TECHNICAL BULLETIN

BacTiter-Glo™ Microbial Cell Viability Assay

Instructions for Use of Products
G8230, G8231, G8232 and G8233

Revised 6/19
TB337

BacTiter-Glo™ Microbial Cell Viability Assay

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1. Description

The BacTiter-Glo™ Microbial Cell Viability Assay[a,b] is a homogeneous method for determining the number of viable bacterial cells in culture based on quantitation of the ATP present. ATP is an indicator of metabolically active cells. The BacTiter-Glo™ Assay is designed for either single-tube or multiwell-plate formats for high-throughput screening (HTS). The homogeneous assay procedure involves adding a single reagent (BacTiter-Glo™ Reagent) directly to bacterial cells in medium and measuring luminescence (Figure 1). Washing cells, removing culture medium and performing multiple pipetting steps are not required.

The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal in a homogeneous "add, mix, measure" format. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of cells in culture (Figure 2). The BacTiter-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) and a proprietary formulation for extracting ATP from bacteria. The assay generates a "glow-type" luminescent signal, produced by the luciferase reaction shown in Figure 3, which has a signal half-life generally over 30 minutes depending on the bacterium and medium. The assay has been shown to detect a variety of bacteria, yeast and fungi (Table 1). The homogeneous format reduces pipetting errors that may be introduced during the multiple steps required by other methods of ATP measurement.

Advantages

- **Simplify your Assay:** The add, mix, measure format reduces the number of handling steps to fewer than that required for similar ATP assays, with no injectors required.
- **Get Results Quickly:** Data can be recorded 5 minutes after adding and mixing reagent, and sensitivity allows you to detect growth sooner.
- **Increase your Sensitivity:** Measures ATP from as few as 10 bacterial cells.
- **Choose your Format:** Can be used with various multiwell or single-use formats. Data can be recorded by luminometer or CCD camera.
- **Achieve Robust Signal:** Luminescent signal is stable, with a 30-minute half-life.

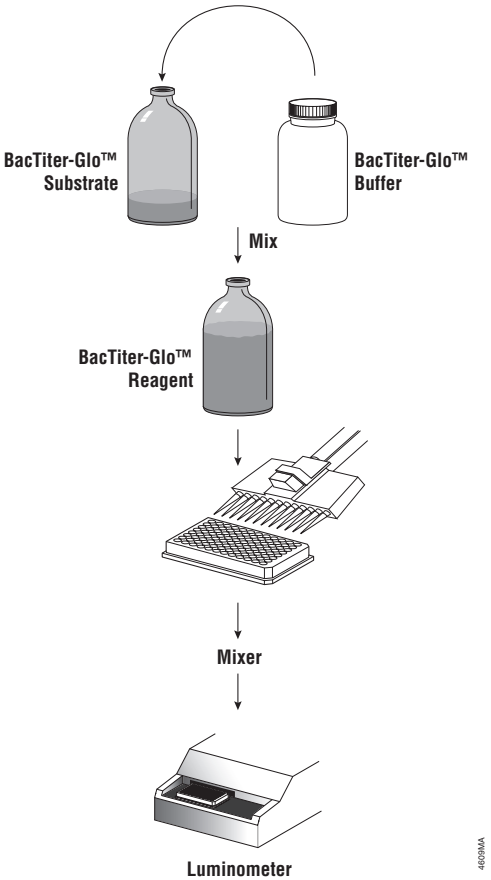

Figure 1. Diagram of the BacTiter-Glo™ Microbial Cell Viability Assay protocol. The assay is suitable for single-tube or multiwell-plate formats shown here.

