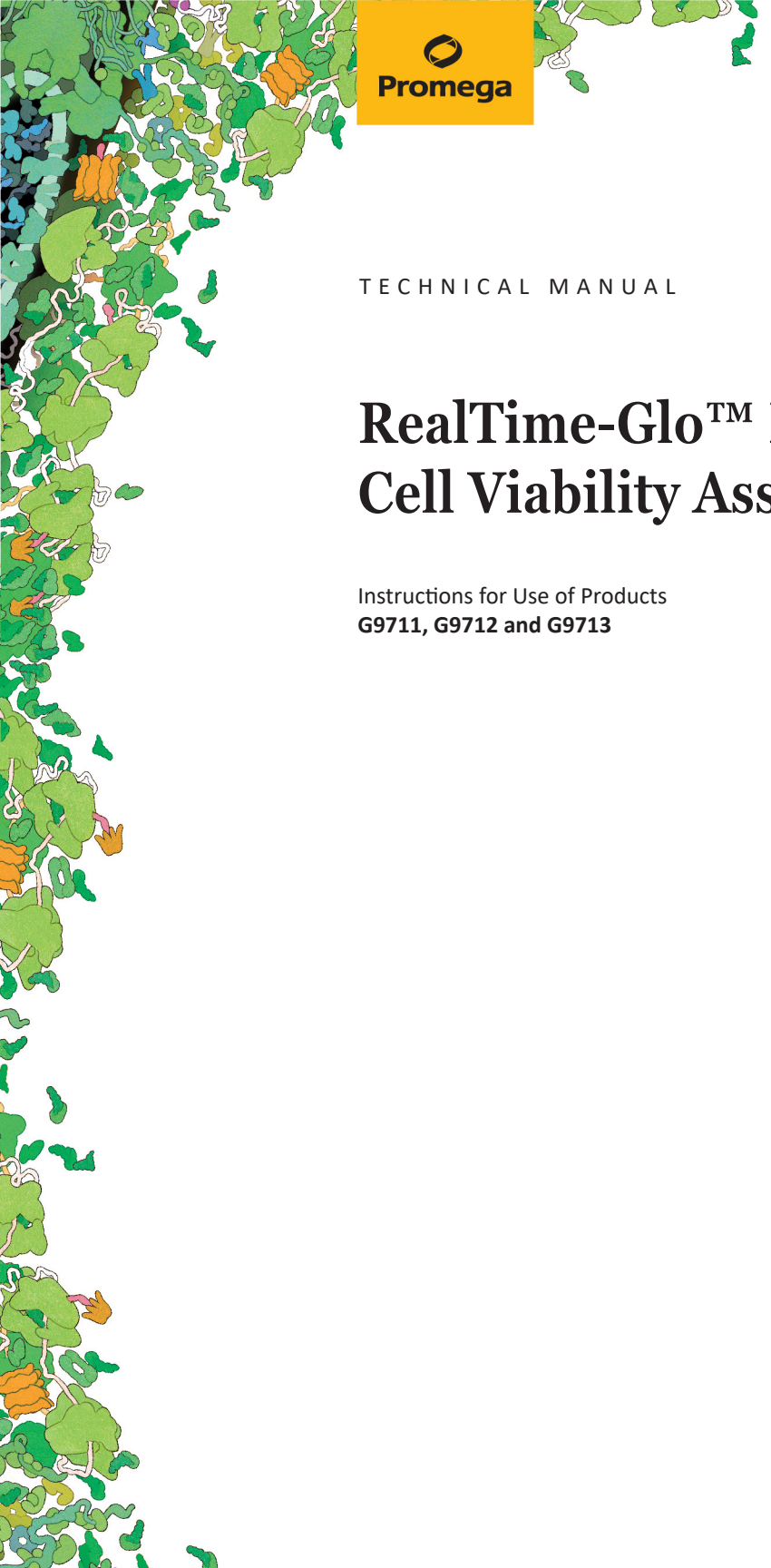


TECHNICAL MANUAL

RealTime-Glo™ MT Cell Viability Assay

Instructions for Use of Products
G9711, G9712 and G9713

Revised 12/21
TM431

RealTime-Glo™ MT Cell Viability Assay

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

1. Description

The RealTime-Glo™ MT Cell Viability Assay[a] is a nonlytic, homogeneous, bioluminescent method to measure cell viability in real time using a simple, plate-based method. The nonlytic nature of this assay allows the cells to be used for further downstream applications, including multiplexing with a variety of assay chemistries. The RealTime-Glo™ MT Cell Viability Assay determines the number of viable cells in culture by measuring the reducing potential of cells and thus metabolism (MT). The assay involves adding NanoLuc® luciferase and a cell-permeant prosubstrate, the MT Cell Viability Substrate, to cells in culture. Viable cells reduce the proprietary prosubstrate to generate a substrate for NanoLuc® luciferase. This substrate diffuses from cells into the surrounding culture medium, where it is rapidly used

1. Description (continued)

by the NanoLuc® Enzyme to produce a luminescent signal. The signal correlates with the number of viable cells, making the assay well suited for cytotoxicity studies. Both the MT Cell Viability Substrate and NanoLuc® Enzyme are stable in complete cell culture medium at 37°C for at least 72 hours. No cell washing, removal of medium or further reagent addition is required to determine the number of viable cells. Figure 1 shows an overview of the assay.

