



Use of the BacTiter-Glo™ Microbial Cell Viability Assay to Study Bacterial Attachment in Biofilm Formation

ABSTRACT Biofilms are complex, sessile communities of bacteria that form on solid surfaces or at air-liquid interfaces. The formation of such biofilms follows a path of controlled steps, the first being attachment to the surface. To rigorously study the physiological changes that allow the bacteria to perform this first step, a quantitative assay that accurately determines the biomass of surface-attached bacteria is required. Here, we describe the use of the BacTiter-Glo™ Microbial Cell Viability Assay to measure biofilm attachment. This 96-well-format assay monitors intracellular ATP concentrations. Since bacterial cells maintain a relatively constant intracellular ATP concentration, this assay provides a reproducible relative measure of attached cells.

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INTRODUCTION

Biofilms, sessile communities of bacteria that form on surfaces, develop in an ordered series of steps (for a review, see reference 1). Each step is characterized by the involvement of a distinct surface organelle. Flagella mediate reversible attachment, type 1 pili mediate irreversible attachment, and capsules allow the formation of the mature biofilm. The synthesis of each organelle requires the coordinated expression of numerous genes. In *Escherichia coli*, the *flg*, *flh*, and *fli* genes, organized as four loci, encode flagella, the *fim* locus encodes type 1 pili, and the *cps* locus encodes an extracellular polysaccharide capsule called colanic acid. Such coordinated expression of genes depends upon the interplay of many regulators (for a review, see reference 2). Part of the network of transcriptional regulation is OmpR, a two-component response regulator that negatively regulates flagella (3) and type 1 pili (4).

Genetic studies that investigate the underlying physiology of biofilm formation not only require microscopy techniques for visualization but also a quantitative assay that determines the amount of cells within the biofilm. The conventional crystal violet assay detects polysaccharides in the extracellular matrix and does not distinguish between living and dead cells (5). To study the early stages of biofilm formation, we need an assay that strictly reports on the attachment of viable cells. Since cells maintain a relatively constant concentration of ATP (6), this concentration has been used to estimate the amount of bacterial biomass in drinking water (7–9). Here, we describe the use of the BacTiter-Glo™ Assay^(a,b) to detect ATP and determine the biomass of attached bacteria. We present calibration experiments that can be used to convert bioluminescence to either ATP concentration or bacterial cell number. Using this assay, we present a time course for attachment of viable cells to a plas-

tic surface. Finally, we demonstrate the use of the assay with mutants defective for the biogenesis of either flagella (*fliA*) or pili (*fimH*) or defective for a two-component response regulator (*ompR*) that controls the biosynthesis of flagella and pili. The quantitative results are compared to scanning electron micrographs.

PERFORMANCE OF THE ATP ASSAY

The general protocol for the determination of ATP and biomass via the BacTiter-Glo™ Assay is detailed in the *BacTiter-Glo™ Microbial Cell Viability Assay Technical Bulletin #TB337*. Table 1 describes the modified protocol for measuring attached bacteria in a 96-well-plate format. We use one row of 12 wells for each bacterial strain; thus, we can process up to eight bacterial strains with one plate (Step 2). All wells are incubated, washed with PBS, and air-dried together (Steps 3–7). To obtain reproducible results, it is crucial that cells be incubated with the BacTiter-Glo™ Reagent for the same duration (Steps 8–11). Because our luminometer, a TD 20/20 (Turner BioSystems), detects light in a single-sample tube, we performed this incubation one row of 12 wells at a time. However, if a plate reader capable of measuring bioluminescence is available, the entire 96-well plate can be incubated simultaneously with the BacTiter-Glo™ Reagent.

ATP CALIBRATION

Relative Light Units (RLU) that result from the BacTiter-Glo™ Assay can be converted to ATP concentration using an ATP calibration curve. To obtain this curve, an ATP solution of 10 μmol/ml (Cat.# P1132) was serially diluted 1:5 eight times. A maximum of 100 nmol was used for the individual reaction in a total of 100 μl of PBS. Each sample was mixed with 100 μl of BacTiter-Glo™ Reagent and incubated for 5 minutes before biolu-

ATP concentration, measured using the BacTiter-Glo™ Assay, can quantitatively determine the biomass of attached bacteria.

