MICROBIAL VIABILITY

DETERMINING MICROBIAL VIABILITY USING A HOMOGENEOUS LUMINESCENT ASSAY

FRANK FAN, PH.D., BRAEDEN BUTLER, B.S., TERRY RISS, PH.D., AND KEITH WOOD, PH.D., PROMEGA CORPORATION

The BacTiter-Glo™ Microbial Cell Viability Assay determines the number of viable cells based on quantitation of ATP. This simple assay can provide results in as little as five minutes, and its excellent sensitivity allows you to detect microbial growth sooner than with conventional O.D. measurements. This assay is easily adaptable to automated and multiwell formats, and automated reagent injectors are not required.

Introduction

ATP-based detection of microbial cells represents one of the first applications of firefly bioluminescence. However, the limited stability of the firefly luciferase has precluded combining it with effective lysis reagents as a single mixture. Therefore, conventional methods require multiple processing steps, making them less suited to analysis of multiple samples. We have developed the BacTiter-Glo™ Microbial Cell Viability Assay[a,b], which combines the lytic reagent with luciferase/luciferin and allows sensitive detection of microbial cells in a single step.

> The "add-mix-measure" format and the stable luminescent signal make the BacTiter-Glo™ Assay an ideal choice for automation and high-throughput screening.

The assay system utilizes a proprietary reagent formulation containing a highly stabilized luciferase, Ultra-Glo™ Recombinant Luciferase, to extract ATP from bacterial cells and support a stable "glow-type" luminescent signal. Other commercial reagents use luciferase purified from *Photinus pyralis* (1–3), which has only moderate stability in vitro and is sensitive to factors such as pH and detergents. We developed a stable form of 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. The combination of these two essential elements in the BacTiter-Glo™ Reagent enabled the design of a single-reagent system for performing ATP assays on cultured microbial cells. In addition, the lysis and luminescent kinetics are optimized for applications that use multiwell plates and lab automation. The reagent is physically robust and provides a sensitive and stable luminescent output.

These characteristics are particularly useful in the discovery and development of new antibiotics that are critical to combat infectious diseases caused by microbial pathogens and to address rapid spreading of antibiotic resistance. The "add-mix-measure" format and the stable luminescent signal make the BacTiter-Glo™ Assay an ideal choice for high-throughput screening.

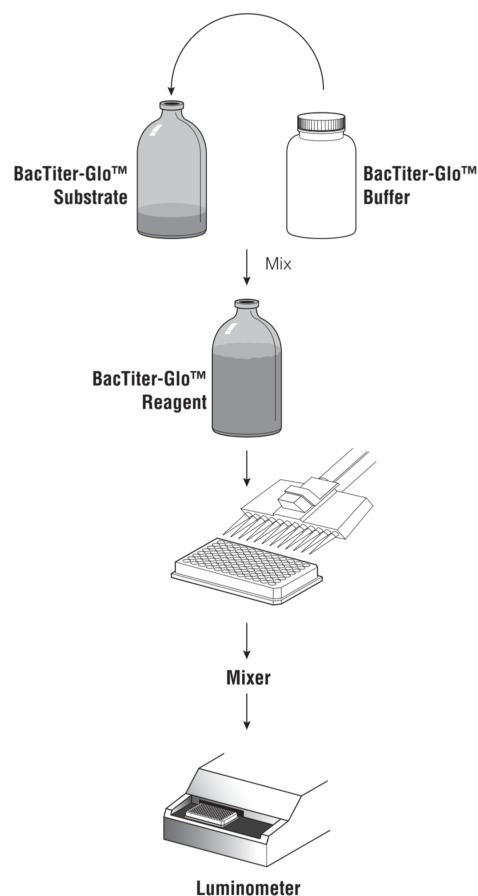


Figure 1. Diagram of the BacTiter-Glo™ Microbial Cell Viability Assay protocol. The assay is suitable for the multiwell-plate assays for high-throughput screening.

