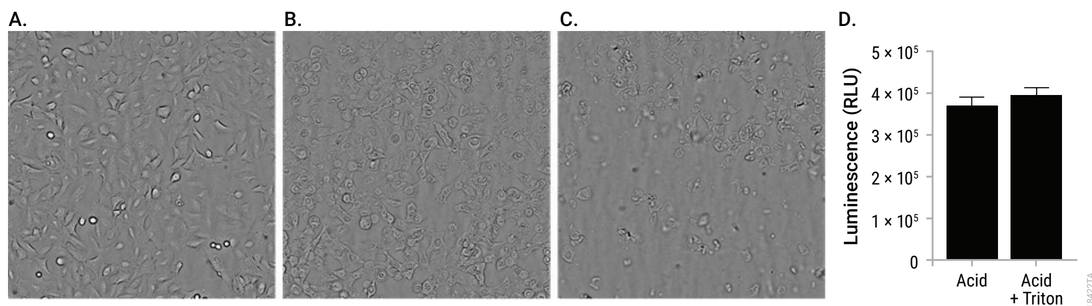

Figure 4. Cell imaging after acid addition. A549 cells were plated at 25,000 cells/well in DMEM (GIBCO™ Cat.# A14430) and 10% dialyzed FBS (GIBCO™ Cat.# A3382001) in a 96-well plate (Greiner Cat.# 655094) overnight. Cells were washed three times with 100µl of PBS. PBS (**Panel A**), PBS premixed with 0.6N HCl at 1:0.2 ratio (**Panel B**) or PBS premixed with 0.6N HCl at 1:0.2 ratio with Triton®X-100 (0.2% final; **Panel C**), was added to each well in triplicate for each condition. The plate was shaken and wells were imaged at 10X with a Tecan Spark® Cyto instrument. Cells containing acid treatment were neutralized with Neutralization Buffer. Malate detection reagent was then added to each well in equal volume to final volume (1:1). After 60 minutes at room temperature, luminescence was read with a GloMax® Discover System (**Panel D**). The data represent average RLU from triplicate wells.

Table 2. Recommended Sample Preparation.

Sample	Malate Concentration in Sample	Recommendations
Cell lysates (intracellular)	0.2–2µM for 20,000 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	0.5–5µM if 10mg of wet tissue is 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	5–50µM depending on serum origin	• Serum samples diluted 20- to 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).

Note: No significant difference in malate concentration was measured in samples treated with 0.6N HCl between 1/5 to 1/2 of the sample volume. Therefore, different ratios can be used to accommodate your experimental setup.

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3.B. Reagent Preparation

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

1. Thaw all components on ice or at room temperature. 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 malate detection reagent required. You will need 50µl for each assay in a 96-well plate with 50µl of sample. We recommend preparing additional reagent to compensate for pipetting error.
3. Prepare malate detection reagent by combining components as shown below.

   Note: Prepare only the volume of malate detection reagent calculated in Step 2. Unused malate 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	0.25µl	25µl
Malate Dehydrogenase	0.25µl	25µl

4. Mix by gently inverting five times.

   Note: Store the remaining Luciferin Detection Solution, Reductase, NAD and Malate Dehydrogenase at less than –65°C or at –30°C to –10°C. Store the Reductase Substrate at less than –65°C protected from light. We recommend a maximum of three freeze-thaw cycles. Store all components in aliquots to avoid freeze-thawing.

3.C. Protocol

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

Information on preparing and using appropriate positive and negative controls for the Malate-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 malate 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 (malate standards 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 malate detection reagent to each well.
5. Mix by shaking the plate for 30–60 seconds.
6. Incubate for 60 minutes at room temperature.

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

3.D. Assay Controls and Data Analysis

There is a linear relationship between luminescence signal and malate 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.

To calculate malate concentration and determine if your samples are within a linear range of the assay, a standard curve using a titration of 10mM malate, included in the kit, can be used (see Table 1). If the sample RLU values fall outside the linear range of the malate standard curves, the sample dilutions should be adjusted and reassayed.