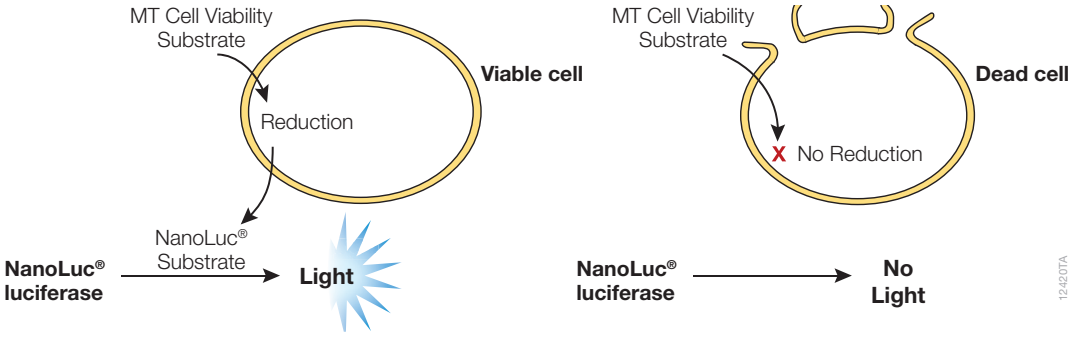


Figure 1. RealTime-Glo™ MT Cell Viability Assay overview. The assay involves adding NanoLuc® luciferase and a cell-permeant prosubstrate, the MT Cell Viability Substrate, to cells in culture. The MT Cell Viability Substrate is reduced to a NanoLuc® substrate by metabolically active cells. The NanoLuc® substrate diffuses from cells into the surrounding culture medium and is rapidly used by NanoLuc® Enzyme to produce a luminescent signal. The signal correlates with the number of viable cells. Dead cells do not reduce the substrate and produce no signal.

The assay can be performed in two formats: continuous-read measurement or endpoint measurement (Figure 2). When the assay is set up in the continuous-read format, the RealTime-Glo™ reagents (i.e., MT Cell Viability Substrate and NanoLuc® Enzyme) can be added to the wells at the same time as the cells or test compound or at any point during the assay to start obtaining viability measurements. In the continuous-read format, the luminescent signal can be continually monitored from the same wells over an extended period of time to analyze cell viability in real time. In the endpoint format, cells are cultured and treated as appropriate for the experimental protocol, and at the end of treatment, the RealTime-Glo™ reagents are added. The endpoint format of the RealTime-Glo™ MT Cell Viability Assay is much more sensitive and rapid compared to currently available viability assays that measure the reducing potential of cells (e.g., MTT, MTS and resazurin-based assays).

Advantages of the RealTime-Glo™ MT Cell Viability Assay include:

Real-time cell viability measurements: Monitor cell viability in real time to determine onset of toxicity, analyze potency versus efficacy over time and analyze differential cell growth with a simple, plate-based protocol.

Superior sensitivity: The assay provides a greater signal-to-background ratio and higher sensitivity in less time compared to colorimetric or fluorometric viability assays that are based on the reducing potential of cells.

Assay setup flexibility: Perform real-time measurements by adding reagents when cells are plated, test compound is added to the cells or at any time point when cell viability measurements are needed. Alternatively, set up the assay for an endpoint cell viability determination.

Nonlytic assay format: The RealTime-Glo™ MT Cell Viability Assay does not require cell lysis, allowing you to use the cells to multiplex the RealTime-Glo™ Assay with other assays, including other luminescent assays without the need for spectral filters, or to use the cells later in a variety of downstream applications (see Section 7.D). This allows you to use less sample and provides more informative data points per sample.

Well established marker of cell viability: The assay chemistry is based on the reducing potential of the cell, which is a trusted metabolic marker of cell viability.

Compatibility with automation: The assay is compatible with automated and high-throughput protocols. Reactions are scalable and can be performed at low volumes in 96-, 384- and 1,536-well plates.

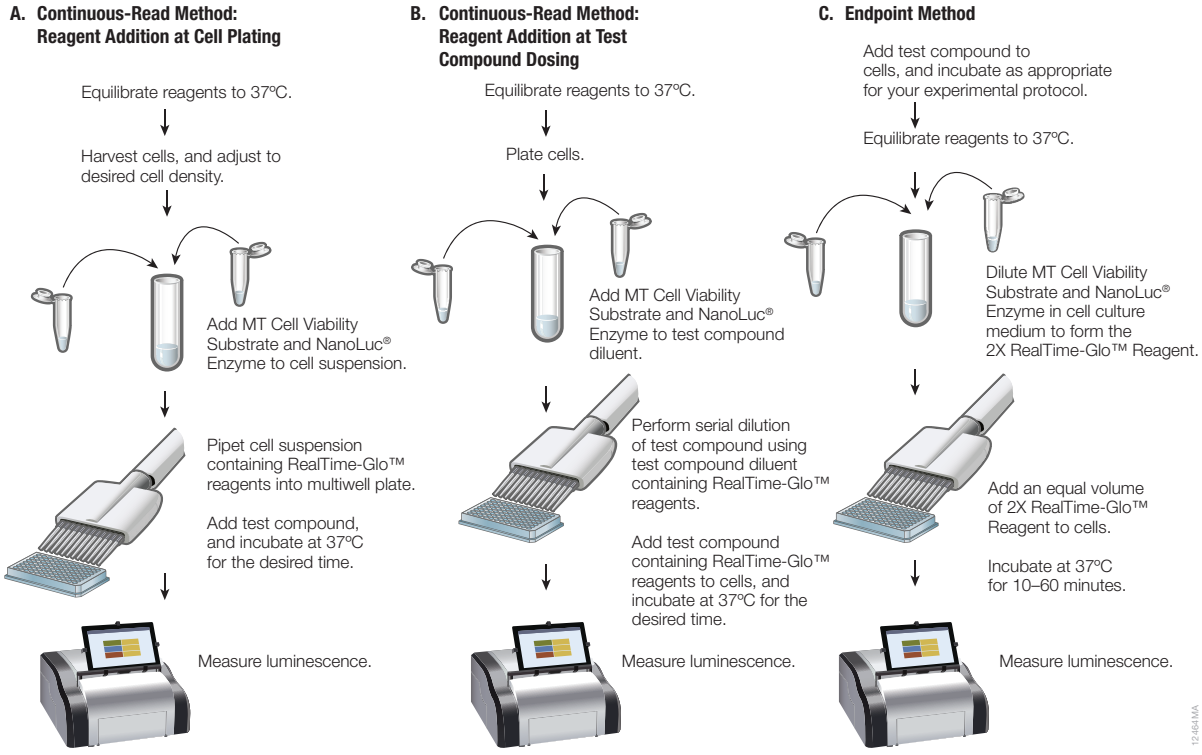


Figure 2. Overview of the different methods for delivering the RealTime-Glo™ Reagents to assay wells. The diagram shows the protocols for performing the assay in a 96-well plate. **Panel A.** Continuous-read protocol with reagent addition at cell plating. **Panel B.** Continuous-read protocol with reagent addition during test compound preparation. **Panel C.** Endpoint protocol with reagent addition at the end of test compound treatment.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
RealTime-Glo™ MT Cell Viability Assay	**100 reactions**	**G9711**