Simply Add, Mix and Measure

The assay is simple to use and requires only a single addition of reagent directly to cells in the culture medium. The procedure does not require additional handling steps, such as removing medium or washing cells, thus reducing errors that may be introduced during a multiple-step procedure.

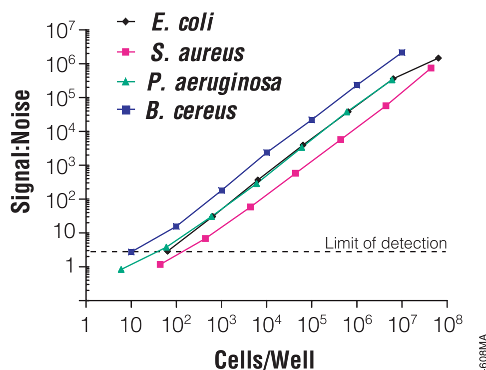


Figure 2. Bacterial cell numbers correlate with luminescent signal. Four bacterial strains: *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853) and *Bacillus cereus* (ATCC10987), were grown in Mueller Hinton II (MH II) Broth (see Technical Bulletin #TB337 for growth medium recommendations) overnight at 37°C. 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 Technical Bulletin #TB337. The reconstituted BacTiter-Glo™ Reagent was equilibrated for 1.5 hours at room temperature to achieve increased sensitivity. Luminescence was recorded on a Veritas™ Microplate Luminometer (Cat.# E6501). Signal values represent the mean of three replicates for each measurement. Bacterial cell numbers were determined by plate counting of colony forming units on Luria-Bertani (LB) agar plates. The signal-to-noise ratio was calculated: S:N = [mean of signal – mean of background]/standard deviation of background. The limits of detection drawn from this experiment for *E. coli*, *S. aureus*, *P. aeruginosa* and *B. cereus* are approximately 40, 150, 70 and 10 cells, respectively.

A diagram of the assay protocol is shown in Figure 1. To prepare BacTiter-Glo™ Reagent, the lyophilized BacTiter-Glo™ Substrate is reconstituted using BacTiter-Glo™ Buffer and equilibrated to room temperature for at least 15 minutes to reduce background luminescence (see Technical Bulletin #TB337 for details). To perform the assay, an equal volume of reagent is added to the microbial cell culture, mixed and incubated for 5 minutes. The emitted luminescence is detected using a luminometer or a charged coupled device (CCD) imager.

Detect as Few as Ten Cells

The BacTiter-Glo™ Microbial Cell Viability Assay is sensitive and linear (Figure 2). The data in Figure 2 demonstrate that the assay can detect as few as 10 *Bacillus cereus* cells, which gave signal levels greater than three standard deviations above the background signal from medium without cells. Luminescent signals correlated well with the cell numbers, showing excellent linearity and a dynamic range typically over 5 logs.

We have demonstrated the utility of the BacTiter-Glo™ Assay on a variety of microbes as shown in Table 1. These microbes include Gram-positive and Gram-negative bacteria and yeast.

Table 1. Organisms Successfully Tested with the BacTiter-Glo™ Assay.

Gram– Bacteria	Gram+ Bacteria	Others
Escherichia coli[1]	*Staphylococcus aureus*[2]	*Saccharomyces cerevisiae*[1]
Psuedomonas aeruginosa[2]	*Enterococcus faecalis*[2]	*Candida albicans*[2]
Enterobacter cloacae	*Streptococcus pneumoniae*[2]	
Flavobacterium okeanokoites	*Bacillus subtilis*[1]	
Haemophilus influenzae[2]	*Bacillus cereus*[3]	
Proteus vulgaris	*Arthrobacter luteus*	
Salmonella typhimurium		
Yersinia enterocolitica[3]		
Francisella philomiragia[3]		

[1]*Model organism* [2]*Drug discovery* [3]*Biodefense*

They also include some common pathogens of interest for antimicrobial drug discovery, biothreat pathogen surrogates of interest for biodefense, model microbial organisms as well as some random collections.