Alternatively, instead of running a full standard curve, 2–4 concentrations of malate standard can be used and sample malate concentrations calculated based on RLU from the standard. We recommend trying a high concentration (25µM) and low concentration (1–2µM) of malate. These amounts can be adjusted based on concentrations expected in the sample.

Malate concentration in the sample can be calculated using the following formula if using one malate standard concentration:

$$[\text{Malate}] = \frac{[\text{Malate standard}]\ (\mu\text{M}) \times (\text{RLU}_{\text{Sample}} - \text{RLU}_{\text{Background}})}{(\text{RLU}_{\text{Malate standard}} - \text{RLU}_{\text{Background}})}$$

To determine if samples contain NAD(P)H or other interfering substances that increase the background signal, samples can be assayed with malate detection reagent lacking Malate Dehydrogenase.

4. Example Protocols and Data for Various Sample Types

4.A. Mammalian Cells

Metabolism is 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. FBS will also contain variable levels 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 Malate-Glo™ Assay can be used for monitoring changes in intracellular malate 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 malate measurement.

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

1. Plate 5,000–50,000 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 once with 200µl of cold PBS.
   Note: Work quickly to minimize changes in malate 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.
5. Add 12.5µl of Neutralization Buffer. Mix by shaking the plate for 30–60 seconds.
6. Add 50µl of malate 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.

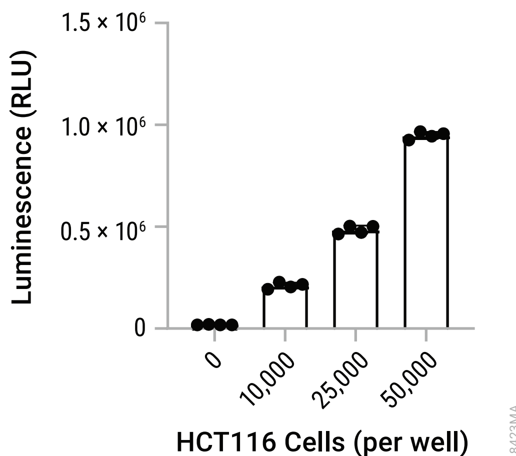

Figure 5. Intracellular malate. HCT116 cells in DMEM supplemented with 10% serum were plated at 10,000, 25,000 and 50,000 cells/well. After overnight incubation, the medium was removed, cells were washed with 200µl of cold PBS and malate was measured as described in the preceding protocol. The average luminescence for four replicates (replicates indicated by dots) is shown in relative light units (RLU). The data show that the intracellular malate was measured with >10-fold signal above background with 10,000 HCT116 cells plated per well, and signal proportionally increased with increasing cell numbers.

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

1. Collect cells, wash with cold PBS, and resuspend at a concentration of 0.5×10^5–1×10^6 cells/ml.
2. Add 1/5 volume of 0.6N HCl (e.g., add 200µl of 0.6N HCl per 1ml 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.

   Note: Aliquots can be removed for protein measurements or samples can be stored below –20°C.
4. Premix PBS, 0.6N HCl and Neutralization Buffer at 5:1:1 ratio. Use it as negative assay background control and to prepare 1µM malate standard as positive control.
5. Transfer 50µl of cell lysates, negative and positive controls to the assay plate.