For the continuous-read protocol, where reagents are added directly to the cell culture or test compound dilution, this kit contains sufficient reagents for 100 reactions containing 50µl of 2X RealTime-Glo™ reagent per well of a 96-well plate or 400 reactions containing 12.5µl of 2X RealTime-Glo™ reagent per well of a 384-well plate. Includes:

- 10µl MT Cell Viability Substrate, 1,000X
- 10µl NanoLuc® Enzyme, 1,000X

PRODUCT	SIZE	CAT.#
RealTime-Glo™ MT Cell Viability Assay	**10 × 100 reactions**	**G9712**

For the continuous-read protocol, where reagents are added directly to the cell culture or test compound dilution, this kit contains sufficient reagents for 1,000 reactions containing 50µl of 2X RealTime-Glo™ reagent per well of a 96-well plate or 4,000 reactions containing 12.5µl of 2X RealTime-Glo™ reagent per well of a 384-well plate. Includes:

- 10 × 10µl MT Cell Viability Substrate, 1,000X
- 10 × 10µl NanoLuc® Enzyme, 1,000X

PRODUCT	SIZE	CAT.#
RealTime-Glo™ MT Cell Viability Assay	**1,000 reactions**	**G9713**

For the continuous-read protocol, where reagents are added directly to the cell culture or test compound dilution, this kit contains sufficient reagents for 1,000 reactions containing 50µl of 2X RealTime-Glo™ reagent per well of a 96-well plate or 4,000 reactions containing 12.5µl of 2X RealTime-Glo™ reagent per well of a 384-well plate. Includes:

- 100µl MT Cell Viability Substrate, 1,000X
- 100µl NanoLuc® Enzyme, 1,000X

Storage Conditions: Store the RealTime-Glo™ MT Cell Viability Assay reagents at –20°C , protected from light. Avoid prolonged exposure to light of the MT Cell Viability Substrate, 1,000X. Avoid multiple freeze-thaw cycles. See product label for expiration date.

3. Reagent Preparation

We recommend preparing only the volume of MT Cell Viability Substrate and NanoLuc® Enzyme required for a single experiment. Be sure that the cell suspension, test compound diluent or cell culture medium used to prepare the RealTime-Glo™ reagent is equilibrated to 37°C prior to addition of the MT Cell Viability Substrate and NanoLuc® Enzyme. Add the diluted reagents to the assay plate within 24 hours of reagent preparation.

The MT Cell Viability Substrate and NanoLuc® Enzyme are provided at 1,000 times (1,000X) the final concentration in the assay. For all assay protocols, the final concentration of each reagent in the assay well must be 1X.

3.A. Continuous-Read Assays

Before use, equilibrate the MT Cell Viability Substrate and NanoLuc® Enzyme to 37°C in a water bath. The MT Cell Viability Substrate and NanoLuc® Enzyme can be delivered to test wells by multiple methods, depending on the desired assay format. For the continuous-read protocols in this manual, the MT Cell Viability Substrate and NanoLuc® Enzyme are added to the cell suspension or 37°C test compound diluent at a concentration of 2X. Alternatively, you can dilute the reagents in 37°C cell culture medium such that the final concentration of each reagent is 1X and use this medium directly in assay wells with or without cells. You also can prepare the RealTime-Glo™ reagent as a more concentrated solution (up to 5X each reagent) in 37°C test compound diluent or cell culture medium and add the reagent to assay wells to a final concentration of 1X.

3.B. Endpoint Assays

Before use, equilibrate the MT Cell Viability Substrate and NanoLuc® Enzyme to 37°C in a water bath. In the endpoint protocol, the MT Cell Viability Substrate and NanoLuc® Enzyme are added to the cells as the 2X RealTime-Glo™ reagent. To prepare the 2X RealTime-Glo™ reagent, equilibrate cell culture medium to 37°C, then add the MT Cell Viability Substrate and NanoLuc® Enzyme to the cell culture medium to a final concentration of 2X for each reagent. For example, for 50µl of cells in culture, add 50µl of 2X RealTime-Glo™ reagent. You also can prepare the RealTime-Glo™ reagent as a more concentrated solution (up to 5X) in 37°C cell culture medium and add the reagent to assay wells to a final concentration of 1X.

4. Determining Assay Linearity for the Endpoint or Continuous-Read Format

Materials To Be Supplied by the User

- white, opaque-walled tissue culture plates (with a clear or solid bottom) compatible with a luminometer
- 37°C water bath
- cells and cell culture medium
- plate-reading luminometer

Each cell line has a different basal reducing potential and thus varying capacities to reduce the MT Cell Viability Substrate, so it is important to characterize assay linearity with regard to cell density for each cell line of interest. For assays in the continuous-read format, the growth rate of the cells also affects assay linearity. We strongly recommend plating cells at a range of cell densities to determine the linear range. This linear range will be different between an endpoint analysis and a continuous-read experiment because cells will proliferate during the continuous-read time course and will constantly consume prosubstrate over time.

In the example shown in Figure 3, K562 cells were plated at increasing cell densities in the presence of 1X RealTime-Glo™ reagent, and cell viability was monitored by measuring luminescence every hour for 72 hours. The luminescent signals at 6,000 cells/well and 3,000 cells/well decreased before the 72-hour time point (Panel A), indicating that the assay was not linear under these conditions. At the 72-hour time point, only the luminescent signals at 1,500 cells/well and 750 cells/well were still linear. To analyze cell viability in K562 cells for 72 hours under these particular cell culture conditions, cells would need to be plated at 1,500 cells/well or less. The signal at 6,000 cells/well was linear to about 24 hours (Panel B).

