Detection of living *Salmonella* cells using bioluminescence

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Abstract

ATP-based bioluminescence using mutant firefly luciferase was combined with an immunochromatographic lateral flow test strip assay for *Salmonella enteritidis* detection. In this combination method, the Salmonella-antibody-gold complex captured at the test line on the test strip was lysed by heat-treatment, and the ATP released from the cells was measured using mutant luciferase. This method resulted in approximately 1000 times higher sensitivity in the detection of Salmonella cells compared to immunochromatographic lateral flow assay.

Introduction

Demands for a rapid bacterial inspection method have increased because of higher production safety requirements in various industries, such as food, cosmetics, and pharmaceuticals, as well as because of increased use of point-of-care tests in clinical diagnoses. Immunochromatographic lateral flow assays are popular diagnostic tools for bacteria, such as *Salmonella* spp., *Listeria* spp., and *Escherichia coli*. However, this assay has a detection limit of approximately 10^6–10^8 cells/ml, which is insufficient for some industrial and clinical applications. Therefore, improvement in immunochromatographic lateral flow assay sensitivity is required.

The use of firefly luciferase to detect ATP is an established technique for monitoring microorganism growth (Bautista et al. 1994; Brovko et al. 1999). Previously, we constructed a genetically modified *Photinus pyralis* luciferase that generates 10-fold or higher luminescent intensity than the wild-type enzyme (Noda et al. 2008). In this study, we report that ATP-based bioluminescence combined with an immunochromatographic lateral flow test provides high sensitivity in the detection of living Salmonella cells.

Materials and methods

Detection of bacteria cells

Salmonella enteritidis NBRC3313 and *Escherichia coli* JM109 were precultured in 3 ml of tryptone-yeast extract medium (2 × TY) at 37°C for 18–24 h. After cultivation, the cells were harvested by centrifugation (12 000 × *g*, 5 min, 20°C) from 1 ml of culture. In addition, they were resuspended in 1 ml of phosphate buffered saline containing 1 mg bovine serum albumin/ml (PBS-BSA-buffer; Nacalai Tesque, Inc., Kyoto, Japan), and diluted to 10–10^8 cells/ml by the same buffer. The colony forming unit (c.f.u.) was determined by the number of colonies appearing on Luria Bertani (LB) agar medium after incubation at 37°C for 18–24 h. Immunochromatographic lateral flow assay was performed with DuPont™ Lateral Flow System™ Salmonella test kits (DuPont Qualicon, Wilmington, DE, USA), according to manufacturer's instruction. In brief, 400 µl of cell suspension was transferred to a tube. A test strip was inserted into the tube, incubated for 10 min at room temperature, transferred to a new tube containing 400 µl of PBS-BSA-buffer, and incubated at room temperature for further 10 min. Test strip dose responses were quantitatively determined by utilizing one of the two detection methods. For photometric measurement, an image of the test strip portion containing the produced color signal was captured with a scanner (ES-2200, Epson, Nagano, Japan) using the software provided by the manufacturer. The color within the fixed area of the image was converted to optical density using a computer program (Image J, National Institutes of Health, Bethesda, MD, USA).

For bioluminescence detection, a 4 × 4 mm region of the test strip around the test line

was removed and inserted in a microcentrifuge tube (Fig. 1). Non-bacterial ATP was reduced by the addition of 170 µl of ATP-deletion buffer (Kikkoman, Chiba, Japan). After incubation at room temperature for 30 min, the ATP-deletion buffer was removed and the test strip portion resuspended with 150 µl of PBS (Nacalai Tesque, Inc.). Cells captured at the test line were lysed by heat treatment at 100°C for 3 min. The sample was then transferred to a new microcentrifuge tube and quantification of intracellular ATP was performed by luciferase reaction.