   Note: If cells need to be diluted, use buffer prepared in Step 4.
6. Add 50µl of malate 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)

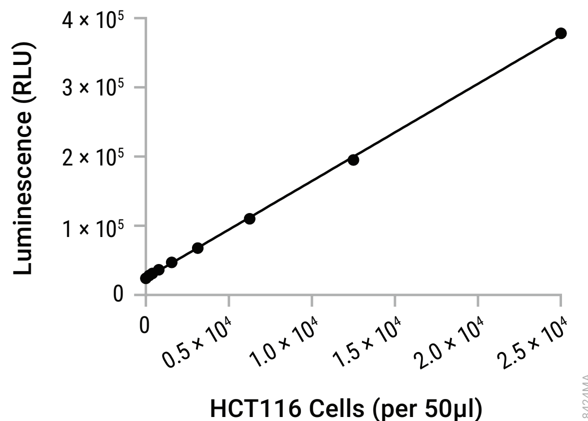

Figure 6. Measuring malate in HCT116 cell lysates. HCT116 cells (0.5×10^6 cells/ml) were prepared as described in the preceding protocol. Aliquots were removed for protein measurement. To determine the linear range of malate in HCT116 cell lysates, twofold serial dilutions were prepared in PBS premixed with 0.6N HCl and Neutralization Buffer at 5:1:1 ratio. Fifty microliters (50µl) of each sample was transferred to a 96-well assay plate. Buffer only and 1µM malate standard was included in the plate as negative and positive controls, respectively, and used for calculating malate concentration. Malate detection reagent (50µl) was added to the samples and after a 60-minute incubation at room temperature, luminescence was recorded using a GloMax® Luminometer. Data represent the average of three replicates. The data show a linear relationship between light signal and cell density, indicating that malate measurements are within the linear range of the assay. The assay sensitivity and wide linearity allows for measurement of malate using 390–25,000 cells/50µl. The calculated malate concentration in the HCT116 cells corresponded to approximately 2fmol/cell or 7.6fmol/ng protein.

4.B. Tissues

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

Example protocol for measuring malate in tissues.

1. Weigh 5–15mg of tissue and add 1ml of PBS.
2. 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).
3. Add 200µl of Neutralization Buffer to homogenate.
   Note: To determine protein concentration in tissue lysate, remove an aliquot of the sample; see Section 5.A.
4. Transfer 50µl of each prepared sample into a 96-well assay plate. Include negative control (buffer only) and positive controls (malate 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 malate 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.

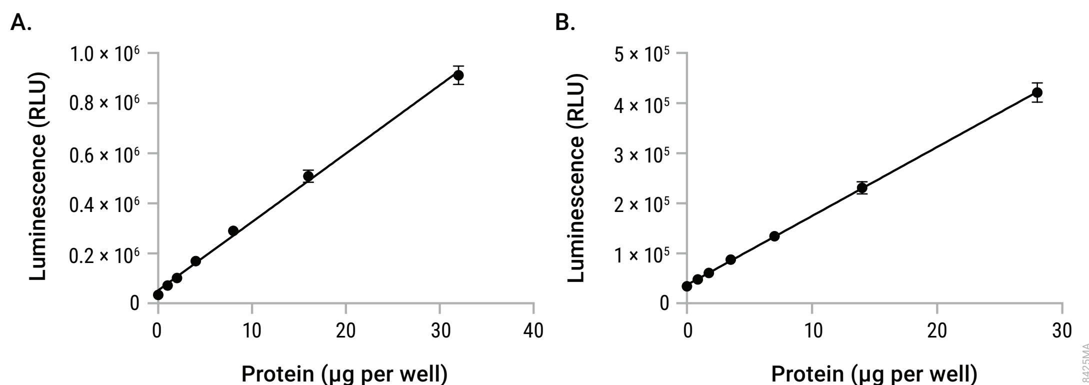

Figure 7. Measuring malate in tissues. A sample (10.7mg/ml) of frozen mouse liver (**Panel A**) and brain (**Panel B**) tissue were homogenized as described in the protocol. An aliquot of each sample was removed for protein measurement. The protein concentration was 1.14mg/ml for brain and 1.28mg/ml for liver. For malate detection, samples were serially-diluted twofold in homogenization buffer and 50µl was transferred to the assay plate. Wells containing 50µl of homogenization buffer with and without 1µM malate were included as the positive and negative controls, respectively. Fifty microliters (50µl) of malate detection reagent was added, and after a 60-minute incubation at room temperature, luminescence was recorded.

