

Use of a Light-Addressable Potentiometric Sensor for the Detection of *Escherichia coli* O157:H7¹

We describe the development of an immunoligand assay (ILA) in conjunction with a light-addressable potentiometric sensor (LAPS) for the rapid detection of *Escherichia coli* O157:H7 cells in buffered saline. The ILA protocol consists of "sandwiching" bacterial analyte between biotinylated and fluoresceinated antibodies, indirect enzyme labeling of the bacteria with urease-labeled anti-fluorescein antibody, and active capture of the immune complex at a biotinylated bovine serum albumin-blocked nitrocellulose filter membrane with streptavidin. Using live *E. coli* O157:H7, the efficiency of the ILA was compared using various ratios of the biotinylated and fluoresceinated antibodies. Simultaneous addition of equimolar biotinylated and fluoresceinated antibodies effected optimal urease labeling and subsequent active capture of the bacteria in the ILA. Equimolar concentrations of the antibodies were varied to achieve optimal LAPS detection response for the live bacteria. Using ILA with LAPS, a minimum detectable level of ca. 7.1×10^2 cells/ml of heat-killed or ca. 2.5×10^4 cells/ml of live *E. coli* O157:H7 bacteria was achieved in Tris-buffered saline in an assay time of ca. 45 or ca. 30 min, respectively.

The contamination of food by pathogenic microorganisms results in poisoning cases that occur in the millions per year in the United States. One heavily cited pathogen is the bacterium *Escherichia coli* O157:H7. Although *E. coli* O157:H7 has not been implicated in nearly as many cases as other food pathogens such as *Salmonella*, *Campylobacter jejuni*, or *Listeria monocytogenes*, it has been associated with a high frequency

of deaths relative to the other pathogens. Deaths associated with *E. coli* O157:H7 have been attributed to the development of hemolytic uremic syndrome and subsequent kidney damage and failure in those infected by the bacteria (1).

Over the past several years, a variety of "rapid" methods have been introduced in an effort to replace conventional culture techniques, which, although highly sensitive and specific for target microorganisms, require days to weeks for completion (2–4). Many of these rapid methods have been based upon the sensitivity and specificity of nonisotopic enzyme immunoassays in combination with diverse capture and/or detection technologies with the intention of improving total assay time and further amplification of signal (5–7). One approach has been to utilize the rapid and efficient technique of filtration for the concentration and, in some cases, active capture of bacteria (8–12). The advantages of enzyme immunoassay and filtration have been exploited in the development of the Threshold System, which combines an immunoligand assay (ILA³; Fig. 1) with a light-addressable potentiometric sensor (LAPS; Fig. 2). The Threshold System employs porous filter membranes that, upon vacuum filtration, utilize avidin–biotin chemistry for the active capture of immuno-complexed analyte by the filter membranes, followed by close contact of the membrane with an n-type silicon semiconductor-based sensor coated with a pH-sensitive insulator. The instrument uses a common electrochemical circuit to measure the alternating photocurrent associated with the illumination of discrete areas of the silicon sensor using an array of intermittently flashing light-emitting diodes. Essentially, changes in pH associated with enzyme reactions occurring in submicroliter volumes are transduced into voltage per time differentials by the instrument (13, 14). Although typi-

¹ Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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³ Abbreviations used: b-Ab, biotinylated anti-*Escherichia coli* O157:H7 antibody; f-Ab, fluoresceinated anti-*E. coli* O157:H7 antibody; ILA, immunoligand assay; LAPS, light-addressable potentiometric sensor; LOD, limit of detection.

cally applied in either an ILA or a probe hybridization format for the detection of analytes with multiple binding sites, including proteins, carbohydrates, and nucleic acids, LAPS has been used to detect intact viruses (15), bacterial spores (16), and bacteria (16, 17).