Table 1. Protocol for the Quantification of Biomass in Attached Bacteria Using the BacTiter-Glo™ Assay.	
Prepare the BacTiter-Glo™ Reagent	
Prepare the BacTiter-Glo™ Reagent as described in the <i>BacTiter-Glo™ Microbial Cell Viability Assay Technical Bulletin #TB337</i> .	
<ul style="list-style-type: none"> • Thaw one vial each of BacTiter-Glo™ Substrate and BacTiter-Glo™ Buffer. • Combine the two solutions and mix well. • Divide the mixed solution into aliquots if necessary (reagent is sufficient for 100 reactions). • Freeze unused portion of reagent before experiment. 	
Quantify Biomass	
1.	Pipette 100 µl of tryptone broth (1% tryptone, 0.5% NaCl) into each well of a 96-well plate.
2.	Pipette 1 µl of an overnight culture (one bacterial strain) into each well of one row (12 wells) of a 96-well plate. Repeat this step with the remaining bacterial strains until plate is full (maximum of eight strains).
3.	Incubate the 96-well plate at 37 °C for the desired time without shaking.
4.	Remove liquid on top of the attached bacteria.
5.	Carefully rinse attached bacteria with 100 µl of PBS.
6.	Remove excess PBS.
7.	Air-dry attached bacteria for about 15 minutes.
8.	Add 100 µl of PBS to each each well of one row (12 wells) of a 96-well plate.
9.	Add 100 µl of BacTiter-Glo™ Reagent to the same 12 wells and mix well.
10.	Incubate for 5 minutes at room temperature.
11.	Measure the relative light units (RLU) in a luminometer.
12.	Repeat Steps 8–11 for each row until the whole plate is processed.

luminescence was detected. RLU was plotted versus the amount of ATP (Figure 1, Panel A). From this standard curve, we obtained the following equation: $y = 506.4x + 20.996$, where x represents ATP in nmol, and y represents the luminescence in RLU. Using this equation, we converted luminescence to ATP.

BACTERIAL CELL NUMBER CALIBRATION

Similarly, RLU that result from the BacTiter-Glo™ Assay can be converted to bacterial cell numbers with a bacterial calibration curve. To obtain this curve, a bacterial suspension of an *E. coli* wild-type strain that had been grown for 38 hours without aeration in tryptone broth (TB; 1% tryptone, 0.5% NaCl) was serially diluted 1:2 for a total of nine dilutions. Luminescence (RLU/ml) was determined for each of these dilutions, and then each dilution was further diluted 1:10 in series (up to three times) and plated onto LB-agar plates to determine the number of colony forming units (CFU/ml). RLU/ml was plotted versus CFU/ml (Figure 1, Panel B). From this standard curve, we obtained the following equation: $y = 0.0012x + 20984$, where x represents bacterial cell number in CFU/ml and y represents luminescence in RLU/ml. Using this equation, we converted luminescence to bacterial cell number.

TIME COURSE OF ATTACHMENT

The time course of attachment was performed following the protocol in Table 1. Wild-type cells were grown in TB at 37 °C without shaking for 72 hours. At regular intervals, one row of 12 wells was assayed as described

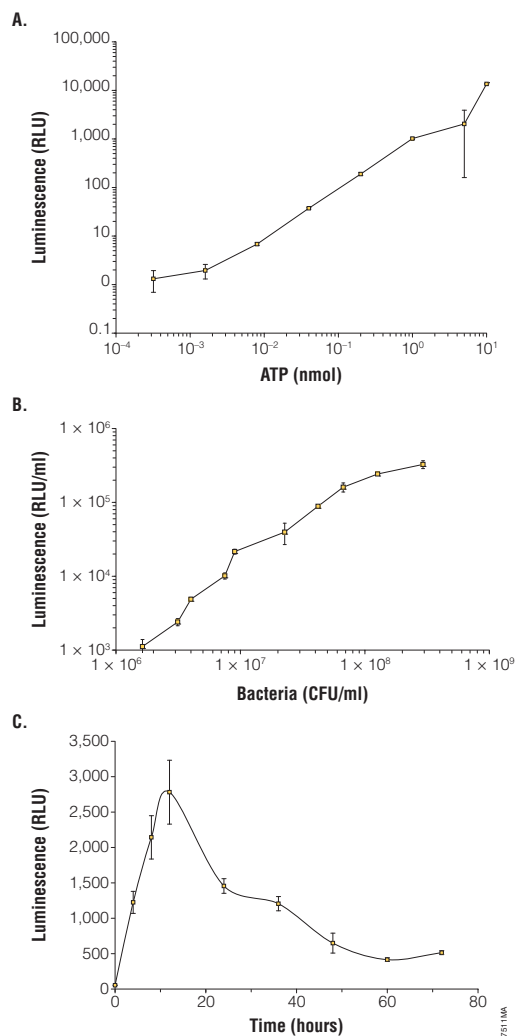


Figure 1. Characterization of the BacTiter-Glo™ Assay. Panel A illustrates the ATP standard curve between 10⁻³ and 10¹ nmol ATP per reaction. Panel B represents the bacterial dilution curve between 2 × 10⁶ CFU/ml and 3 × 10⁸ CFU/ml. Panel C shows attachment of wild-type cells grown in TB at 37 °C without shaking for 72 hours. For each panel, the values represent the mean ± standard deviation of three independent experiments.

in Table 1. The biomass of attached bacteria increased steadily, peaking at 12 hours, after which it first declined rapidly and then more slowly. Because the change in the number of attached bacteria per hour was reproducibly smaller from 22 to 38 hours than during the earlier stages of biofilm formation, end-point analyses were performed after 38 hours of incubation.

