Application of the BacTiter-Glo™ Assay for Rapid Enumeration and Screening of Antimicrobial Compounds for *Mycobacterium avium* Complex Bacteria

ABSTRACT | Experiments were performed to determine if the BacTiter-Glo™ Microbial Cell Viability Assay could be used as a rapid method to count *Mycobacterium avium* complex (MAC) organisms. Using the BacTiter-Glo™ Assay, the amount of ATP measured was linear ($r^2 < 0.99$) with respect to MAC cell number between 10^7 and 10^3 cells; the limit of detection was 10^4–10^3 cells per 100 µl. Freezing cell suspensions at –80 °C prior to the assay significantly increased the luminescent signal in the assay. Exposure to antimicrobial compounds azithromycin and chlorine resulted in a significant decrease in both viable cell numbers and ATP levels.

Alice Yuroff[1], Frank Fan[2], Braeden Butler[2] and Michael Collins[1], [1]Department of Pathobiological Sciences, University of Wisconsin-Madison, [2]Promega Corporation

INTRODUCTION

The *Mycobacterium avium* complex (MAC) organisms are environmental, opportunistic pathogens of both humans and animals. Human infections with these mycobacteria commonly present as lymphadenitis in children, respiratory infections in adults and disseminated disease in immune-compromised patients (1). MAC bacteria are acid-fast, slow-growing, rod-shaped bacteria with complex, lipid-rich cell walls. Because of their slow growth rate and tendency to form clumps, traditional plate count and densitometry methods are not always effective or efficient ways to enumerate MAC organisms (2). Other methods have been developed to enumerate MAC organisms in samples, including the BACTEC™ 460 radiometric liquid culture system (2) and the Mycobacterial Growth Indicator Tube System (MGIT™ System; BD Biosciences; 3). However, these methods require several weeks to generate estimates of viable cell numbers in a sample.

The primary advantage of using the BacTiter-Glo™ Assay with mycobacteria is that data can be generated within minutes, as opposed to weeks with mycobacterial culture-based methods.

The BacTiter-Glo™ Microbial Cell Viability Assay[(a,b)] is a quantitative ATP assay for bacteria. The assay contains a proprietary cell lysis buffer and chemically stable luciferase and luciferin reagents. The reagent is added to cell samples in a microplate, and the resulting relative light units (RLU) are measured on a luminometer. In the assay, ATP standards may be included to allow conversion of RLU to amount of ATP. This assay has been used with a variety of Gram-negative and Gram-positive bacteria to quantify viable cell numbers and can be used to screen for antimicrobial compounds (4,5). However, the BacTiter-Glo™ Assay has not been validated for mycobacteria quantitation. The primary advantage of using the BacTiter-Glo™ Assay with mycobacteria is that data can be generated within minutes, as opposed to weeks with mycobacterial culture-based methods. Given the thick, lipid-rich cell wall of mycobacteria, the goal of these experiments was to determine if the BacTiter-Glo™ Assay buffer effectively lysed MAC cells to release ATP and whether additional cell lysis steps would be required.

INVESTIGATING LYSIS METHODS

Initial experiments investigated the effect of buffers, boiling and freezing of MAC cells to potentially enhance cell lysis and the resulting luminescent signal. Two MAC strains, *Mycobacterium avium ssp. avium* (*M. avium*) ATCC 35712 and *Mycobacterium avium ssp. paratuberculosis* (*M. paratuberculosis*) ATCC 19698, were used in these trials. For both MAC strains, the luminescent signals generated in the BacTiter-Glo™ Assay were significantly greater than background signals. For example, in one assay the no-cell control gave a luminescent signal of $2.3 \pm 0.2 \times 10^3$ relative light units (RLU). For samples containing MAC cells, approximately 10^7 *M. avium* and *M. paratuberculosis* cells gave a luminescent signal of $1.2 \pm 0.1 \times 10^7$ and $6.0 \pm 0.5 \times 10^6$ RLU, respectively (Figure 1), indicating that the BacTiter-Glo™ Assay reagent released ATP from the MAC cells without additional lysis.