In this study, we initially demonstrated the feasibility of applying ILA/LAPS to the rapid detection of whole bacterial cells using dead (heat-killed) *E. coli* O157:H7. Avidin-biotin-mediated capture (active capture) of immune complexes by biotinylated filter membranes was employed in the application of a simultaneous ILA. The active capture necessitated optimization of the concentration and ratio of the sandwiching antibodies (biotinylated and fluoresceinated) utilized since a limited number of binding sites (biotin molecules) are present on the filter membrane. Subsequently, an ILA/LAPS method for the detection of live *E. coli* O157:H7 cells in buffered saline was developed and is presented. Detection of both dead and live cells exhibits broad applicability for the method.

MATERIAL AND METHODS

Materials

Materials used in this research included biotinylated goat anti-*E. coli* O157:H7 antibody [b-Ab; custom produced; an affinity-purified polyclonal antibody that

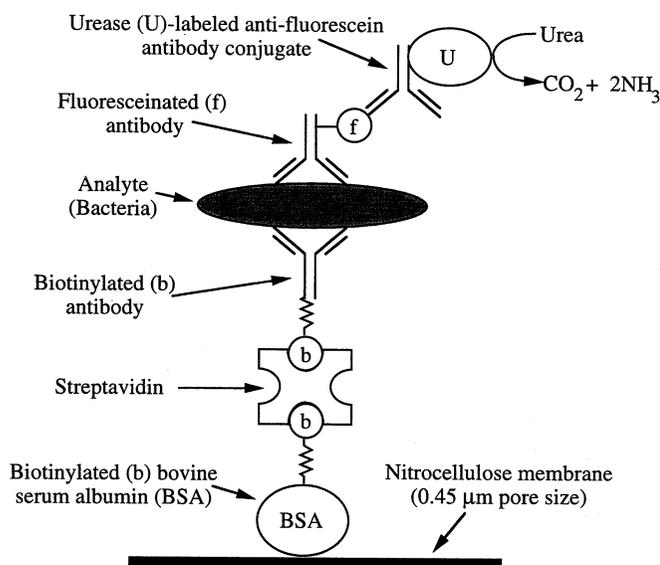


FIG. 1. Schematic representation of the immune complex formed during the immunoligand assay (ILA). The sequence of the ILA reactions was as follows: (i) sample containing multiantigenic analyte (bacteria) was simultaneously sandwiched between biotinylated and fluoresceinated antibodies in the presence of urease-labeled anti-fluorescein antibody; (ii) streptavidin was added; (iii) the sample was vacuum filtered through a biotinylated bovine serum albumin-blocked nitrocellulose membrane; (iv) close contact between the membrane and a sensor was made in the presence of enzyme substrate (urea); and (v) an assay of urease activity was achieved as the light-addressable potentiometric sensor detected the presence of the product ammonia as a change in pH.