APPLICATION OF THE ASSAY

To test the BacTiter-Glo™ Assay for its ability to quantify attachment, we compared the attached biomass of a set of mutant bacteria to that of their isogenic wild-type parent (Figure 2). To verify the quantification, we also subjected each strain to scanning electron microscopy. Each mutant lacked a surface organelle, a critical component of an organelle, or a regulator of

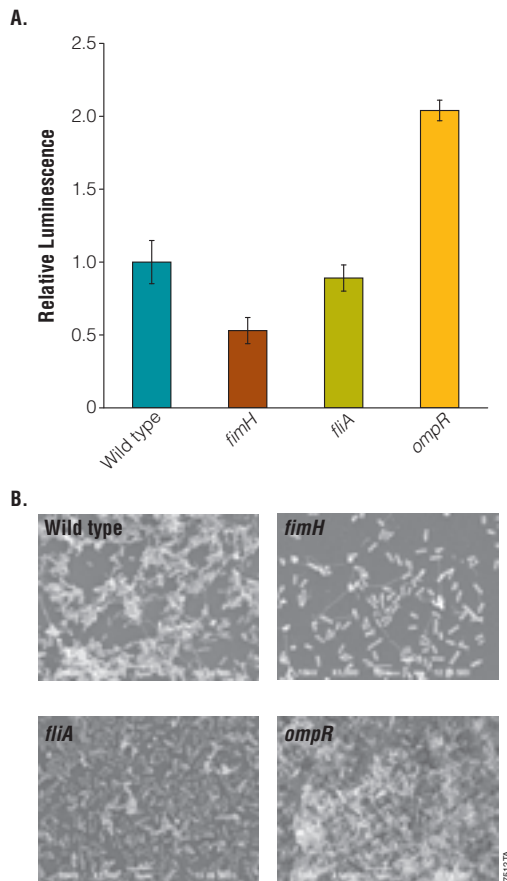


Figure 2. Comparison of attachment between different strains of *E. coli*. Cells were grown in TB at 37 °C for 38 hours without shaking. **Panel A** shows the relative luminescence (RLU) for the strains using the wild-type strain as a reference. **Panel B** contains scanning electron micrographs for the same strains taken with a JSM-6490 V scanning microscope from JEOL USA (Peabody, MA) under conditions that were identical to the quantitative biofilm experiment (Table 1).

those surface organelles. The *fliA* mutant cannot synthesize σ_{28} , the flagellar-specific sigma factor (10), and thus lacks flagella. The *fimH* mutant lacks the adhesion subunit of the type 1 pilus that mediates autoaggregation (11). *OmpR* inhibits expression of genes required for the biogenesis of flagella (3) and type 1 pili (4), and thus, the *ompR* mutant overexpresses both organelles.

The BacTiter-Glo™ Assay (Figure 2, Panel A) and scanning electron microscopy (Figure 2, Panel B) yielded similar results: the *fliA* mutants attached to surfaces similar to their wild-type parent, while the *fimH* mutants attached more poorly, and the *ompR* mutants attached better. The combination of both a quantitative assay and a visual assay provided details that each assay alone could not provide. The BacTiter-Glo™ Assay provided statistically significant differences in attachment by *fimH* and *ompR* mutants, while the electron micrographs showed clear differences in morphology. For example, although the *fliA* mutant attached to the plastic surface with approximately the same biomass as its wild-type parent, it did

so with a much less complex morphology. Furthermore, the electron micrographs show a complex array of surface structures that almost certainly facilitate attachment.

CONCLUSION

In summary, our data indicate that the BacTiter-Glo™ Assay can be used to quantify attached biomass. Therefore, we consider it to be a suitable assay to study early stages of biofilm formation. The method requires only simple analytical equipment and can be performed rapidly. The assay can be performed entirely in a 96-well format, depending on available instruments.

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PROTOCOL

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

ORDERING INFORMATION

Product	Size	Cat.#
BacTiter-Glo™ Microbial Cell Viability Assay*	10 ml	G8230
GloMax® 20/20 Luminometer**	1 each	E5311
GloMax® 96 Microplate Luminometer**	1 each	E6501

*For Laboratory Use. Also available in larger sizes.

**Available with single or dual injectors.

©U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312 and 785294 and other patents and patents pending.

©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.

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The BacTiter-Glo™ Assay and scanning electron microscopy complement each other when examining biomass and attachment of organelle mutants.