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4.C. Serum

The Malate-Glo™ Assay can be used to measure malate in serum or plasma. Undiluted serum interferes with the Malate-Glo™ Assay. For that reason, serum must be diluted prior to measuring malate. The optimal dilution will vary depending on the serum source and malate concentration. We recomend evaluating a series of serum dilutions to determine the dilution factor that results in sensitive detection of malate with minimal interference. The sample interference can be tested by measuring a 'spike' recovery. A 'spike' is a known concentration of malate standard added to the sample. It is important when doing spike experiments to know the concentration you are adding to each well, and to run the sample with and without spike, treating the wells the same. Interference can be measured based on the recovery of the spike added relative to a malate only control as shown in Figure 8.

Example protocol for determining interference and measuring malate in serum.

The protocol below is provided for acid/base treated samples. For samples without acid/base treatment, Steps 5 and 6 are omitted and 20µl of PBS is added (see Figure 8).

1. Make serum serial dilutions in PBS. Include negative control (buffer only).
2. Prepare 4µM malate spike by diluting the Malate, 10mM standard in PBS.
3. Combine diluted serum samples and negative control with either PBS (no spike control) or 4µM malate spike at a 1:1 ratio (final [malate spike]:2µM).
4. Transfer 30µl of each prepared samples 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 malate detection reagent prepared as described Section 3.B.
8. Mix by shaking the plate for 30–60 seconds.
9. Incubate at room temperature for 60 minutes.
10. Record luminescence.
11. Calculate percent recovery using this formula:

$$\text{Percent Recovery} = \frac{(\text{RLU}_{\text{serum + malate spike}} - \text{RLU}_{\text{serum}})}{(\text{RLU}_{\text{malate standard}})} \times 100$$

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

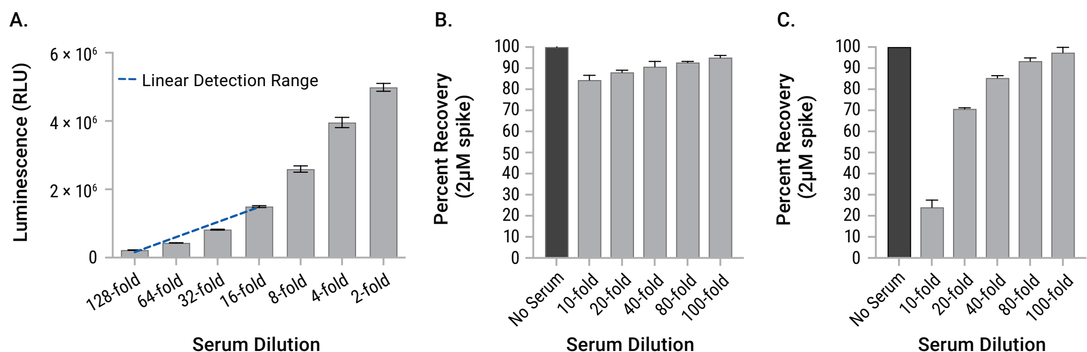

Figure 8. Optimization of malate detection in serum. Serial dilutions of fetal bovine serum were prepared in PBS (**Panel A**) or PBS mixed 1:1 with 4µM malate standard for spike samples **(Panels B and C)**. Dilutions were transferred to a white 96-well plate (30µl per well). Samples were acidified with 0.6N HCl (10µl per well; **Panels A** and **B**) for 5 minutes, then Neutralization Buffer (10µl per well) was added. In **Panel C**, 20µl of PBS was added to the samples. Malate detection reagent was added (50µl per well) and incubated for 60 minutes. The blue dotted line (**Panel A**) shows the linear range of the assay. Percent recovery, shown in **Panel B** and **Panel C**, was calculated as described in the protocol. Fetal bovine serum without acid treatment had to be diluted at least 40-fold for >80% recovery of a known amount of malate spiked into the sample (**Panel C**). Treating serum with acid improved ability to measure malate in less diluted samples (**Panel B**). In acid treated samples (**Panels A** and **B**) >80% recovery was achieved with 10-fold diluted samples and malate detection was linear with samples diluted from 16-fold to 128-fold. In 128-fold diluted serum, malate was measured with 10-fold signal above background, indicating that malate concentration in serum can be measured with high sensitivity and wide assay window. Luminescence was measured using a GloMax® Discover System. Data is the average of three replicates.