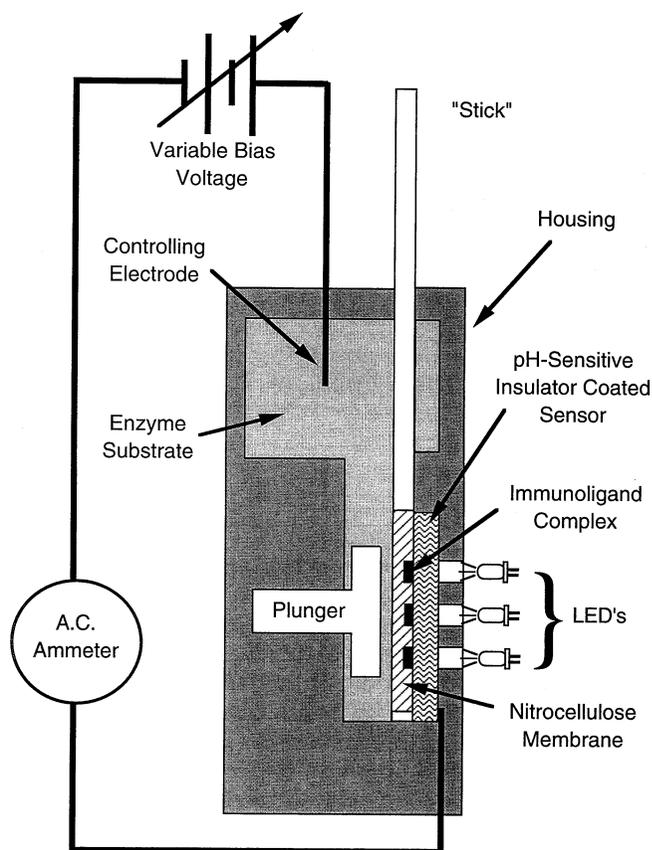


FIG. 2. Schematic representation of the Threshold System's LAPS reader chamber. Threshold "sticks" (nitrocellulose membranes containing immobilized immune complexes) are immersed into the chamber that holds ~30 ml of 100 mM urea. With the aid of a plunger, close contact between the membrane and a pH-sensitive insulator-coated n-type semiconducting silicon sensor chip can be established. Incident light from a light-emitting diode (LED) generates a photocurrent and allows independent measurements of captured immune complexes using common electrodes (reference or controlling electrodes in the electrolyte/substrate solution and the sensor contact). Conversion of the pH change associated with the enzymatic reaction (which occurs in a <math><1-\mu\text{l}</math> volume) to voltage per time (rate) by the instrument's electronics achieves assays of up to eight enzyme-labeled immune complexes in less than 2 min.

was isolated from pooled serum of goats immunized with whole, heat-killed, *E. coli* O157:H7 cells; potential cross-reactivity was minimized by further purification of the antibody through extensive adsorption using non-O157:H7 serotypes of *E. coli*; biotin incorporation was determined to be 5–10 molecules per biotinylated antibody conjugate; this antibody was verified to not cross-react with numerous bacterial genera including non-O157:H7 *E. coli* strains (11); furthermore, we observed no cross-reactivity between the antibody and a live or γ -ray irradiation-killed non-O157:H7 *E. coli* serotype ATCC 25922 or heat-killed *Salmonella typhimurium* (data not shown); fluorescein-labeled goat anti-*E. coli* O157:H7 antibody (f-Ab: this was the same antibody used for the preparation of b-Ab; fluorescein

incorporation was determined to be 6 ± 2 molecules per fluoresceinated antibody conjugate); heat-killed *E. coli* O157:H7 (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD); live *E. coli* O157:H7 B1409 (Centers for Disease Control, Atlanta, GA); streptavidin (Pierce, Rockford, IL); brain–heart infusion (Difco Laboratories, Detroit, MI); and components of an ILA detection kit, including enzyme reagent (urease-labeled anti-fluorescein antibody conjugate), Threshold sticks (biotinylated-bovine serum albumin-blocked nitrocellulose membranes with a pore size of $0.45 \mu\text{m}$), assay buffer (phosphate-buffered saline containing bovine serum albumin, Triton X-100, and azide, pH 7.0), wash buffer (phosphate-buffered saline containing Tween 20, EDTA, and azide, pH 6.5), and filter unit blocks and bases (Molecular Devices Corp., Sunnyvale, CA). Other chemicals used were of reagent grade.

Apparatus

Bacteria samples were counted on a Petroff–Hausser bacteria counting chamber (Thomas Scientific, Swedesboro, NJ). Immune complexes formed in ILAs were vacuum filtered through Threshold sticks placed in filter units mounted on the vacuum manifold incorporated in the Threshold System (Molecular Devices Corp.). All LAPS measurements were made with the Threshold System.

Growth and Enumeration of Live *E. coli* O157:H7

A loopful of *E. coli* O157:H7 cells collected from a slant was inoculated into 25 ml of brain–heart infusion broth and incubated at 37°C for 18–19 h. The stationary phase cells were then placed on ice for no longer than 9 h prior to use. A portion of the cells was serially diluted in assay buffer to 1:10 and 1:100 dilutions. Bacteria in an aliquot ($6 \mu\text{l}$) of the 1:100 dilution were enumerated using only the center $0.2 \times 0.2\text{-mm}$ grid of a Petroff–Hausser counting chamber. Enumeration was repeated three times using additional $6\text{-}\mu\text{l}$ aliquots of the 1:100 dilution and a mean (\pm standard deviation) was determined for the four obtained values. The 1:100 dilution was used for further serial dilution in the preparation of samples for the generation of calibration curves. Where appropriate, the subsequent error (standard deviation) for the determined bacteria concentration (mean value for the 1:100 dilution) and subsequent dilutions thereof was propagated taking into account an estimated error of 5% for volumetric measurements.