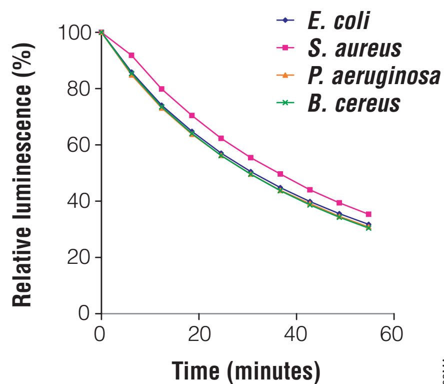
Gain Flexibility With Stable Signal

The BacTiter-Glo™ Microbial Cell Viability Assay generates a glow-type luminescent signal that has a half-life generally over 30 minutes depending on the microbe and medium (Figure 3). High sensitivity and signal stability make the BacTiter-Glo™ Assay amenable for high-throughput screening. To further assess this capability, we determined the Z´-factor value for both 96-well and 384-well formats. Z´-factor is a characteristic statistical parameter of the assay (5). A Z´-factor value closer to 1.0 indicates a robust assay. The number of bacteria cells/well used for this analysis is typical for conducting antimicrobial screening (6). The BacTiter-Glo™ Assay has Z´-factor values of 0.90 and 0.87 for 96-well and 384-well formats, respectively (Figure 4).

The BacTiter-Glo™ Assay is compatible with typical microbial growth media and commonly used organic solvents (Figure 5). In general, we recommend cation-adjusted Mueller Hinton Broth (MH II Broth, BD Cat.# 297963). It supports growth for most commonly encountered aerobic and facultative anaerobic bacteria and has been selected for use in food testing and antimicrobial susceptibility testing by the Food and Drug Administration (FDA) and the National Committee for Clinical Laboratory Standards (NCCLS) (6,7). In addition, this medium has low luminescence background and good batch-to-batch reproducibility. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, had little effect on luminescent signal when tested at a final concentration of 3%.

Screen Antimicrobial Compounds

We used the BacTiter-Glo™ Assay to screen a panel from the Library of Pharmacologically Active Compounds from Sigma (LOPAC, #8, enzyme inhibitors, total of 80 compounds) for antimicrobial activity against *Staphylococcus aureus*. The



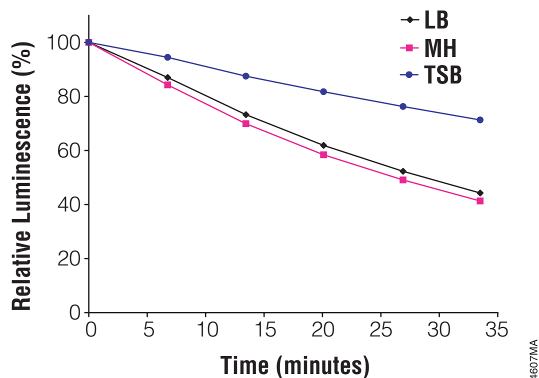


Figure 3. BacTiter-Glo™ Assay generates a glow-type luminescent signal. Bacterial cells were grown and assayed as described in Figure 2. Approximately 10^6 cells were used in each assay. The stability of the luminescent signal was monitored over time. Luminescence was recorded on a Veritas™ Microplate Luminometer (Cat.# E6501). **Panel A.** Four different bacteria: *E. coli, S. aureus, P. aeruginosa* and *B. cereus,* were grown in Mueller Hinton II Broth and assayed. **Panel B.** *E. coli* cells were grown in three media: Luria-Bertani (LB), Mueller Hinton II Broth (MH) and Trypticase Soy Broth (TSB), and assayed.

results are shown in Figure 6. 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. Their anti-*S. aureus* activities were reported in the literature (8–10).

Evaluate Antimicrobial Compounds

We further examined the dosage effects of standard antibiotics and three LOPAC hits on *S. aureus* using the BacTiter-Glo™ Assay and compared with minimal inhibitory concentration (MIC) values determined by following the NCCLS protocol (7). All tested samples showed anti-*S. aureus* activity in a dosage dependent fashion (Figure 7). The anti-*S. aureus* activities measured by BacTiter-Glo™ Assay not only correlated well with the MIC values but also were more quantitative.