ATP quantification by luciferase reaction

A novel mutant North American firefly (*Photinus pyralis*) luciferase that generates 10-fold or higher luminescence intensity than wild-type luciferase has enabled detection of ATP at 10^{-18} mol (Thore et al. 1975), approximately equal to the quantity contained in a single bacteria cell (Noda et al. 2008). This mutant luciferase was used for ATP-based bioluminescence production in this study. Luminescence intensity was measured in LumiTubes using Lumitester C-1000 (Kikkoman, Chiba, Japan), and was expressed as a relative light unit (RLU) for 5 s. The reaction was initiated by the addition of a 50 µl luciferase solution containing 100 µg mutant luciferase and 0.8 mM D-luciferin (Acros Organics, Geel, Belgium) in TMAT buffer containing ((20 mM Tricine (pH 7.8), 20 mg trehalose/ml, and 0.4 mM magnesium acetate). After mixing for 1 s, luminescence was measured. The signals represent the mean of three replicates for each measurement.

Results and discussion

Immunochromatographic lateral flow assay

Immunochromatographic lateral flow assay results, including Salmonella cell numbers are shown in Figure 2. Cell numbers of Salmonella were determined by plate counting of c.f.u. on LB agar. The limits of detection of the immunochromatographic lateral flow assay was determined for 1.0×10^6 c.f.u./ml. In the absence of Salmonella, the test line did not turn red. At the upstream control line, reagents that captured excess gold while passing through the test line caused the control line to turn red, indicating that the materials flowed correctly along the strip. Quantification of the colloidal gold captured in the immune complex was transformed to optical density values using scanning photometry (Nilsson et al., 1995; Laitinen and Vuento, 1996). The photometric signal measured was proportional to the color perceived by human eyes as well as to the number of Salmonella cells on the strip (Figs. 2 and 3). No color was perceived by the eye and no photometric signals were detected in an immunochromatographic lateral flow assay using anti-Salmonella antibodies applied to *E. coli* (data not shown).

Combination of ATP assays

Without the ATP assay, the immunochromatographic lateral flow assay Salmonella detection limit was 1.0×10^6 c.f.u./ml. After mutant luciferase based ATP assay of the test line portion of the test strip, the Salmonella cell detection limit was reduced to 1.0×10^3 c.f.u./ml. This level corresponds to approximately 400 Salmonella cells per

analyte, and is 1000 times more sensitive than using only the immunochromatographic lateral flow assay (Fig. 4). Furthermore, the detection limit of the immunochromatographic lateral flow and ATP combination assay using wild type luciferase was 5.0×10^4 c.f.u./ml cells (data not shown). Figure 4 shows the linear relationship between luminescent signal intensity and the number of Salmonella cells, a relationship valid over several orders of magnitude. Moreover, the bioluminescence intensity of the immunochromatographic lateral flow and ATP combination assay when applied to *E. coli* was below that of the negative control of Salmonella. Therefore, this combined assay showed specific detection of Salmonella cells.

Many rapid specific bacteria detection methods have been proposed to replace enumeration of plate colonies, which requires several days. An optical surface plasmon resonance sensor using gold surface immobilized with anti-salmonella antibody can detect Salmonella at 10^5 c.f.u./ml (Son et al. 2007), while an enzyme-linked immunosorbent assay (ELISA) is capable of detecting 10^4 c.f.u./ml of Salmonella (Durant et al. 1997), and. PCR-ELISA and LightCycler real-time PCR assays have a Salmonella detection limit of 10^3 c.f.u./ml (Prelle et al. 2004). However, these methods cannot fundamentally distinguish between live and dead cells. Therefore, Salmonella cells inactivated by previous sterilization processes cannot be detected by these methods, and may affect decisions on food production safety. On the other hand, because ATP exists only in live cells, an ATP-based method selectively detects living Salmonella cells that have the capacity to reproduce. An immunochromatographic lateral flow and ATP combination assay is particularly useful in the selective detection of live cells that may result in food poisoning.