ILA/LAPS Detection of Heat-Killed *E. coli* O157:H7

Heat-killed *E. coli* O157:H7 (lyophilized cells initially reconstituted in 50% glycerol in phosphate-buffered saline to a concentration of $7.0 \times 10^9/\text{ml}$) were twofold serially diluted from 1.0×10^5 to 1.6×10^3 and

0 (blank) cells/ml in assay buffer. The b-Ab and f-Ab stocks were diluted in assay buffer and the final working solution contained mixtures of the two at equimolar concentrations of 200 ng/ml. Aliquots ($100 \mu\text{l}$) of the b-Ab/f-Ab working solutions were added to the bacteria samples (1 ml each) followed by the addition of $100 \mu\text{l}$ of enzyme reagent (lyophilized urease conjugate initially reconstituted with 4 ml of assay buffer as recommended by Molecular Devices Corp.). The mixture was vortexed and reacted at room temperature ($\sim 25^\circ\text{C}$) for 30 min. Aliquots ($100 \mu\text{l}$) of streptavidin ($20 \mu\text{g}/\text{ml}$ in assay buffer) were added to the mixtures, the mixtures were vortexed, and the samples were filtered through Threshold sticks mounted in filter units on the Threshold System's vacuum manifold ("low" vacuum setting; complete filtration in ~ 10 min). Wash buffer (2 ml) was added to each filter well, and the vacuum was reapplied (on the "high" setting; complete filtration in ~ 5 min). The Threshold sticks were placed into the reader chamber (Fig. 2), containing 100 mM urea in wash buffer, of the instrument so that enzyme activity could be assayed (< 2 min).

Comparison of the Ratio and Optimization of the Concentrations of b-Ab and f-Ab in an ILA for Live *E. coli* O157:H7

The concentrations and/or ratios of b-Ab and f-Ab were varied and reacted with constant amounts of live *E. coli* O157:H7 in a manner similar to the ILA/LAPS used in the detection of heat-killed bacteria (above), but with the following changes: (1) $25 \mu\text{l}$ of b-Ab/f-Ab as used to achieve the amount of antibody/test as indicated in Tables 1 and 2, (2) $25 \mu\text{l}$ of enzyme reagent (lyophilized urease conjugate initially reconstituted with 1 ml of assay buffer) was used, (3) reaction of the bacteria with b-Ab/f-Ab and the enzyme reagent occurred for 15 min, and (4) $25 \mu\text{l}$ of streptavidin ($80 \mu\text{g}/\text{ml}$ in assay buffer) was added to the reaction mixtures.

ILA/LAPS Detection of Live *E. coli* O157:H7

ILA/LAPS was applied to the detection of live *E. coli* O157:H7 using the same procedure as indicated in the previous section on comparison/optimization of ratios and concentrations of b-Ab and f-Ab. Twofold serial dilutions from 5.1×10^5 to 1.0×10^3 and 0 (blank) cells/ml of bacteria in assay buffer were tested using a final optimized amount of 40 ng/test for both b-Ab and f-Ab.

RESULTS AND DISCUSSION

Prior to development of an ILA for *E. coli* O157:H7, a preliminary experiment investigated the feasibility of using LAPS for the detection of the bacteria. It was expected that as with relatively smaller analytes (molecules), the rod-shaped bacterial cells ($\sim 1 \mu\text{m}$ in