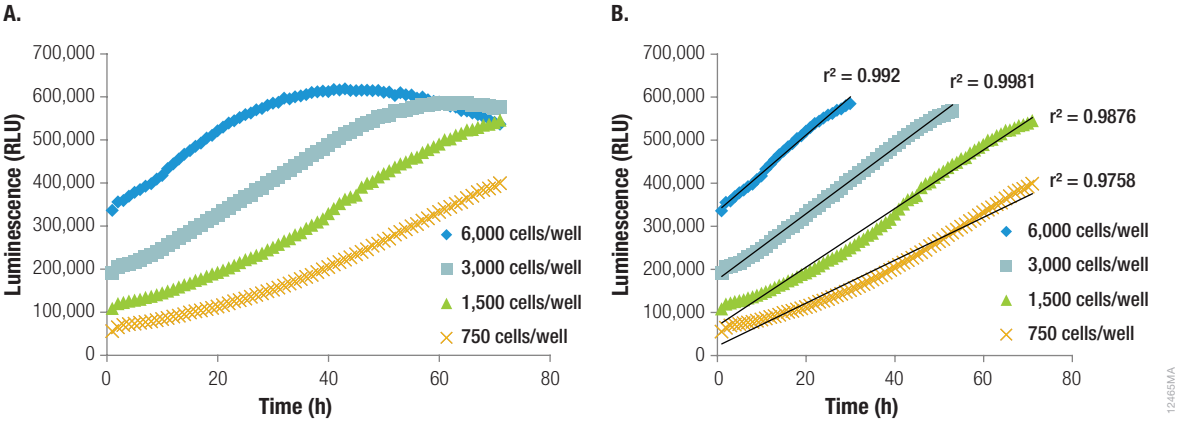


Figure 3. Incubation time and cell density affect assay linearity. K562 cells were plated at 6×10^3, 3×10^3, 1.5×10^3 or 750 cells/well in 80µl in the presence of the 1X RealTime-Glo™ reagent in a 384-well plate. Luminescence was monitored every hour for 72 hours using a Tecan Infinite® 200 Multimode Reader with Gas Control Module (37°C and 5% CO_2). **Panel A.** The luminescent signals measured at each cell density over the 72-hour time course are shown. **Panel B.** Only luminescent signals within the linear range are shown. These data represent the linear range of the RealTime-Glo™ MT Cell Viability Assay at the indicated cell densities.

1. Equilibrate the MT Cell Viability Substrate, NanoLuc® Enzyme and cell culture medium to 37°C.
2. Plate the cell line of interest at various cell densities in a white assay plate.

   Note: For a 384-well plate, we recommend testing initial cell densities of 200 to 10,000 cells/well in 40µl of cell culture medium. The optimal initial cell density should be determined for each cell type and may fall outside of the 200–10,000 cells/well range. We recommend analyzing at least three replicate samples at each cell density.
3. Add the MT Cell Viability Substrate and NanoLuc® Enzyme to 37°C cell culture medium to a 2X concentration to form the 2X RealTime-Glo™ reagent. For example, to prepare 1ml of 2X RealTime-Glo™ reagent, add 2µl of MT Cell Viability Substrate, 1,000X, and 2µl of NanoLuc® Enzyme, 1,000X, to 996µl of cell culture medium. Mix well with a vortex mixer.
4. Add an equal volume of the 2X RealTime-Glo™ reagent to the cells.

   For example, if cells are plated in 40µl, then add 40µl of 2X RealTime-Glo™ reagent to the cells.
5. Incubate cells in a cell culture incubator at 37°C and 5% CO_2 for 1 hour.
6. Measure luminescence using a plate-reading luminometer, and return cells to the cell culture incubator.

   Notes:

   1. Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.
   2. These measurements will be used in Step 8 to determine the linear range of cell densities for endpoint assays.
   3. Be sure to control the temperature of the plate during the measurement because the NanoLuc® reaction is affected by temperature (see Section 7.B for more information).
7. To determine the linear range of cell densities for the continuous-read format, continue measuring luminescence at various times over the desired time course (e.g., 8 hours, 24 hours, 72 hours).

   Notes:

   1. These measurements will be used to determine the linear range of cell density at each time point. The optimal cell density range will change over time for the continuous-read format, so determining assay linearity over the desired time course is important.
   2. Be sure to control the temperature of the plate at each measurement because the NanoLuc® reaction is affected by temperature (see Section 7.B for more information).
8. Calculate the average luminescence for each replicate group. Plot luminescence versus cell number, and apply linear curve fit. We recommend an r^2 value >0.95 as acceptable. If the linear fit yields an r^2 value <0.95, remove the average luminescence for assays with the highest cell density from the data set and recalculate the r^2 value. If the linear fit yields an r^2 value <0.95, continue to remove the average luminescence for assays with the highest cell density until an r^2 value >0.95 is achieved. The cell density range that yields an r^2 value >0.95 is the linear range of the assay for the cell type of interest over the desired time course. An example is shown in Figure 3.

5. Protocols for the RealTime-Glo™ MT Cell Viability Assay

Materials To Be Supplied by the User

- white, opaque-walled tissue culture plates (with a clear or solid bottom) compatible with a luminometer
- 37°C water bath
- cells and cell culture medium
- plate-reading luminometer

This assay has been tested with multiple assay plate formats including low-volume 384-well plates, 384-well plates and 96-well plates.

The RealTime-Glo™ MT Cell Viability Assay is nonlytic and can be multiplexed with a variety of downstream applications (see Section 7.D for more information).

Precipitate may form during preparation of the RealTime-Glo™ reagent. See Section 7.E for a simple precipitate removal protocol to use prior to addition to cells.