Based on these results we suggest that a mutant firefly luciferase ATP assay is as effective as other high sensitivity immunological methods for detecting the presence of

S. enteritidis, especially living cells. Detection of bacterial contamination at levels as low as one c.f.u. in *E. coli* and *Bacillus subtilis* using mutant firefly luciferase has been achieved (Noda et al., 2008). Therefore, we suggest that, by improving the selectivity of the antibody, specific bacteria detection at the single living cell level is fundamentally possible.

Acknowledgments

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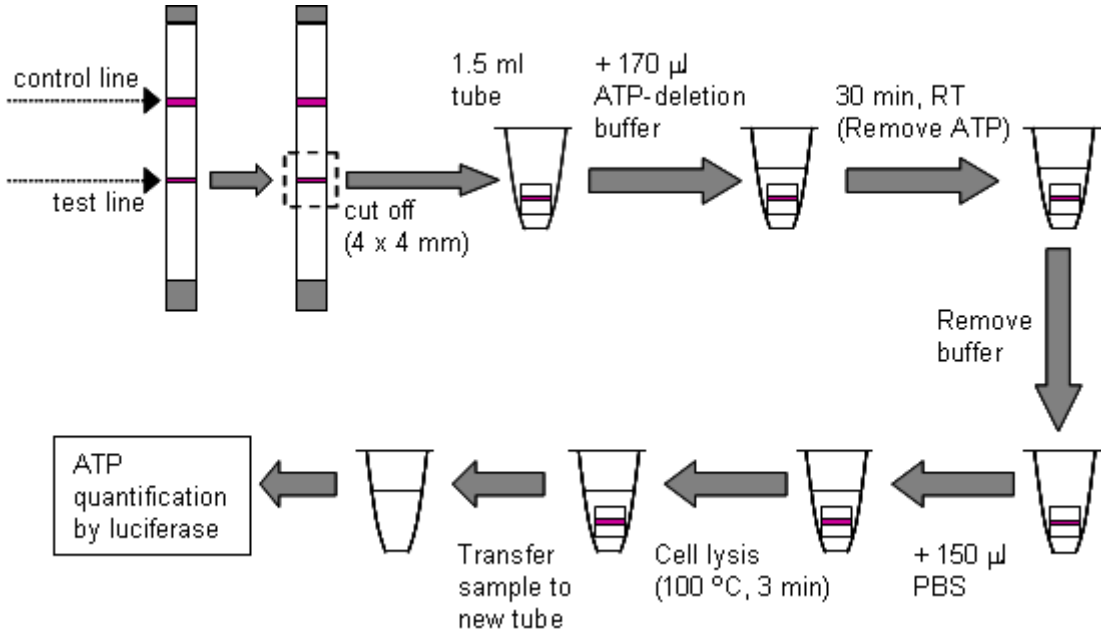
Figure Legends

Fig. 1 Steps in the ATP-bioluminescence assay following the immunochromatographic lateral flow assay. The region around the test line on the lateral flow assay test strip was removed and prepared for ATP quantification using the steps indicated.

Fig. 2 Immunochromatographic lateral flow assay for detection of *Salmonella enteritidis* NBRC3313. As a negative control, PBS-BSA-buffer that did not contain any Salmonella cells was used (Control). The areas enclosed in the broken lines are the locations of the test line on the assay test strip.

Fig. 3 Relationship between optical density and colony number in *Salmonella enteritidis* ($^{\Delta}$).

Fig. 4 Relationship between bioluminescence intensity and colony number in *Salmonella enteritidis* (●) and *Escherichia coli* (○).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test line
cut off
(4 x 4 mm)
1.5 ml
tube
+ 170 µl
ATP-deletion
buffer
30 min, RT
(Remove ATP)
Remove
buffer
+ 150 µl
PBS
Cell lysis
(100 °C, 3 min)
Transfer
sample to
new tube
ATP
quantification
by luciferase