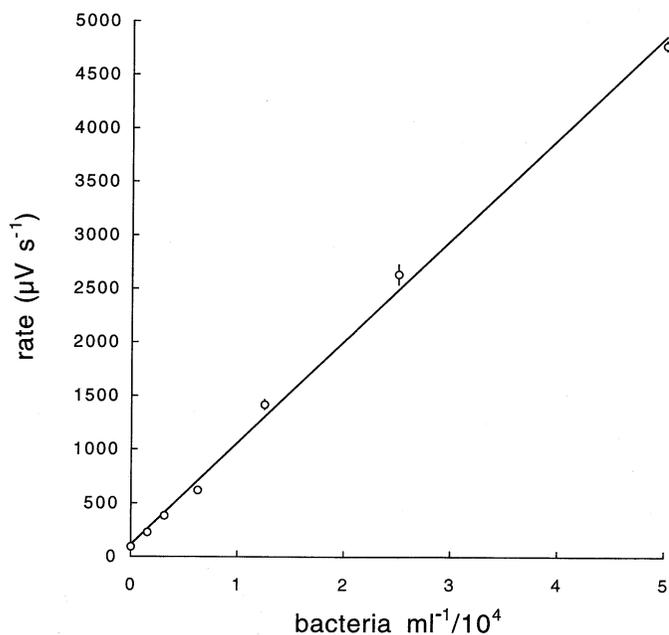


FIG. 3. ILA/LAPS detection of heat-killed *E. coli* O157:H7 cells (bacteria) in Tris-buffered saline. The plot represents averaged responses of duplicate measurements \pm standard deviation.

length) are capable of passing through the 0.45- μ m nitrocellulose membrane used in the ILA if the cells assume an “on end” orientation. Although filtration rates may have been dramatically affected, smaller pore sized membranes could have been employed to achieve greater bacterial capture efficiency. However, smaller pore sized membranes are not yet available from the manufacturer of the LAPS Threshold System. Therefore, avidin–biotin-mediated active capture of the bacteria was employed, thus taking advantage of the prebiotinylated nitrocellulose supplied by the manufacturer. Heat-killed *E. coli* O157:H7 were twofold serially diluted in assay buffer and immobilized to a nitrocellulose membrane in an ILA as described under Materials and Methods. The membrane was then placed into the Threshold System and subjected to LAPS detection. A linear response (rate) was observed for the range (\sim 1.5 orders of magnitude) of bacteria analyte tested (Fig. 3).

Although only duplicate samples were tested, a minimum detectable limit of bacteria concentration can be conferred from analyte and background responses using the method suggested by the manufacturer of the Threshold System,

$$X_{\text{antigen}} > X_{\text{blank}} + 2 \text{SD}_{\text{blank}} + 2 \text{SD}_{\text{antigen}}, \quad [1]$$

where X_{antigen} is the mean sample (multiantigenic bacteria) response, X_{blank} is the mean background response, and SD is the standard deviation from the mean. Upon substitution into the above equation, the sum of the blank response (96.5 ± 31.2) and the two doubled standard deviations was revealed to be considerably less than the mean response (230.8 ± 14.5) for the lowest concentration (1.6×10^3 cells/ml) of heat-killed bacteria tested. Furthermore, combination of the above equation with the equation for a line (in particular, that of the calibration curve)

$$\text{LOD} \cong [(X_{\text{blank}} + 2 \text{SD}_{\text{blank}} + 2 \text{SD}_{\text{antigen}}) - b]m^{-1} \quad [2]$$

where m and b are the slope and y intercept of the standard curve, respectively, allows for incorporation of the slope of a standard curve and interpolation of the theoretical limit of detection (LOD). Therefore, subsequent linear regression of the data and substitution into Eq. [2] result in a calculated LOD of $\sim 7.1 \times 10^2$ bacteria/ml.

In the development of an ILA for live bacteria, initial investigations focused on studying the effects of varying the ratio and concentration of b-Ab and f-Ab on the LAPS detection of live *E. coli* O157:H7. As presented in Table 1, the largest amount of equimolar b-Ab/f-Ab elicited the highest response at either level of bacteria concentration tested. Interestingly, the observed responses demonstrate that f-Ab was more responsible than b-Ab for its contribution to the overall response. This result indicates that labeling of the analyte with enzyme and not necessarily the active capture of the analyte to the membrane results in a more efficient ILA signal.