5.A. Protocol for Continuous-Read Format: Reagent Addition at Cell Plating

To continuously monitor cell viability in real time, the MT Cell Viability Substrate and NanoLuc® Enzyme can be added at the same time as the cells. In this protocol, the MT Cell Viability Substrate and NanoLuc® Enzyme are included in the cell culture suspension at 2X concentrations and an equal volume of 2X test compound is added to the cells at Step 5. The final concentration of each reagent is 1X after test compound addition.

If the concentration of test compound added at Step 5 is not 2X (i.e., the volume used to deliver the test compound is not the same as the volume of cells in the well), adjust the concentration of each RealTime-Glo™ reagent in the cell suspension in Step 3 to ensure that the final concentration after test compound addition is 1X. Alternatively, you can use 1X RealTime-Glo™ reagent as the vehicle used to deliver the test compound.

1. Equilibrate the MT Cell Viability Substrate and NanoLuc® Enzyme to 37°C.
2. Harvest cells, and adjust cells to twice the final desired cell density.

   Note: Be sure that the final cell density in each well is within the linear range determined in Section 4.
3. Add the MT Cell Viability Substrate and NanoLuc® Enzyme to the cell suspension to a final concentration of 2X for each reagent. For example, to prepare 1ml of cell suspension, add 2µl of MT Cell Viability Substrate, 1,000X, and 2µl of NanoLuc® Enzyme, 1,000X, to 996µl of cell suspension. Mix gently.

   Note: If the test compound added at Step 5 is not 2X (i.e., the volume used to deliver the test compound is not the same as the volume of cells in the well), adjust the concentration of each reagent in the cell suspension to ensure that the final concentration of each reagent after test compound addition is 1X.
4. Dispense the desired volume of cells into each well of an opaque-walled assay plate. (We recommend white plates; see Section 7.B.)
5. Add an equal volume of 2X test compound of interest, and incubate at 37°C as appropriate for your experimental protocol.

   Note: If the incubation time is not sufficient to allow plate contents to reach 37°C, equilibrate the 2X test compound to 37°C before adding. This ensures that the temperature of the plate at each measurement is constant.

6. Monitor luminescence at the desired time points using a plate-reading luminometer. Return the plate to the cell culture incubator between measurements to maintain a consistent temperature at each measurement. The NanoLuc® reaction is affected by temperature. See Section 7.B for more information.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

5.B. Protocol for Continuous-Read Format: Reagent Addition at Test Compound Dosing

To continuously monitor cell viability in real time, the MT Cell Viability Substrate and NanoLuc® Enzyme can be added at the same time as the test compound. In this protocol, the MT Cell Viability Substrate and NanoLuc® Enzyme are included in the test compound diluent at 2X concentrations and an equal volume of 2X test compound containing RealTime-Glo™ reagent is added to the cells at Step 6. After test compound containing RealTime-Glo™ reagents is added, the final concentration of each reagent is 1X. If the concentration of test compound added at Step 6 is not 2X, adjust the concentration of each RealTime-Glo™ reagent added to the test compound diluent in Step 4 so that the final concentration of each reagent is 1X in the sample well.

1. Equilibrate the MT Cell Viability Substrate, NanoLuc® Enzyme and test compound diluent to 37°C.
2. Harvest cells, and adjust cells to twice the final desired cell density.

   Note: Be sure that the final cell density in each well is within the linear range determined in Section 4.
3. Dispense the desired volume of cells into each well of an opaque-walled assay plate. (We recommend white plates; see Section 7.B.)
4. Add the MT Cell Viability Substrate and NanoLuc® Enzyme to the test compound diluent to a final concentration of 2X each reagent. For example, to prepare 1ml of test compound diluent, add 2µl of MT Cell Viability Substrate, 1,000X, and 2µl of NanoLuc® Enzyme, 1,000X, to 996µl of test compound diluent. Mix well with a vortex mixer.

   Note: If the test compound added at Step 6 is not 2X, adjust the concentration of each reagent in the diluent to ensure that the final concentration of each reagent after test compound addition is 1X.
5. Use the test compound diluent prepared in Step 4 to prepare the 2X test compound solution(s).
6. Add an equal volume of 2X test compound containing RealTime-Glo™ reagents to the cells.

   Note: If the plate contents will not reach 37°C by the first desired time point, we recommend equilibrating the 2X test compound to 37°C prior to addition. This ensures that the temperature at each measurement is constant.
7. Monitor luminescence at the desired time points using a plate-reading luminometer. Return the plate to the cell culture incubator between measurements to maintain a consistent temperature at each measurement. The NanoLuc® reaction is affected by temperature. See Section 7.B for more information.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

5.C. Protocol for Endpoint Assay Format

Endpoint measurements of cell viability can be performed when real-time measurements are not needed. This endpoint assay method provides greater sensitivity and speed than other cell viability assays that measure reducing potential of the cell. Because the RealTime-Glo™ MT Cell Viability Assay is nonlytic, it can be multiplexed with a variety of downstream applications. See Section 7.D for more information.

1. Harvest cells, and adjust cells to the final desired cell density.

   Note: Be sure that the final cell density in each well is within the linear range determined in Section 4.

2. Dispense the desired number of cells into opaque-walled assay plates. (We recommend white plates; see Section 7.B).

3. Treat cells with the test compound of interest, and incubate as appropriate for your experimental protocol.

4. Equilibrate the MT Cell Viability Substrate, NanoLuc® Enzyme and cell culture medium to 37°C.

5. Prepare the 2X RealTime-Glo™ reagent by diluting the MT Cell Viability Substrate and NanoLuc® Enzyme in 37°C cell culture medium to a 2X concentration for each reagent. For example, to prepare 1ml of 2X RealTime-Glo™ reagent, add 2µl of MT Cell Viability Substrate, 1,000X, and 2µl of NanoLuc® Enzyme, 1,000X, to 996µl of cell culture medium to form the 2X RealTime-Glo™ reagent. Mix well with a vortex mixer.