Standard culture medium for MAC bacteria (Middlebrook 7H9) did not interfere with the BacTiter-Glo™ Assay; luminescent signals were comparable between cells suspended in 7H9 culture medium and Phosphate Buffered Saline (PBS; data not shown). However, cells resuspended in a Tris-EDTA buffer (100 mM Tris, 2 mM EDTA [pH 7.75]) had significantly decreased luminescent signal compared to cells resuspended in PBS for both *M. avium* and *M. paratuberculosis* (data not shown).

Boiling *M. avium* cells resuspended in PBS for 1 minute significantly decreased the luminescent signal compared to the signal from untreated cells (Figure 1). Freezing the cells at –80 °C and thawing them just prior to the assay increased the

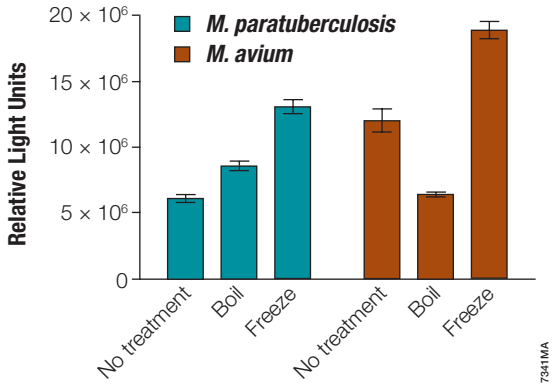

Figure 1. Effect of boiling and freezing MAC cells on BacTiter-Glo™ Assay luminescence signal. Log phase cultures of two MAC strains (*M. avium* ATCC 35712 and *M. paratuberculosis* ATCC 19698) were washed and resuspended in PBS. Approximately 10^7 resuspended cells were used directly in the BacTiter-Glo™ Assay ("no treatment"), boiled for 2 minutes and immediately frozen at –80 °C ("boil") or frozen at –80 °C for 30 minutes ("freeze"). Frozen samples were thawed at room temperature just prior to the assay. The BacTiter-Glo™ Assay was performed according to the instructions in the *BacTiter-Glo™ Assay Technical Bulletin*, #TB337. Luminescence (Relative Light Units) was measured on a Veritas™ microplate luminometer. Data are the mean ± standard deviation of triplicate samples.

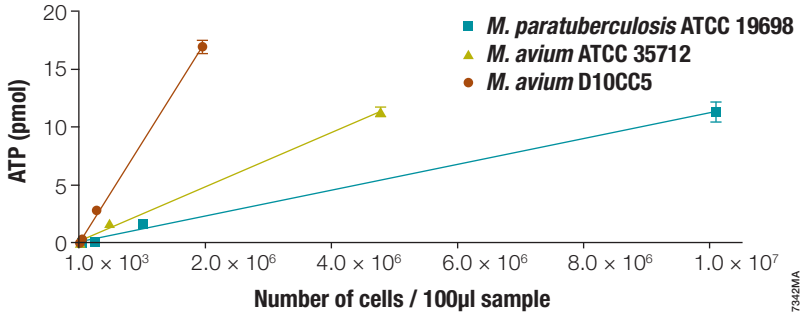

Figure 2. Measured ATP amounts were linear with respect to MAC bacteria cell numbers. Log-phase cultures of three MAC strains (*M. avium* ATCC 35712, *M. paratuberculosis* ATCC 19698, and *M. avium* D10CC5) were washed, resuspended and serially diluted in PBS buffer. Diluted cell suspensions were frozen at –80 °C until the assay. The number of cells in each dilution was determined using the MGIT™ System (3). Samples contained approximately 10^3 to 10^6 cells per 100 µl sample for *M. avium* and 10^3 to 10^7 cells per 100 µl sample for *M. paratuberculosis*. Relative light units were converted to amount of ATP using a standard curve as recommended in the *BacTiter-Glo™ Assay Technical Bulletin*, #TB337. Data are the mean ± standard deviation of 6 replicates.

luminescent signal compared to the signal from untreated cells (Figure 1). Boiling *M. paratuberculosis* slightly increased the luminescent signal compared to the signal from the untreated cells (Figure 1). Similar to *M. avium* results, freezing *M. paratuberculosis* samples at –80 °C resulted in an increased luminescent signal (Figure 1). Based on these results, all subsequent samples were prepared in PBS, frozen at –80 °C (for at least 30 minutes and up to 3 weeks) and thawed at room temperature just prior to performing the BacTiter-Glo™ Assay.