TABLE 1

Comparison of Biotinylated (b-) and Fluoresceinated (f-) Antibody Ratios in the Immunoligand Assay for the Detection of Whole Live *E. coli* O157:H7 Cells with the Threshold System

b-Ab/f-Ab (ng/test)	Blank (0 cells/ml)	20,000 cells/ml	200,000 cells/ml
2/2	$80.8^a \pm 1.3^b$	82.2 ± 5.8	171.4 ± 13.9
2/20	72.8 ± 2.6	103.0 ± 1.91	364.7 ± 9.05
20/2	84.4 ± 2.1	99.8 ± 6.8	277.2 ± 3.68
20/20	99.0 ± 3.5	148.8 ± 4.74	745.9 ± 35.1

^a Rate (μ V/s).

^b Standard deviation of duplicate samples.

TABLE 2

Optimization of Biotinylated (b-) and Fluoresceinated (f-) Antibody Amounts in the Immunoligand Assay for the Detection of Whole Live *E. coli* O157:H7 Cells with the Threshold System

b-Ab/f-Ab (ng/test)	Blank (0 cells/ml)	20,000 cells/ml	200,000 cells/ml
0.5/0.5 ^I	71.2 ^a ± 3.4 ^b	72.9 ± 1.0	89.1 ± 3.7
5/5 ^I	73.4 ± 5.4	111.9 ± 4.97	444.4 ± 15.6
10/10 ^I	68.8 ± 0.64	122.9 ± 6.10	635.6 ± 29.6
25/25 ^{II}	77.1 ± 8.8	121.3 ± 2.84	598.4 ± 7.33
40/40 ^I	107.1 ± 2.97	164.2 ± 6.25	755.2 ± 39.8
75/75 ^{II}	88.4 ± 10	124.5 ± 2.93	556.8 ± 15.4
125/125 ^{II}	89.5 ± 9.2	119.6 ± 1.55	477.9 ± 20.9
200/200 ^{II}	110.9 ± 8.20	128.7 ± 3.03	420.4 ± 12.3

Note. The data listed represent the average values for triplicate measurements (duplicate for blank samples). The samples labeled with either a "I" or a "II" belonged to two different groups that were run on 2 separate days of experimentation.

^a Rate ($\mu\text{V/s}$).

^b Standard deviation.

Since the data from Table 1 suggest that equal amounts of b-Ab and f-Ab result in a more efficient (higher signal response) ILA for bacteria, further studies were performed on the optimization of b-Ab/f-Ab concentrations with live bacteria. Table 2 displays the data obtained for the ILA/LAPS detection of 0, 2.0×10^4 , and 2.0×10^5 cells/ml of live *E. coli* O157:H7

reacted with b-Ab/f-Ab equimolar ratios ranging from 0.5 to 200 ng per test. At both of the bacterial levels tested, the ILA/LAPS response trend rose and then declined while the blank response tended to rise with increasing concentrations of b-Ab/f-Ab. This relationship was expected for an immunoassay that is constrained by a limited number of active membrane bind-