   Note: When multiplexing applications, a more concentrated solution of the MT Cell Viability Substrate and NanoLuc® Enzyme (up to 5X each reagent) can be prepared in cell culture medium. Be sure that the final concentrations of the MT Cell Viability Substrate and NanoLuc® Enzyme in the assay wells are 1X.

6. Add an equal volume of 2X RealTime-Glo™ reagent to the cells.

7. Incubate the cells for 10–60 minutes in the cell culture incubator.

   Note: For many cell types, an incubation time of 10 minutes is sufficient to yield a strong signal above background. Some cell lines may require a longer incubation time (e.g., 1 hour). We recommend measuring luminescence after 10 minutes and, if the signal is insufficient, measuring luminescence again at later time points until a strong signal is obtained.

8. Measure luminescence using a plate-reading luminometer.

   Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

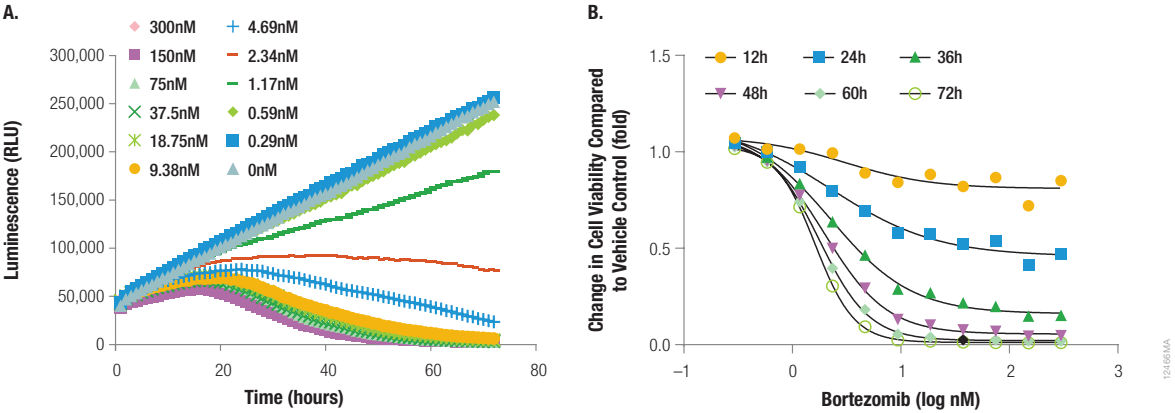
6. Representative Data

6.A. EC_{50} Determination

The luminescent signal from the RealTime-Glo™ MT Cell Viability Assay correlates with the number of metabolically active cells. The sensitivity of this assay to detect viable cells in culture makes it well suited for cytotoxicity studies. When performed as an endpoint assay, the RealTime-Glo™ MT Cell Viability Assay allows robust, rapid and sensitive cell viability measurements in a live-cell format, which allows the cells to be used in additional downstream applications including multiplexed assays.

The RealTime-Glo™ MT Cell Viability Assay allows you to monitor the effect of a test compound on cells over time. Half-maximal effective concentrations (EC_{50}) can be determined at multiple time points from the same sample wells when the assay is performed in continuous-read format (Figure 4).

The EC_{50} value can be determined at any time point. By comparing the viability of treated cells to that of untreated cells and calculating the fold change in viability at each test compound concentration, the cytotoxicity of the test compound can be monitored and compared over time. Analysis of cytotoxicity in this format allows you to determine the rate at which different test compounds affect cells.



Incubation Time

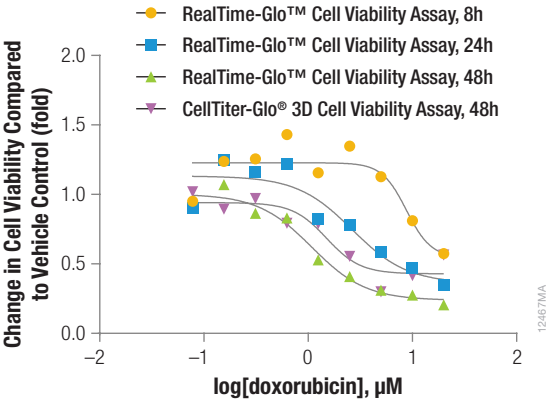
EC_{50} Value (nM)	12h	24h	36h	48h	60h	72h
	3.07	2.29	2.27	1.99	1.78	1.62

Figure 4. EC_{50} determination using the continuous-read assay format. Five hundred A549 cells/well were plated in a 384-well plate in 40µl of cell culture medium containing 2X RealTime-Glo™ reagent. An equal volume of 2X bortezomib was added to achieve the indicated concentrations. Luminescence was monitored for 72 hours. **Panel A.** Cell viability was monitored every hour on a Tecan Infinite® 200 Multimode Reader with Gas Control Module (37°C/5% CO_2). **Panel B.** The fold change in cell viability of bortezomib-treated cells compared to the vehicle control at each concentration is shown. EC_{50} values were determined using GraphPad Prism® software, version 5.03.

6.B. EC_{50} Determination in 3-Dimensional (3D) Cell Culture

The RealTime- Glo™ MT Cell Viability Assay can be used to measure cell viability in 3D cell culture. To generate the data shown in Figure 5, we used the hanging-drop method for 3D cell culture and produced spheroids using the GravityPLUS™ 3D Culture and Assay Platform (InSphero AG). Results obtained using the RealTime-Glo™ MT Cell Viability Assay were compared to results generated using the CellTiter-Glo® 3D Cell Viability Assay, which measures ATP as an indicator of cell viability and has increased lytic capacity, making the assay well suited for use with microtissues produced through 3D cell culture.

The RealTime-Glo™ MT Cell Viability Assay showed decreasing EC_{50} values over time. The EC_{50} value at 48 hours corresponded well with the EC_{50} value determined using the CellTiter-Glo® 3D Cell Viability Assay.