Fig. 1

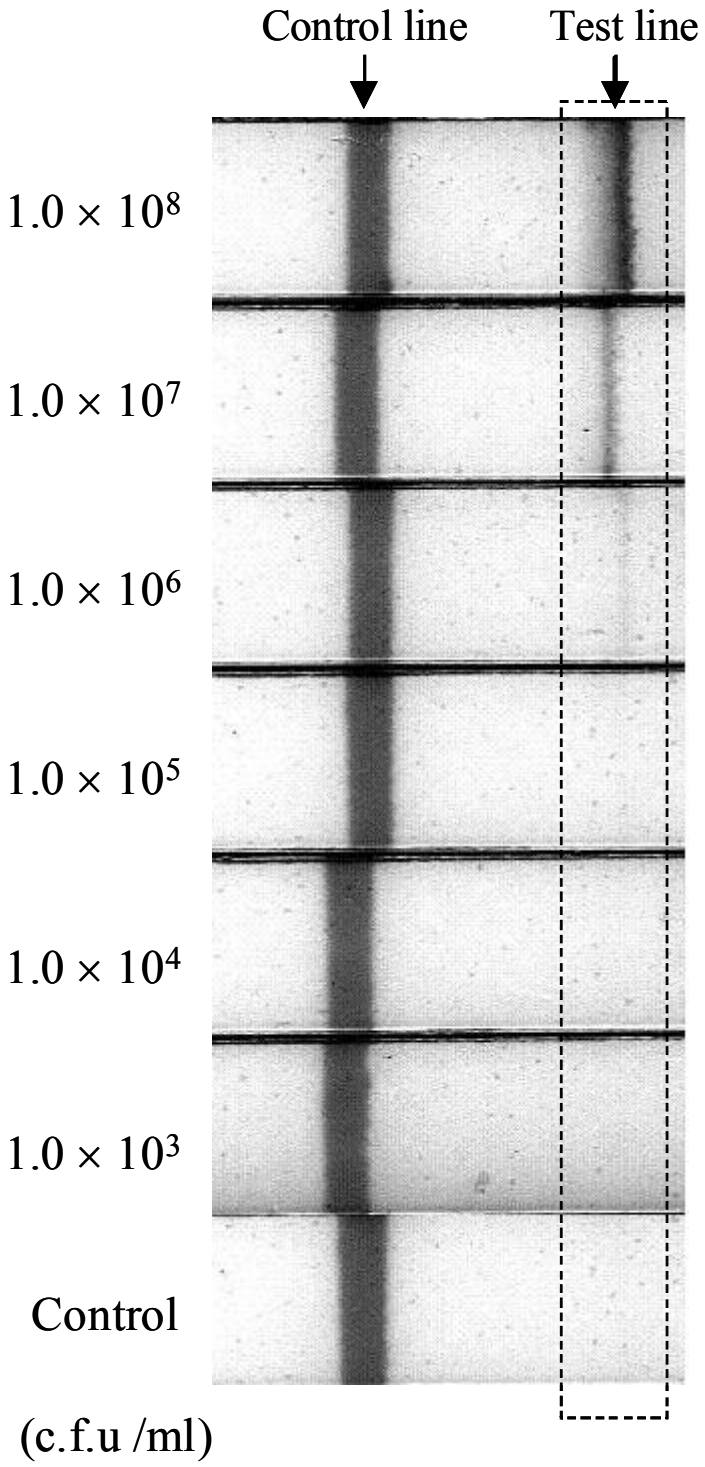
Control line
Test line
1.0 × 10^8
1.0 × 10^7
1.0 × 10^6
1.0 × 10^5
1.0 × 10^4
1.0 × 10^3
Control
(c.f.u /ml)