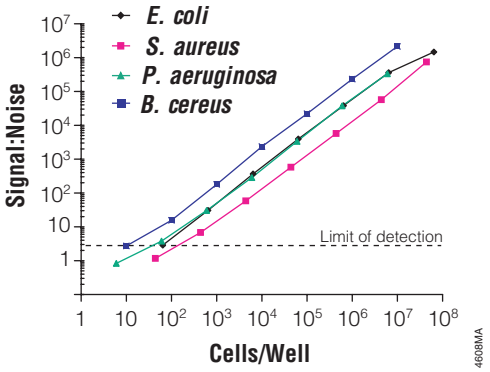

Figure 2. Bacterial cell numbers correlate with luminescent signal. Four bacterial strains [*Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853) and *Bacillus cereus* (ATCC10987)] were grown in Mueller Hinton II (MH II) Broth (BD Cat.# 297963; see Section 4.B for growth medium recommendations) at 37°C overnight. The overnight culture was diluted 50-fold in fresh MH II Broth and then incubated for several hours to reach log phase. Samples of the culture were serially diluted using MH II Broth in a 96-well plate. The assay was performed according to the protocol described in Section 3. The reconstituted BacTiter-Glo™ Reagent was equilibrated for 1.5 hours at room temperature to achieve better sensitivity (see Reagent Background in Section 4.B). Luminescence was recorded on a GloMax® 96 Microplate Luminometer (Cat.# E6501). Signals represent the mean of three replicates for each measurement. Bacterial cell numbers were determined by plate counting of colony forming units on Luria-Bertani agar plates. The signal-to-noise ratio was calculated: S:N = [mean of signal – mean of background]/standard deviation of background. There is a linear correlation between luminescent signal and the number of cells over five orders of magnitude. The limits of detection drawn from this experiment for *E. coli, S. aureus, P. aeruginosa* and *B. cereus* are approximately 70, 150, 40 and 10 cells, respectively.

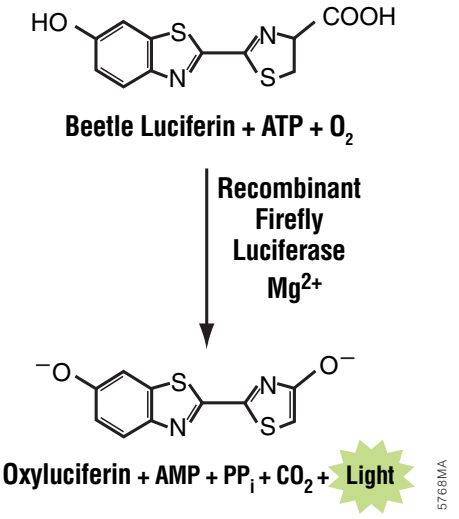

Figure 3. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP and molecular oxygen.

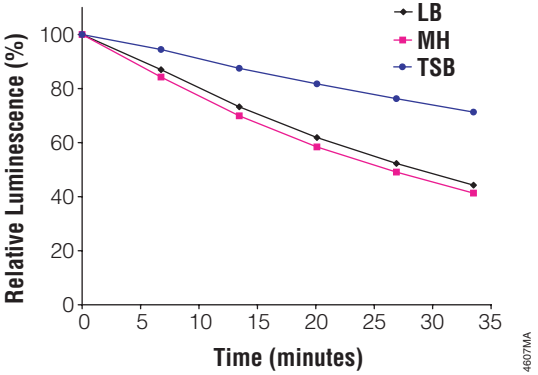

Figure 4. BacTiter-Glo™ Reagent generates a glow-type luminescent signal. *E. coli* cells were grown and assayed as described in Figure 2. Three media were tested: Luria-Bertani Broth, Mueller Hinton II (MH II) Broth (BD Cat.# 297963), and Trypticase Soy Broth (TSB, BD Cat.# 299113). Approximately 10^6 *E. coli* cells were used for the assay. The stability of the luminescence signal was monitored over time. Luminescence was recorded on a GloMax® 96 Microplate Luminometer (Cat.# E6501). The half-lives of the luminescence signals in MH II, LB and TSB were 26, 28 and 68 minutes, respectively.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
BacTiter-Glo™ Microbial Cell Viability Assay	**10ml**	**G8230**

Substrate is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10ml BacTiter-Glo™ Buffer
- 1 vial BacTiter-Glo™ Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
BacTiter-Glo™ Microbial Cell Viability Assay	**10 × 10ml**	**G8231**

Each vial of substrate is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10 × 10ml BacTiter-Glo™ Buffer
- 10 vials BacTiter-Glo™ Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
BacTiter-Glo™ Microbial Cell Viability Assay	**100ml**	**G8232**

Substrate is sufficient for 1,000 assays at 100µl/assay in 96-well plates or 4,000 assays at 25µl/assay in 384-well plates. Includes:

- 100ml BacTiter-Glo™ Buffer
- 1 vial BacTiter-Glo™ Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
BacTiter-Glo™ Microbial Cell Viability Assay	**10 × 100ml**	**G8233**

Each vial of substrate is sufficient for 1,000 assays at 100µl/assay in 96-well plates or 4,000 assays at 25µl/assay in 384-well plates (10,000 to 40,000 total assays). Includes:

- 10 × 100ml BacTiter-Glo™ Buffer
- 10 vials BacTiter-Glo™ Substrate (lyophilized)

Storage Conditions: For long-term storage, the lyophilized BacTiter-Glo™ Substrate and BacTiter-Glo™ Buffer should be stored at –20°C. For frequent use, the BacTiter-Glo™ Buffer can be stored at 4°C or at room temperature for 48 hours without loss of activity. For optimal performance, reconstituted BacTiter-Glo™ Reagent (buffer plus substrate) should be used within eight hours when the reagent is kept at room temperature. The reconstituted BacTiter-Glo™ Reagent can be stored at 4°C for four days, at –20°C for one week or at –70°C for one month with less than 20% loss of activity.

3. Protocol for Performing the BacTiter-Glo™ Assay

Materials to Be Supplied by the User

- opaque-walled multiwell plates
- multichannel pipette or automated pipetting station for delivering reagent
- plate shaker or other device for mixing contents of multiwell plates
- luminometer (e.g., GloMax® Navigator System [Cat.# GM2000] or GloMax® 20/20 Luminometer [Cat.# E5311]), or CCD camera capable of reading multiwell plates
- optional: ATP for generating a standard curve

Caution: Skin contains ATP. Because this assay is so sensitive, we recommend wearing gloves to avoid contamination.

3.A. Reagent Preparation

1. Thaw the BacTiter-Glo™ Buffer and equilibrate to room temperature before use. For convenience the BacTiter-Glo™ Buffer may be thawed and stored at room temperature for up to 48 hours before use.
2. Equilibrate the lyophilized BacTiter-Glo™ Substrate to room temperature.
3. Transfer the appropriate volume (10ml for Cat.# G8230, G8231 or 100ml for Cat.# G8232, G8233) of BacTiter-Glo™ Buffer into the amber bottle containing BacTiter-Glo™ Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the BacTiter-Glo™ Reagent.
4. Mix by gently vortexing, swirling or by inverting the bottle to obtain a homogeneous solution. The BacTiter-Glo™ Substrate should go into solution easily, in less than one minute.
5. Equilibrate Reagent at room temperature for at least 15 minutes before use. To achieve maximum sensitivity, additional equilibration time may be required. See “Reagent Background” in Section 4.B for more information.

3.B. Protocol for Measuring ATP From Bacteria

Note: All steps are performed at room temperature (22–25°C).

1. Prepare an opaque-walled multiwell plate with microbial cells in culture medium (e.g., 100µl for each well of a 96-well plate or 25µl for each well of a 384-well plate).
2. Prepare control wells containing medium without cells to obtain a value for background luminescence.
3. Equilibrate the plate and its contents to room temperature.
4. Add a volume of BacTiter-Glo™ Reagent equal to the volume of cell culture medium present in each well (e.g., add 100µl of reagent to 100µl of medium containing cells for the 96-well plate format or 25µl of reagent for the 384-well plate format).
5. Mix contents briefly on an orbital shaker and incubate for five minutes.
6. Record luminescence.

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

3.C. Protocol for Generating an ATP Standard Curve (optional)

Note: All steps are performed at room temperature (22–25°C).

1. Prepare 1µM ATP in culture medium (100µl of 1µM ATP solution contains 10^{-10} moles ATP).
2. Prepare 10-fold serial dilutions of ATP in culture medium (1µM to 10pM; 100µl volumes would contain 10^{-10} to 10^{-15} moles of ATP).
3. Prepare a multiwell plate with varying concentrations of standard ATP solution in 100µl medium.
4. Add a volume of BacTiter-Glo™ Reagent equal to the volume of ATP standard present in each well (1:1 ratio).
5. Mix contents briefly on an orbital shaker and incubate for one minute. Since there is no lysis required to release ATP, longer incubations are not required.
6. Record luminescence.