	RealTime-Glo™ Assay at 8 hours	RealTime-Glo™ Assay at 24 hours	RealTime-Glo™ Assay at 48 hours	CellTiter-Glo® Assay at 48 hours
EC_{50} Value (µM)	8.77	2.65	1.09	1.50

Figure 5. Analysis of doxorubicin-treated HCT116 spheroids with RealTime-Glo™ MT Cell Viability and CellTiter-Glo® 3D Cell Viability Assays. HCT116 colon cancer spheroids were generated by plating 4,000 cells into a 96-well GravityPLUS™ hanging-drop plate (InSphero AG); cells were grown for 4 days to form spheroids that were 522 ± 4µm in diameter. All samples were transferred to a standard white assay plate and treated with the indicated concentrations of doxorubicin. The RealTime-Glo™ reagent was added to a subset of wells, and cell viability was monitored at various time points of up to 48 hours. At 48 hours, the CellTiter-Glo® 3D Reagent was added to a parallel set of samples. The graph shows the fold change in cell viability of doxorubicin-treated cells compared to the vehicle control at each concentration. EC_{50} values were determined using GraphPad Prism® software, version 5.03.

7. Additional Considerations

7.A. Assay Linearity Over Time

Different cell types have different basal reducing potentials and therefore varying capacities to reduce the MT Cell Viability Substrate. The assay signal increases over time when the RealTime-Glo™ reagent is incubated with proliferating cells. The assay signal also can decrease at later time points for many reasons, including cell death because the initial cell density was too high, a shift in metabolism because cells reached confluency or waste products accumulated in the medium, or depletion of the MT Cell Viability Substrate. We strongly recommend performing a cell titration experiment to determine assay linearity with regard to cell density and incubation time (see Section 4). We also recommend that you determine assay linearity if you change medium formulation or other cell culture conditions because cell culture conditions can affect reducing potential and cell proliferation.

7.B. Assay Performance Considerations

Temperature

The intensity of the luminescent signal from the RealTime-Glo™ MT Cell Viability Assay depends on the rate of the NanoLuc® luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will change the intensity of light output. Temperature is one factor that affects the rate of this enzymatic assay and thus light output. For consistent results, measure luminescence immediately after removing the plate from the 37°C incubator to prevent significant cooling or equilibrate assay plates to a constant temperature before measuring luminescence. Certain plate-reading luminometers can be set at 37°C to minimize temperature changes. For batch-mode processing of multiple assay plates, take precautions to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require longer equilibration times than plates arranged in a single layer. Insufficient equilibration may result in a temperature gradient between the wells in the center and on the edge of the plates. The temperature gradient pattern also may depend on the position of the plate in the stack.

The overall RLU values will differ at different temperatures; however, if the plate is equilibrated such that all wells within one plate are at the same temperature at each consecutive measurement (i.e., 37°C or 22°C) then the relationship among samples within the plate will follow the same trend. Analyzing the data as percent change compared to the vehicle control samples will normalize the data and allow comparison among different time points.

Chemicals

The chemical environment of the luciferase reaction affects the enzymatic rate and thus luminescence intensity. Differences in luminescence intensity may be observed using different types of culture medium and serum. The presence of phenol red in culture medium should have little impact on luminescent output.

Some test compound diluents may interfere with the luciferase reaction and thus light output. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations of up to 1% in the assay and does not affect light output.

Some reducing agents can reduce the MT Cell Viability Substrate when used at high concentrations. When using medium formulations that include reducing agents, you should determine the effect of the reducing agent on the luminescent background by comparing luminescence in the presence and absence of the reducing agent.

7.B. Assay Performance Considerations (continued)

Plate Recommendations

We recommend using standard white, opaque-walled multiwell plates suitable for luminescence measurements. Opaque-walled plates with clear bottoms also may be used to allow microscopic visualization of cells; however, these plates will have diminished signal intensity and greater cross talk between wells. Opaque white tape may be used to decrease luminescence loss and cross talk. Black multiwell plates also can be used and may be optimal for multiplexing applications that include a fluorescent measurement. Using black multiwell plates will result in a reduced luminescent signal.

Cell Culture System Optimization

Cytotoxicity may be caused by culture conditions, independent of test compound effects, over periods longer than 24 hours due to accumulation of metabolic waste, pH changes and depletion of nutrients. Therefore, make every effort to optimize culture conditions and volumes to ensure observed cytotoxicity is test compound-dependent. Similarly, using cell cultures with poor initial viability will decrease signals in all cell samples. Every effort should be made to deliver the same number of viable cells to each assay well. The CellTox™ Green Cytotoxicity Assay, which consists of a cell membrane-impermeant dye that binds to the DNA of cells with impaired membrane integrity, can be used to assess cytotoxicity due to culture conditions.

7.C. Assay Chemistry Considerations

The uptake mechanism of the MT Cell Viability Substrate is not well established. We have not found any significant interference of the RealTime-Glo™ MT Cell Viability Assay with any of the test compounds that we have tested in various cancer and primary cell lines. It is possible that the test compound and RealTime-Glo™ reagents could have synergistic effects on the cells, and this is something that should be considered during data analysis.

The MT Cell Viability Substrate and NanoLuc® Enzyme are well tolerated by cells in culture. We have tested the RealTime-Glo™ reagents for cytotoxic effects at up to 72 hours in a variety of cell lines and found the reagents to be well tolerated by cells over this time course. In these experiments, we grew multiple cell lines at various densities in medium only or medium supplemented with the RealTime-Glo™ reagents for 72 hours, then removed medium from these samples to measure cytotoxicity using the CytoTox-Fluor™ Assay, an assay that detects the release of a specific protease as a result of compromised membrane intergrity. There was less than a 10% difference in signal in cells that contained the RealTime-Glo™ reagents when compared to signal in cells grown in medium only.

7.D. Multiplexing with the RealTime-Glo™ MT Cell Viability Assay

The RealTime-Glo™ MT Cell Viability Assay is nonlytic and can be conveniently multiplexed with many assay chemistries, both fluorescent and luminescent, without the need for special spectral filters. Because the RealTime-Glo™ MT Cell Viability Assay is well tolerated by cells, cells can be further analyzed using a number of methods, including RNA analysis. Table 1 shows a subset of the assays and applications that can be multiplexed with the RealTime-Glo™ MT Cell Viability Assay for more informative data per sample.