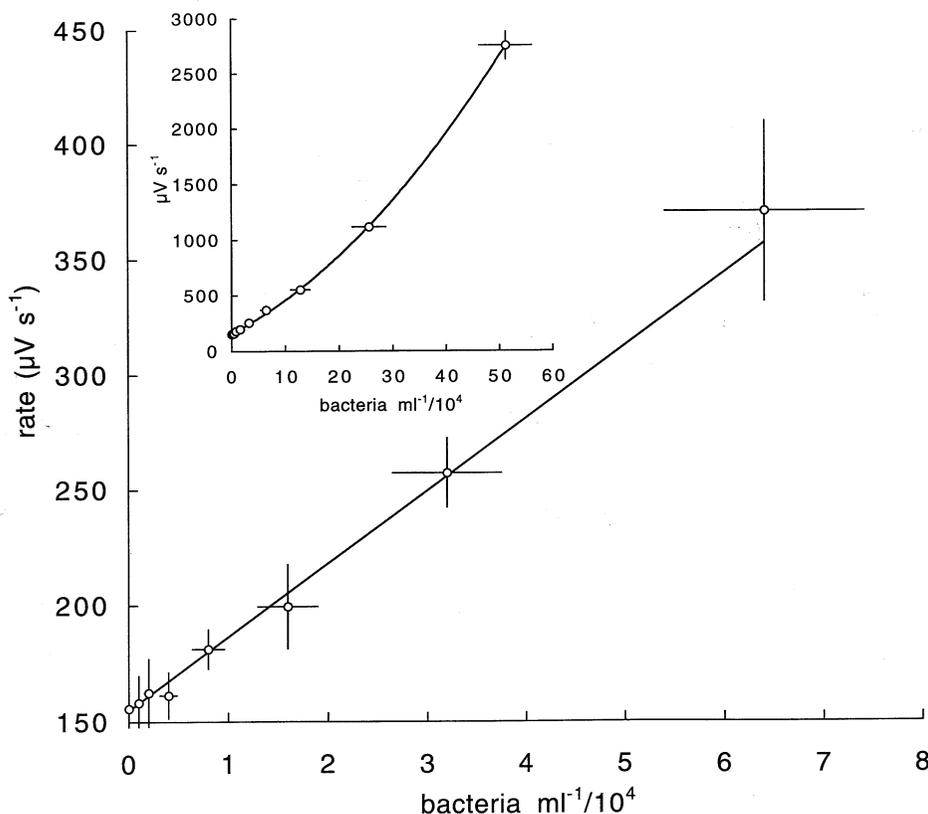


FIG. 4. ILA/LAPS detection of live *E. coli* O157:H7 cells (bacteria) in Tris-buffered saline. The plot is an expanded portion, near the origin, of the inset plot that represents averaged responses of triplicate (duplicate for the most concentrated, 5.1×10^5 bacteria/ml samples) measurements \pm standard deviation.

ing sites and that uses a simultaneous incubation protocol where b-Ab and f-Ab compete for binding to the target analyte (bacteria). There was an apparently anomalous drop in response at both bacterial concentrations tested at the b-Ab/f-Ab ratio of 25/25 relative to the ratios 10/10 and 40/40. Since the former 25/25 ratio sample was tested on a day separate from the latter two, and hence with a different bacteria stock, error (~6-15%) in the bacterial enumeration may account for the observation, whereas the high blank response for the 40/40 ratio may have been an artifact due to the use of fresh urea substrate for the assays on that day of experimentation. The quantitation gain (net $\mu\text{V/s}$ /bacteria concentration) was highest at both bacterial levels when the concentrations of b-Ab/f-Ab were 40 ng/test.

The ratio of 40/40 ng per test for b-Ab/f-Ab was considered to be optimal for and was applied to the ILA/LAPS detection of live *E. coli* O157:H7. Figure 4 displays the rate (background/blank subtracted) for serial dilutions of the bacteria over ~2.7 orders of magnitude in concentration. The error displayed for the rate was the standard deviation from the mean, whereas the error in the bacteria concentrations was derived from combining the error associated with enumeration of the initially diluted bacteria solution with estimated acquired volumetric errors of 5% propagated over the prepared range of serial dilutions. Substituting the standard deviations of the blank and the 1.6×10^4 cells/ml sample (estimated LOD from the plot in Fig. 4) along with the slope and y intercept from the standard curve (not blank subtracted) used to generate Fig. 4 into Eq. [2], a LOD of $\sim 2.5 \times 10^4$ cells/ml was obtained. This result was ~1.5 orders of magnitude higher than that achieved for the heat-killed cells. Three explanations may account for this discrepancy: (i) considering the twofold reduction in b-Ab/f-Ab, bacteria, and enzyme reagent reaction time, the exposure time for the live cells to the unreacted antibody was 15 min less than that for the dead cells during the preliminary test, which may have resulted in incomplete binding of the antibodies; (ii) preliminary results (unpublished data) suggest that bacteria killed by heat readily "release" small, relative to the size of the bacteria, antigenic fragments that exhibit high immunogenic surface area and enhance immunological reaction kinetics and subsequently higher apparent analyte responses in various immunoassay formats;

and/or (iii) the antibodies used in this research were raised against heat-killed bacteria, whereupon heat treatment may evoke antibody formation against antigenic components, perhaps cryptic epitopes, "displayed" by heat-killed and not live bacteria, therefore conferring higher reactivity toward heat-killed than toward live bacteria.

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