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4. Appendix

4.A. Overview of the BacTiter-Glo™ Assay

The BacTiter-Glo™ Assay System utilizes a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) to enable extraction of ATP from bacterial cells and to support a stable "glow-type" luminescent signal. Historically, firefly luciferase purified from *Photinus pyralis* has been used in reagents for ATP assays (1–3). However, this enzyme has only moderate stability in vitro and is sensitive to factors such as pH and detergents, limiting its usefulness in a robust homogeneous ATP assay. Promega has successfully developed a stable form of luciferase (Ultra-Glo™ Recombinant Luciferase) based on the gene from another firefly, *Photuris pennsylvanica*, using an approach to select for characteristics that improve performance in ATP assays (4). In addition, we developed a proprietary formulation to achieve rapid and more efficient extraction of ATP from a variety of microbial cells (Table 1). The combination of these two essential elements in the BacTiter-Glo™ Reagent enabled design of a homogeneous single-reagent system for performing ATP assays on cultured cells. The reagent is physically robust and provides a sensitive and stable luminescent output.

Table 1. BacTiter-Glo™ Reagent Works with a Variety of Microbial Organisms.

Gram-Negative Bacteria	Gram-Positive Bacteria	Others
Escherichia coli	*Staphylococcus aureus*	*Saccharomyces cerevisiae*
Pseudomonas aeruginosa	*Enterococcus faecalis*	*Candida albicans**
Enterobacter cloacae	*Streptococcus pneumoniae*	*Mycobacterium avium*
Flavobacterium okeanokoites	*Bacillus subtilis*	*Mycobacterium paratuberculosis*
Haemophilus influenzae	*Bacillus cereus*	
Proteus vulgaris	*Arthrobacter luteus*	
Salmonella typhimurium		
Yersinia enterocolitica		
Francisella philomiragia		

*For *Candida albicans* a 15-minute incubation time is required for optimal signal, and the limit of detection is around 5×10^3 cells.

4.B. Additional Considerations

Temperature: The intensity and rate of decay of the luminescent signal from the BacTiter-Glo™ Assay depend on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and the stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to room temperature before performing the assay. Insufficient equilibration may result in a temperature gradient effect between the wells in the center and on the edge of the plates.

Growth Medium: Growth medium is another factor that could contribute to the background luminescence and affect the luciferase reaction in terms of signal level and signal stability (Figure 4). We have used MH II Broth (cation-adjusted Mueller Hinton Broth; Becton, Dickinson and Company Cat.# 297963) for all our experiments unless otherwise stated. It supports growth of most commonly encountered aerobic and facultative anaerobic bacteria and is selected for use in food testing and antimicrobial susceptibility testing by the Food and Drug Administration and the National Committee for Clinical Laboratory Standards (NCCLS) (5,6). MH Medium has low luminescence background and good batch-to-batch reproducibility.

Chemicals: The chemical environment of the luciferase reaction will affect the enzymatic rate and thus luminescence intensity. Solvents used for the various chemical compounds tested for their antimicrobial activities may interfere with the luciferase reaction and thus the light output from the assay. Interference with the luciferase reaction can be determined by assaying a parallel set of control wells containing medium without cells. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the assay and has little effect on light output (<5% loss of activity).

Plate and Tube Recommendations: The BacTiter-Glo™ Assay is suitable for multiwell-plate or single-tube formats. Standard opaque-walled multiwell plates suitable for luminescence measurements are recommended for use. Opaque-walled plates with clear bottoms allowing microscopic visualization of cells also may be used; however, these plates will have diminished signal intensity and greater cross-talk between wells. Opaque white tape may be used to reduce luminescence loss and cross-talk. For single-tube assays, the standard tube accompanying the luminometer used should be suitable.

Cellular ATP Content: Different bacteria have different amounts of ATP per cell, and values reported for the ATP level in cells vary considerably (7,8). Factors that affect the ATP content of cells such as growth phase, medium, and presence of metabolic inhibitors, may affect the relationship between cell number and luminescence (7).

Mixing: Optimum assay performance is achieved when the BacTiter-Glo™ Reagent is completely mixed with the sample of cultured cells. For all the bacteria we tested, maximum luminescence signals were observed after efficiently mixing and incubating for 1–5 minutes. However, complete extraction of ATP from certain bacteria, yeast or fungi may take longer. Automated pipetting devices using a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Ensure complete reagent mixing in 96-well plates by using orbital plate shaking devices built into many luminometers. We recommend considering these factors when performing the assay and determining whether a mixing step and/or longer incubation is necessary.

Reagent Background: Despite the rigorous ATP-free manufacturing process, a trace amount of ATP is still present in the BacTiter-Glo™ Substrate and Buffer. In addition, ATP could be introduced by the user during the reconstitution step. When the BacTiter-Glo™ Substrate and Buffer are mixed together to reconstitute BacTiter-Glo™ Reagent, a background luminescence signal is generated that decreases over time as the ATP is being consumed. This process is referred to as "burn-off." Complete burn-off to the lowest achievable background could take up to two hours. However, this is only necessary when the maximum sensitivity is required (e.g., detection of very low numbers of microorganisms).

