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.

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 flh, flh, and flt genes, organized as four loci, encode flagella, the flm 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 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 plastic 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-
minescence 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 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...
those surface organelles. The fliA mutant cannot synthesize σ28, 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.

**REFERENCES**


**PROTOCOL**

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

**ORDERING INFORMATION**

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†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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