5. Appendix

5.A. Multiplexing and Normalization for Mammalian Cells

The Malate-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 provide useful tool for normalizing malate measurements to the number of viable cells and separating immediate effects on malate metabolism from global effects on cell health. To multiplex the cell viability assays and malate detection using the same population of cells, perform the cell viability assay first as described in the appropriate product technical manuals. 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 treatment and neutralization, an aliquot of each sample can be removed for protein measurement and ATP detection using CellTiter-Glo® Cell Viability Assay (Cat.# G9241). To the rest of the sample, add an equal volume of Malate detection reagent and follow protocol described in Section 3.B.

To measure protein concentration, we recommend the Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific Cat.# 23235), 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.# 323235), which has higher sensitivity (>0.05 × 10^6 cells/ml). However, sample aliquots for protein determination must be removed before sample neutralization because Neutralization Buffer interferes with the Micro BCA Protein Assay.

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

Representative data of multiplexing viability assays, ATP detection and malate measurements in HCT116 cancer cells are shown in Figure 9. Linear increase in signal with increasing number of cells was measured with all assays. The data can be normalized by directly comparing fluorescence and luminescence signals or malate concentration can be calculated and normalized to protein amount.

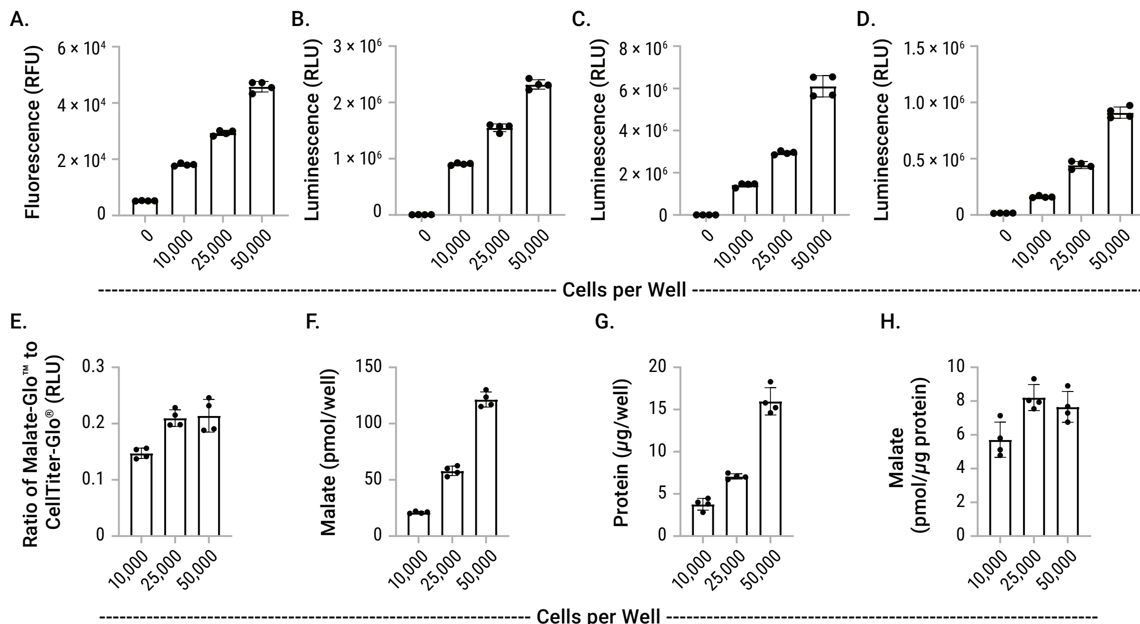

Figure 9. Multiplexing viability, ATP and protein measurements with malate detection. HCT116 cells in 100µl of DMEM supplemented with 10% serum were plated at 10,000, 25,000 and 50,000 cells/well. After an overnight incubation, viability was assessed using (**Panel A**) CellTiter-Fluor™ Assay (Cat.# G6080) and (**Panel B**) RealTime-Glo™ MT Cell Viability Assay (Cat.# G9711). Cells were then prepared per the protocol for plated cells in Section 4.A. After 0.6N HCl and Neutralization Buffer treatment, an aliquot was removed to analyze ATP content (**Panel C**) using the CellTiter-Glo® Assay (Cat.# G7570). Malate (**Panel D**) was then measured using the described protocol and normalized to ATP content (**Panel E**). The amount of malate per well (**Panel F**) was calculated based on the Malate, 10mM standard. Prior to malate detection, an aliquot was removed for protein measurement (**Panel G**) using the Pierce™ Rapid Gold BCA Protein Assay Kit (Cat.# A53225), and the concentration of malate was normalized to total protein (**Panel H**). The calculated amount of malate per microgram (µg) of protein was similar at 25,000 and 50,000 HCT cells/well. Cells plated at 10,000 cells/well contained approximately 30% less malate per µg of protein, indicating the difference in metabolism in the cells plated at different densities.