CORRELATION BETWEEN MAC CELL NUMBER AND LUMINESCENT SIGNAL

To establish if there was a relationship between the BacTiter-Glo™ Assay luminescent signal and the number of MAC cells, approximately 10^6 MAC cells were resuspended in PBS and serially diluted to 10 cells per sample. Both plate count and MGIT™ System methods were used to determine the number of cells in each dilution (3). The BacTiter-Glo™ Assay was done using 100 µl samples of each dilution. This experiment was repeated with three different MAC strains, *M. paratuberculosis* ATCC 19698, *M. avium* ATCC 35712, and *M. avium* D10CC5 (an environmental isolate provided to this project by the USEPA culture collection). For the three strains, the amount of ATP measured was linear ($r^2 < 0.99$) with respect to cell number between 10^3 and 10^6 cells for the two *M. avium* strains and between 10^3 and 10^7 cells for *M. paratuberculosis* (Figure 2). The limit of detection was between 10^3 and 10^4 cells/100 µl sample for the three MAC strains, which was significantly higher than the limit of detection reported for other bacterial species (less than 100 cells/sample; 4). The reduced sensitivity for MAC bacteria as compared to other species was most likely due to incomplete lysis of the MAC cells in the BacTiter-Glo™ Assay buffer.

For the three MAC strains tested, the linear regression of ATP amount vs. cell number resulted in significantly different equations. These results suggest different standard curves should be established for different MAC strains in order to estimate the number of viable cells from the measured amount of ATP.

EFFECT OF ANTIMICROBIALS ON ATP LEVELS

Experiments were done to determine if the BacTiter-Glo™ Assay could be used to monitor the effects of two antibacterial agents, azithromycin and chlorine, on the viability of MAC bacteria. Azithromycin affects mycobacterial survival by binding the 50S ribosomal subunit, thus interfering with protein synthesis. For the first experiment, *M. paratuberculosis* cells were exposed to increasing concentrations of the antibiotic azithromycin (1–16 µg/ml) for three days. After incubation, the number of viable cells in the samples was determined by the MGIT™ System (3), and ATP was quantified using the BacTiter-Glo™ Assay. Both the number of viable cells and ATP levels significantly decreased with increasing azithromycin concentrations. Treatment with 16 µg/ml azithromycin resulted in a 98% decrease in the number of viable *M. paratuberculosis* cells as compared with the untreated control (Figure 3, Panel A). Similarly, the ATP levels decreased by 88% with exposure to 16 µg/ml azithromycin (Figure 3, Panel A).

In a second experiment, M. *paratuberculosis* cells were exposed to the common disinfectant sodium hypochlorite (NaOCl). After a 30-minute exposure to NaOCl, the number of viable M. *paratuberculosis* cells decreased significantly with exposure to increasing amounts of free chlorine (Figure 3, Panel B). As with exposure to azithromycin, ATP levels correlated well with the measured number of viable cells after exposure to chlorine, and ATP levels were significantly decreased compared to untreated controls (Figure 3, Panel B).

Taken together, these experiments suggest the BacTiter-Glo™ Assay can be used as a rapid screening tool for antimicrobial compounds for MAC organisms as has been shown for other bacterial species (5).

Taken together, these experiments suggest the BacTiter-Glo™ Assay can be used as a rapid screening tool for antimicrobial compounds for MAC organisms as has been shown for other bacterial species (5).