4.C. Examples of BacTiter-Glo™ Assay Applications

The BacTiter-Glo™ Assay provides a simple and robust way to quantify bacteria with superb sensitivity and dynamic range. Some examples of its applications are shown below.

Screening for Antimicrobial Compounds

We used the BacTiter-Glo™ Assay to screen one rack of Library of Pharmacologically Active Compounds from Sigma (LOPAC, #8, enzyme inhibitors, total of 80 compounds) for antimicrobial activity against *Staphylococcus aureus*. The results are shown in Figure 5. All positive controls of standard antibiotics (boxed points) and three LOPAC compounds (circled points) exhibited significant anti-*S. aureus* activity. The three LOPAC hits were D6, emodin; D11, sanguinarine chloride; and H7, minocycline. The anti-*S. aureus* activities of these compounds have been reported in the literature (9–11).

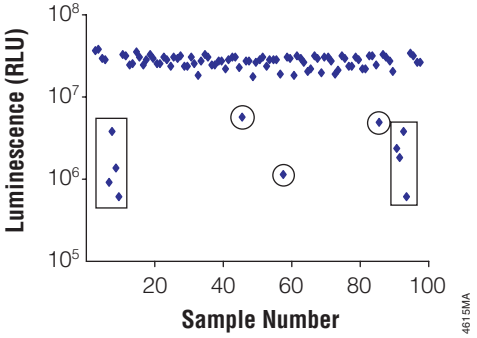

Figure 5. Screening for antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain was grown in Mueller Hinton II (MH II) Broth (BD Cat.# 297963; see Section 4.B for growth medium recommendations) at 37°C overnight. The overnight culture was diluted 100-fold in fresh MH II Broth and used as inoculum for the antimicrobial screen. Working stocks (50X) of LOPAC compounds and standard antibiotics were prepared in DMSO. Each well of the 96-well multiwell plate contained 245µl of the inoculum and 5µl of the 50X working stock. The multiwell plate was incubated at 37°C for 5 hours. One hundred microliters of the culture was taken from each well, and the BacTiter-Glo™ Assay was performed according to the protocol described in Section 3. Luminescence was measured using a GloMax® 96 Microplate Luminometer (Cat.# E6501). The samples and concentrations are: Wells 1–4 and 93–96, negative control of 2% DMSO; wells 5–8 and 89–92, positive controls of 32µg/ml standard antibiotics tetracycline, ampicillin, gentamicin, chloramphenicol, oxacillin, kanamycin, piperacillin, and erythromycin; wells 9–88, LOPAC compounds at 10µM.

Evaluating Antimicrobial Compound Activity

We examined the dosage effects of oxacillin on *S. aureus* using the BacTiter-Glo™ Assay. The results are shown in Figure 6. Oxacillin showed anti-*S. aureus* activity in a dosage-dependent fashion. The reported and observed minimal inhibitory concentration (MIC) values for oxacillin on S. aureus ATCC 25923 in cation-adjusted MH II Broth are 0.125–0.5µg/ml (6), corresponding to approximately IC_{75}–IC_{90} values on the dosage curve determined using the BacTiter-Glo™ Assay.

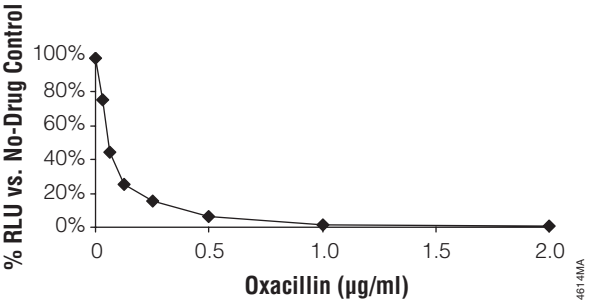

Figure 6. Evaluating antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain and oxacillin were prepared as described in Figure 5 and incubated at 37°C; the assay was performed after 19 hours of incubation as recommended for MIC determination by NCCLS (6). The relative percentage of RLU compared to the no-oxacillin control is shown. Luminescence was recorded on a GloMax® 96 Microplate Luminometer (Cat.# E6501).