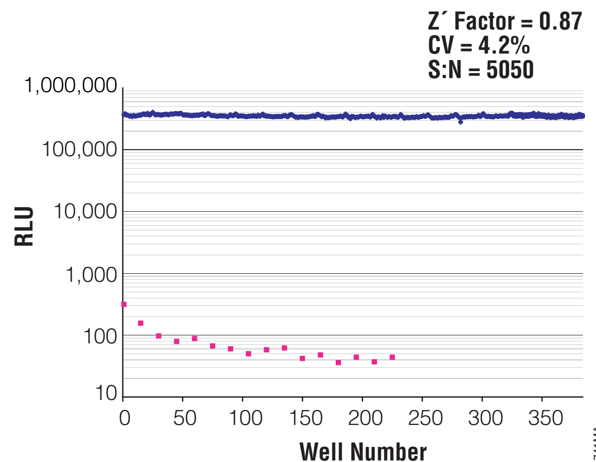
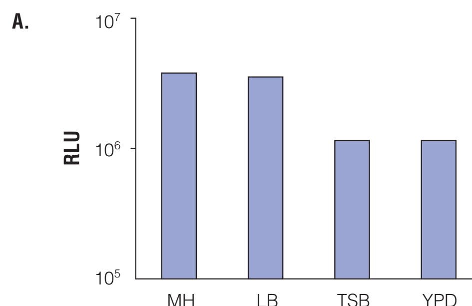


Figure 4. Scattergram of BacTiter-Glo™ Assay performed in a 384-well plate. *E. coli* (ATCC25922) was grown in Mueller Hinton II (MH II) broth at 37ºC overnight. The overnight culture was diluted 100-fold in fresh MH II Broth and grown to log phase. The sample was diluted 1:10, and 25µl was dispensed in a 384-well plate using the Beckman Coulter Biomek® FX Workstation. Reagent was added to the plate in equal volume to the sample, and luminescence was measured using the PHERAstar from BMG Laboratories at 0.5 seconds per well. Wells contained either 1 × 10^6 *E. coli* cells (blue) or no cells (pink) for measuring background luminescence.



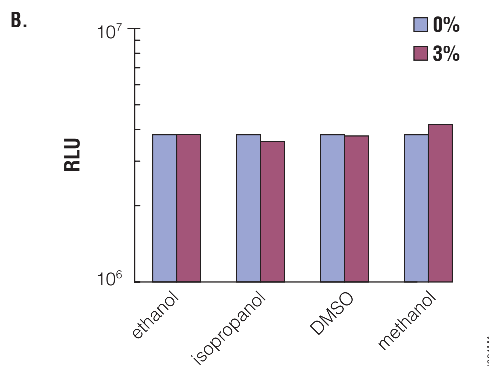


Figure 5. BacTiter-Glo™ Assay is compatible with typical media and solvents. Approximately 1 × 10^{-12} mole of ATP was used in each assay. Luminescence was recorded on a Veritas™ Microplate Luminometer (Cat.# E6501). **Panel A.** Luminescence was measured from samples in four different media: Mueller Hinton II Broth (MH), Luria-Bertani (LB), Trypticase Soy Broth (TSB) or Yeast Peptone Dextrose (YPD). **Panel B.** Luminescence was measured from samples of MH II medium with either 0% or 3% solvent.

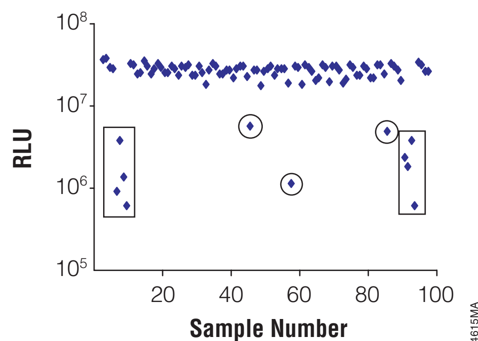


Figure 6. Screening for antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC25923 strain was grown in Mueller Hinton II (MH II) Broth at 37°C overnight. The overnight culture was diluted 100-fold in fresh MH II Broth and used as inoculum for the antimicrobial screen. Working stocks (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 Technical Bulletin #TB337. Luminescence was measured using a Veritas™ 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.