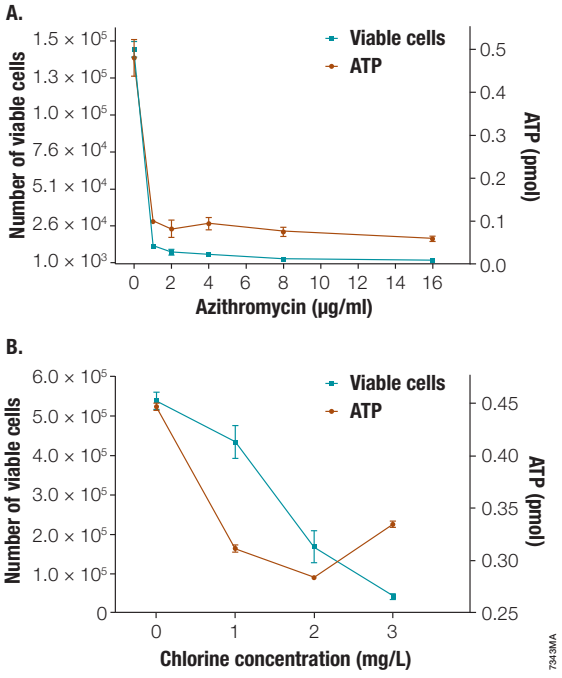

Figure 3. Measured ATP amounts correlate to viable *M. paratuberculosis* cell numbers after exposure to antibiotics and decontaminants. Panel A. Log-phase culture (100 µl) of *M. paratuberculosis* ATCC 19698 was added to 900 µl fresh Middlebrook 7H9 media. Cells were treated with 50 µl azithromycin stock (in ethanol) and incubated for 3 days at 37 °C. After incubation, cells were collected and washed once in PBS to remove residual antibiotic. Viable cell numbers were determined by the MGIT™ System counting method (3). Aliquots of cell suspensions for the BacTiter-Glo™ Assay were stored at –80 °C until the time of the assay. Relative light units were converted to amount of ATP using a standard curve as recommended in the *BacTiter-Glo™ Microbial Cell Viability Assay Technical Bulletin*, #TB337. Data are the mean ± standard deviation of 4 samples. **Panel B.** Log-phase culture of *M. paratuberculosis* ATCC 19698 was washed and resuspended in PBS. Cells were treated with NaOCl to give initial chlorine concentrations of 0, 1, 2 and 3 mg/L. Treated cells were incubated at room temperature for 30 minutes, then chlorine was neutralized with 0.1% sodium thiosulfate. Viable cell numbers were determined by the the MGIT™ System counting method. Aliquots of cell suspensions for the BacTiter-Glo™ Assay were stored at –80 °C until the time of the assay. Relative light units were converted to amount of ATP using a standard curve as for Panel A. Data are the mean ± standard deviation of triplicate samples.

CONCLUSION

These experiments demonstrate the BacTiter-Glo™ Assay provides a quantitative, rapid tool for estimating viable cell numbers for MAC mycobacteria. Despite the fact that MAC bacteria have a higher limit of detection than other bacterial species, the BacTiter-Glo™ Assay provides good dynamic range for the mycobacteria tested, allowing detection and quantification between 10^3 to 10^7 cells per 100 µl sample. One freeze-thaw cycle significantly increased the luminescent signal, conveniently allowing for storage for samples at –80 °C until the time of the assay. Preliminary experiments presented here also suggest that the BacTiter-Glo™ Assay is a useful tool for testing the effects of antimicrobial agents on the viability of MAC bacteria.

REFERENCES

1. Inderlied, C.B. *et al.* (1993) *Clin. Microbiol. Rev.* **6**, 266–310.
2. Lambrecht, R.S. *et al.* (1988) *Appl. Environ. Microbiol.* **54**, 910–16.
3. Shin, S.J. *et al.* (2007). *J. Clin. Microbiol.* **45**, 1941–8.
4. Fan, F. *et al.* (2004) *Promega Notes* **88**, 2–4.
5. Fan, F. *et al.* (2005) *Promega Notes* **89,** 25–7.

ORDERING INFORMATION

Product	Size	Cat.#
BacTiter-Glo™ Microbial Cell Viability Assay	10 ml	G8230
	10 × 10 ml	G8231
	100 ml	G8232
	10 × 100 ml	G8233

For Laboratory Use.

[a]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312, 785294 and other patents and patents 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.