Fig. 2

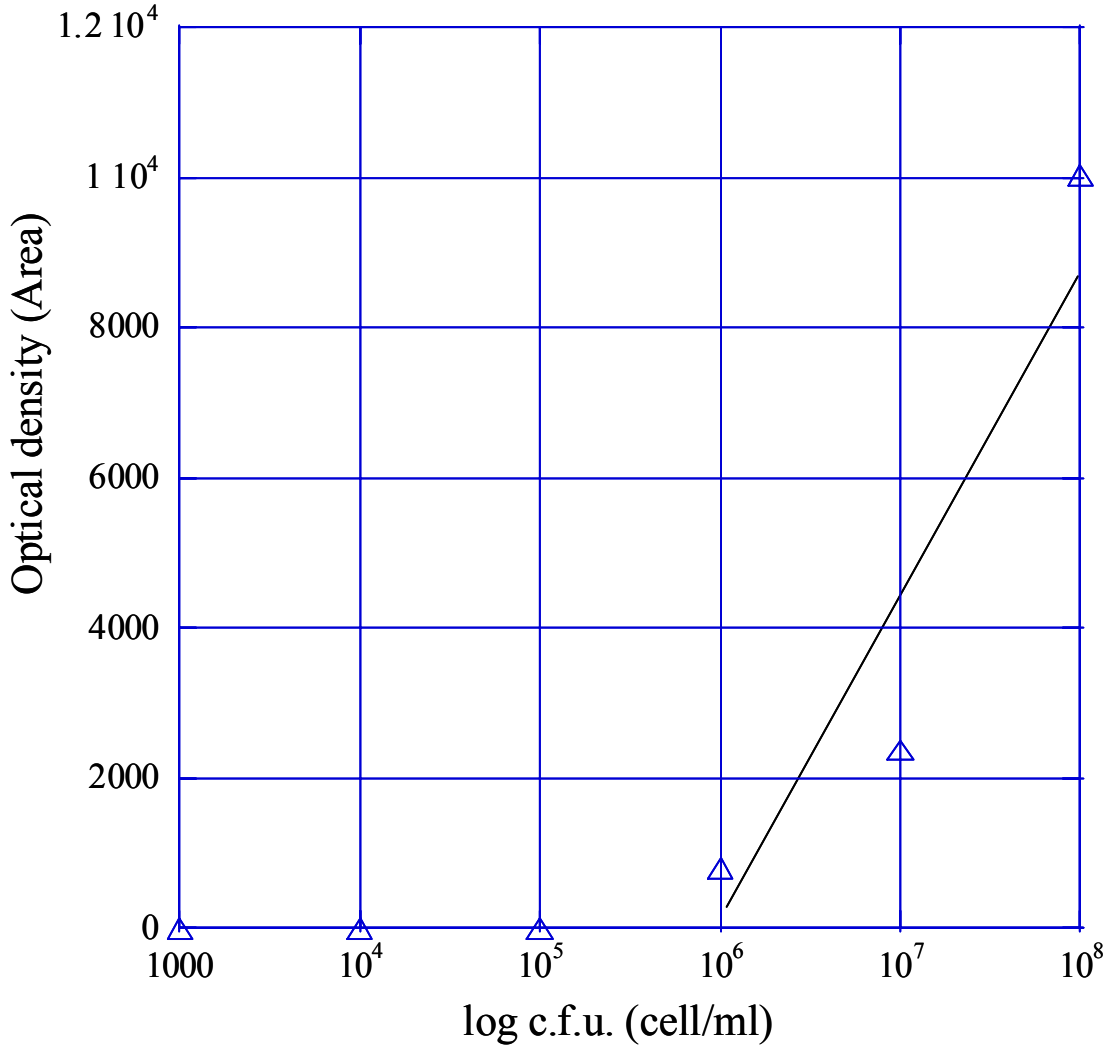
1.2 10^4
1 10^4
8000
6000
4000
2000
0
Optical density (Area)
1000
10^4
10^5
10^6
10^7
10^8
log c.f.u. (cell/ml)