Examining Bacterial Growth with Extended Sensitivity and Range

We examined the growth of *E. coli* using either the BacTiter-Glo™ Assay or optical density (O.D.) measurement. The results are shown in Figure 7. The extended sensitivity and range of the BacTiter-Glo™ Assay allows users to monitor *E. coli* growth immediately after inoculation. When measuring growth by O.D., the first significant measurement (0.025) did not occur until 5 hours after inoculation. The growth curve determined by the BacTiter-Glo™ Assay has a dynamic range over six orders of magnitude compared to the growth curve determined by O.D. measurement, which only has a range of about two orders of magnitude. The increased dynamic range allows researchers to more easily monitor slow-growing bacteria.

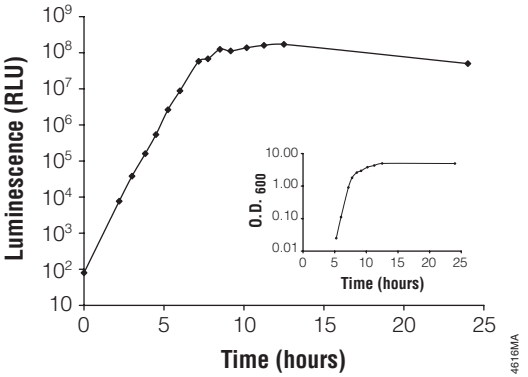

Figure 7. Evaluating bacterial growth using the BacTiter-Glo™ Assay. *E. coli* ATCC 25922 strain was grown in Mueller Hinton II (MH II) Broth (B.D. Cat.# 297963; see Section 4.B for growth medium recommendations) at 37°C overnight. The overnight culture was diluted 1:10^6 in 50ml of fresh MH II Broth and incubated at 37°C with shaking at 250rpm. Samples were taken at various time points, and the BacTiter-Glo™ Assay was performed according to the protocol described in Section 3. Luminescence was recorded on a GloMax® 96 Microplate Luminometer. Optical density was measured at 600nm ($O.D._{600}$) using a Beckman DU650 spectrophotometer. Diluted samples were used when readings of RLU and O.D. exceeded 10^8 and 1, respectively.

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4.D. References

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3. Lundin, A. and Thore, A. (1975) Analytical information obtained by evaluation of the time course of firefly bioluminescence in the assay of ATP. *Anal. Biochem.* **66**, 47–63.
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7. Stanley, P.E. (1986) Extraction of adenosine triphosphate from microbial and somatic cells. *Methods. Enzymol.* **133**, 14–22.
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11. Raad, I. *et al.* (2003) In vitro and ex vivo activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. *Antimicrob. Agents Chemother.* **47**, 3580–5.

4.E. Related Products

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500
GloMax® Navigator System	1 each	GM2000
GloMax® 20/20 Luminometer	1 each	E5311
Disposable Polypropylene Test Tubes	1,000 tubes	E4221

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
(luminescent, ATP)	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
CellTiter-Blue® Cell Viability Assay	20ml	G8080
(colorimetric, resazurin)	100ml	G8081
	10 × 100ml	G8082
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	200 assays	G3582
(colorimetric, MTS)	1,000 assays	G3580
	5,000 assays	G3581
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890
(Fluorometric LDH)	1,000–4,000 assays	G7891
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP	1,000–4,000 assays	G7892
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
(Fluorometric, measure live and dead cells)	5 × 10ml	G9201
	2 × 50ml	G9202
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260
	5 × 10ml	G9261
	2 × 50ml	G9262

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Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
	100ml	G8092
	10 × 10ml	G8093
Caspase-Glo® 8 Assay	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo® 9 Assay	2.5ml	G8210
	10ml	G8211
	100ml	G8212
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
	10ml	G7790
	100ml	G7791

Proteasome Assays

Product	Size	Cat.#
Proteasome-Glo™ Cell-Based Assay	10ml	G8660
	5 × 10ml	G8661
	2 × 50ml	G8662
Proteasome-Glo™ 3-Substrate System	10ml	G8531
	50ml	G8532

5. Summary of Changes

The following changes were made to the 6/19 revision of this document:

1. The Related Products section was updated.

[a]U.S. Pat. Nos. 6,602,677, 7,241,584 and 8,030,017, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

[b]U.S. Pat. No. 7,422,868, European Pat. No. 1763584 and other patents pending.

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