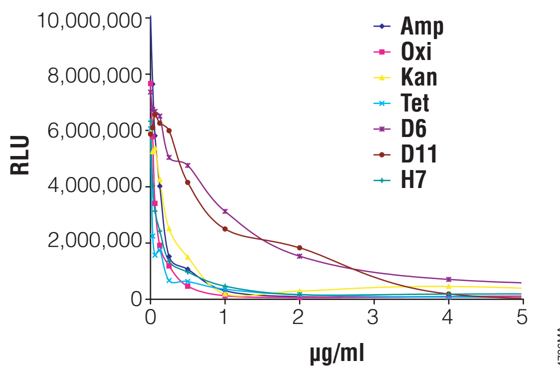


Figure 7. Evaluating antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain and antibiotics were prepared as described in Figure 6 and incubated at 37°C; the assay was performed after 19 hours of incubation as recommended for MIC determination by NCCLS (7). Luminescence was recorded on a Veritas™ Microplate Luminometer (Cat. E6501).

Conclusions

The new BacTiter-Glo™ Microbial Cell Viability Assay is a simple yet highly sensitive method for quantitating viable microbial cells. The assay uses a single reagent to release and measure ATP from microbial cells. It is the only homogeneous single-step assay of its kind. The "add-mix-measure" format and stable luminescent signal of this assay make it an ideal choice for automation and high-throughput applications such as antimicrobial drug screening. ■

References

1. DeLuca, M.A. and McElroy, W.D. (1978) *Meth. Enzymol.* **57**, 3–15.
2. McElroy, W.D. and DeLuca, M.A. (1983) *J. Applied Biochem.* **5**, 197–209.
3. Lundin, A. and Thore, A. (1975) *Anal. Biochem.* **66**, 47–63.
4. Hall, M.P. *et al.* (1998) Stabilization of firefly luciferase using directed evolution. In: *Bioluminescence and Chemiluminescence, Perspectives for the 21st Century*. Roda, A. *et al.* (eds) New York: John Wiley & Sons. 392–5.
5. Zhang, J-H., Chung, T.D.Y. and Oldenburg, K.F. (1999) *J. Biomol. Screen.* **4**, 67–73.
6. National Committee for Clinical Laboratory Standards. (2000) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard-Fifth edition* M7-A5. National Committee for Clinical Laboratory Standards, Wayne, PA.
7. Association of Official Analytical Chemists. (1995) *Bacteriological Analytical Manual*, 8th ed. AOAC International, Gaithersburg, MD.
8. Hatano, T. *et al.* (1999) *Chem. Pharm. Bull.* **47**, 1121–7.
9. Godowski, K.C. *et al.* (1995) *J. Periodontol.* **66**, 870–7.
10. Radd, I. *et al.* (2003) *Antimicrobial Agents and Chemotherapy* **47**, 3580–5.

Protocol

BacTiter-Glo™ Microbial Cell Viability Assay Technical Bulletin #TB337
(www.promega.com/tbs/tb337/tb337.html)

Ordering Information

Product	Size	Cat.#
BacTiter-Glo™ Microbial Cell Viability Assay*	10ml	G8230
	10 × 10ml	G8231
	100ml	G8232
	10 × 100ml	G8233
Veritas™ Microplate Luminometer	1 each	E6501

*For Laboratory Use.

(a)U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents are pending.

(b)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

BacTiter-Glo and Ultra-Glo are trademarks of Promega Corporation.

Biomek is a registered trademark of Beckman Coulter Instruments, Inc. Veritas is trademark of Turner BioSystems, Inc.

MICROBIAL VIABILITY