Fig. 3

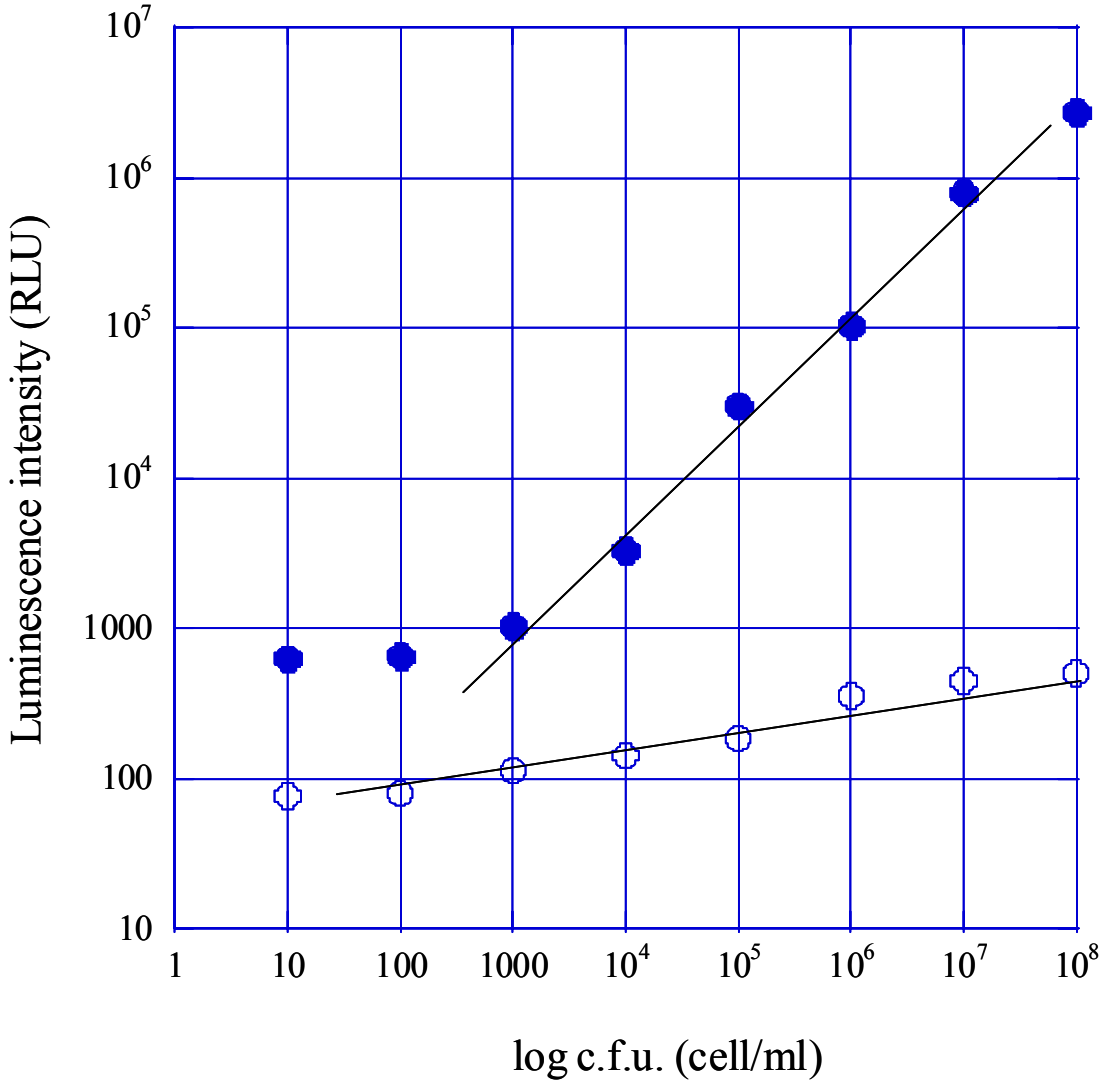
Luminescence intensity (RLU)
10^7
10^6
10^5
10^4
1000
100
10
1
10
100
1000
10^4
10^5
10^6
10^7
10^8
log c.f.u. (cell/ml)

Fig. 4