5.B. 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 using.

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

5.C. Plates and Equipment

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

Use opaque, white-walled multiwell plates that are compatible with your luminometer (e.g., Corning® Costar® 96-well plates, Cat.# 3917, Costar® 384-well plates, Cat.# 3570). For cultured cells, white-walled clear bottom tissue culture plates (e.g., Corning® 96-well plates, Cat.# 3903) are acceptable. Luminescent metabolite assays are well suited for miniaturization. When samples are limited, consider using 96-half area (Corning® Cat.# 3696), 384-well (Costar® Cat.# 3570) or 384-low volume (Corning® Cat.# 4512) plates. We do not recommend black or clear plates. Light signal is diminished in black plates and increased well-to-well cross talk is observed 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.D. References

1. Zhou, W. *et al.* (2014) Self-immolative bioluminogenic quinone luciferins for NAD(P)H assays and reducing capacity-based cell viability assays. *Chembiochem.* **15**, 670–5.
2. Vidugiriene, J. *et al.* (2014) Bioluminescent cell-based NAD(P)/NAD(P)H assays for rapid dinucleotide measurement and inhibitor screening. *Assay Drug Dev.Technol.* **12**, 514–26.
3. Leippe, D. *et al.* (2017) Bioluminescent assays for glucose and glutamine metabolism: High-throughput screening for changes in extracellular and intracellular metabolites. *SLAS Discov.* **22**, 366–77.

5.E. Related Products

Energy Metabolism Assays

Product	Size	Cat.#
BCAA-Glo™ Assay	5ml	JE9300
BHB-Glo™ (Ketone Body) Assay	5ml	JE9500
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190
Dehydrogenase-Glo™ Detection System	5ml	J9010
Glucose-Glo™ Assay	5ml	J6021
Glucose Uptake-Glo™ Assay	5ml	J1341
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycerol-Glo™ Assay	5ml	J3150
Glycogen-Glo™ Assay	5ml	J5051
Lactate-Glo™ Assay	5ml	J5021
Metabolite-Glo™ Detection System	5ml	J9030
Pyruvate-Glo™ Assay	5ml	J4051
Triglyceride-Glo™ Assay	5ml	J3160

Additional sizes available.

Oxidative Stress Assays

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

Additional sizes available.

Cell Viability, Cytotoxicity and Apoptosis Assays

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

Additional sizes available.

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

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