Table 1. Assays that can be Multiplexed with the RealTime-Glo™ MT Cell Viability Assay.

First Assay	Second Assay	Information Obtained by Multiplexing
RealTime-Glo™ MT Cell Viability Assay	CellTox™ Green Cytotoxicity Assay[1]	Cell viability and cytotoxicity (membrane integrity)
RealTime-Glo™ MT Cell Viability Assay	CytoTox-Fluor™ Cytotoxicity Assay	Cell viability and cytotoxicity (protease release)
RealTime-Glo™ MT Cell Viability Assay	CellTiter-Glo® Luminescent Cell Viability Assay	Two independent markers of cell viability (reducing potential and ATP)
RealTime-Glo™ MT Cell Viability Assay	NAD/NADH-Glo™ Assay	Cell viability and measurement of NAD^+ and NADH
RealTime-Glo™ MT Cell Viability Assay	NADP/NADPH-Glo™ Assay	Cell viability and measurement of $NADP^+$ and NADPH
RealTime-Glo™ MT Cell Viability Assay	Reporter assays[2]	Cell viability and reporter gene activity
RealTime-Glo™ MT Cell Viability Assay	RNA isolation	Cell viability and RNA analysis

[1]The RealTime-Glo™ MT Cell Viability Assay and CellTox™ Green Cytotoxicity Assay can be multiplexed by adding both reagents simultaneously to measure both live cells and dead cells kinetically.

[2]The RealTime-Glo™ MT Cell Viability Assay is compatible with reporter assays that measure Ultra-Glo™ and firefly luciferase activities. We do not recommend multiplexing the RealTime-Glo™ MT Cell Viability Assay with NanoLuc® or *Renilla* luciferase-based assays. We have not tested compatibility of the RealTime-Glo™ MT Cell Viability Assay with other reporter assays.

7.D. Multiplexing with the RealTime-Glo™ MT Cell Viability Assay (continued)

To demonstrate the ability to use cells assayed using the RealTime-Glo™ MT Cell Viability Assay for downstream applications such as qRT-PCR, we plated multiple cell lines in medium only or medium containing 1X RealTime-Glo™ reagent. After taking cell viability measurements, we isolated RNA from all cell samples using the Maxwell® 16 LEV simplyRNA Cells Kit and performed qRT-PCR to detect the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1). The cycle threshold (C_t) values for RNA isolated from cells incubated with the RealTime-Glo™ reagents were comparable to those for RNA isolated from cells exposed to medium only (Table 2).

Table 2. C_t Values Generated from RNA Isolated from Different Cell Types With or Without RealTime-Glo™ Reagent.

	C_t Value	
Cell Type	**With Medium Only**	**With RealTime-Glo ™ Reagent**
A549	30.9	30.3
K562	31.3	30.2
MCF7	30.9	30.1
THP-1	29.9	29.9

7.E. RealTime-Glo™ Reagent Precipitate Removal

Precipitate may occur when preparing the RealTime-Glo™ reagent. The precipitate is not contamination. To remove precipitate, you can filter the RealTime-Glo™ reagent before adding to samples, using the following protocol.

Note: Filtering the RealTime-Glo™ reagent will consume a small amount of MT Cell Viability Substrate, thereby decreasing the length of time the assay can be performed.

1. Warm the RealTime-Glo™ reagent and cell culture medium to 37°C.
2. Prepare a 2X stock of RealTime-Glo™ reagent in warmed medium. Mix thoroughly.
3. Load syringe with the 2X RealTime-Glo™ reagent.
4. Filter the RealTime-Glo™ reagent through a 0.2µm filter, such as Fisherbrand® 25mm syringe filter, 0.2µm nylon sterile (Fisher Scientific Cat.# 09-719C).
5. Add the filtered solution to your cell sample at a 1:1 volume to bring the final concentration of RealTime-Glo™ reagent to 1X.
6. Perform assay as described in Section 5.

8. Related Products

Viability Assays

Product	Size	Cat.#
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
CellTiter-Blue® Cell Viability Assay	20ml	G8080

Other sizes are available.

Cytotoxicity Assays

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

Other sizes are available.

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.

Mechanism-Based Viability and Cytotoxicity Assays

Product	Size	Cat.#
ApoTox-Glo™ Triplex Assay	10ml	G6320
ApoLive-Glo™ Multiplex Assay	10ml	G6410

Other sizes are available.

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay	10ml	G8091
Caspase-Glo® 8 Assay	10ml	G8201
Caspase-Glo® 9 Assay	10ml	G8211
Apo-ONE® Homogeneous Caspase-3/7 Assay	10ml	G7790
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011

Other sizes are available.

Promega

8. Related Products (continued)

Inflammation Assays

Product	Size	Cat.#
Caspase-Glo® 1 Inflammasome Assay	10ml	G9951
	5 × 10ml	G9952

Metabolism Assays

Product	Size	Cat.#
NAD(P)H-Glo™ Detection System	10ml	G9061
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081

Other sizes are available.

Mitochondrial Toxicity

Product	Size	Cat.#
Mitochondrial ToxGlo™ Assay	10ml	G8000
	100ml	G8001

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.

Cytochrome P450 Cell-Based Assays

Product	Size	Cat.#
P450-Glo™ CYP1A2 Induction/Inhibition Assay	10ml	V8421
P450-Glo™ CYP3A4 Assay with Luciferin-IPA	10ml	V9001
P450-Glo™ CYP2C9 Assay	10ml	V8791

Other sizes are available.

Detection Instrumentation

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000

9. Summary of Changes

The following changes were made to the 12/21 revision of this document:

1. Updated Sections 5 and 7.E to reinforce steps to remove precipitate if it occurs in RealTime-Glo™ reagent.
2. The cover image was updated.

[a]Patent Pending.