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Ultra-sensitive Detection Using Integrated Waveguide Technologies

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1. Introduction

There is a pressing need to detect analytes at very low concentrations, such as food- and water-borne pathogens (e.g. *E. coli* O157:H7) and biothreat agents (e.g., anthrax, toxins). Common fluorescence detection methods, such as 96 well plate readers, are not sufficiently sensitive for low concentrations. We describe here a novel detection principle---integrating waveguide technology (IWT)---that allows for greater sensitivity, and report on the sensitivity of a new instrument (Signalyte™-II) based on IWT, and relevant assays.

When fluorescent labels emit light, the emission is typically in all directions such that only a small fraction of the light is collected by the detector as signal. This is the case with 96 well plate readers. Simultaneously, light from the excitation source, auto fluorescence from the sample, Raman emission from water, and electronic noise of the detector contribute to background noise. Consequently, a detection technology that captures the majority of emitted light while eliminating background is inherently more sensitive.

2. Integrating Waveguide Technology (IWT)

Creatv MicroTech, Inc. has developed assays based on IWT which achieves high sensitivity by maximizing the signal while minimizing background noise. IWT assays can be conducted either in a solid-phase or liquid-phase format. In the solid-phase IWT format, analytes are first captured on the inner surface of the capillary tube or cuvette; while in the liquid-phase IWT format, the analyte is in solution inside of the cuvette.

The basic principle of liquid-phase IWT detection is shown in Figure 1. In this configuration, the sample containing fluorescent dye is placed in a glass capillary cuvette. The closed end of the capillary cuvette forms a half-ball lens that focuses the emitted light. Detection and quantitation are achieved by illuminating the cuvette at a 90-degree angle relative to the length of the cuvette. The glass walls of the tube together with the sample act as a waveguide, efficiently gathering and propagating the fluorescent signal from the entire sample to the end of the cuvette and exits through the half-ball lens. The half-ball lens

focuses the signal and together with additional optics, allows efficient application of bandpass or longpass filters before sending the signal to the detection.

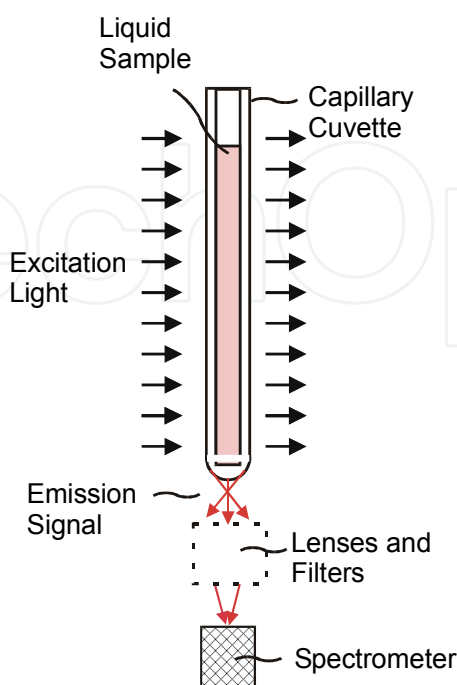


Fig. 1. Integrating Waveguide Technology detection concept.

We have previously reported applications using solid-phase IWT, where analytes are captured on the inner surface of an open ended capillary tube and analyzed using an instrument called Signalyte™ (Li, 2005a). This format has been used to detect and quantify *Escherichia coli* O157 (Zhu et al., 2005) and *Bacillus anthracis* spores (Hang et al., 2008), where detection limits were 10 cells and 1000 spores, respectively.

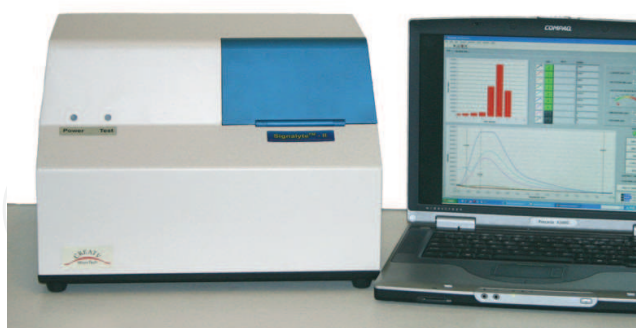


Fig. 2. Signalyte™-II instrument.

Based on these previous studies, a new generation instrument was developed to optimize assays based on liquid phase IWT, called Signalyte™-II; the instrument is shown in Figure 2. Signalyte™-II allows for multiplex assays by providing up to four fluorescence excitation wavelengths from 365 nm – 635 nm. This is achieved by a combination of the excitation source and band pass filters. To allow for maximum flexibility of high power excitation source, LEDs are used. High power LEDs are typically available at peak wavelengths of 365 nm, 470 nm, 530 nm, 590 nm and 635 nm. Because their bandwidths overlaps, these LEDs

are applicable for most commercially available fluorescent dyes. The excitation wavelength can be altered, depending on the application.

Bandpass filters for the excitation source and long pass filters for the emission signal are selected to match the absorption and emission spectra for each specific dye. These filters are easily installed or changed. A band pass filter is needed to select the excitation LED light in the wavelength region of the emission signal of interest. This filtered excitation light impinges on the sample. Under ideal situations, all the excitation light leaves. Because of scattering of the excitation light by the sample and cuvette, a long pass filter is needed to eliminate or minimize the signal caused by the excitation source.

The detection system for Signalyte™-II is a spectrometer that is sensitive from 350 nm – 800 nm. This spectrum range is applicable for most common fluorescence applications. A spectrometer, rather than photomultiplier tubes (PMT), is used because the spectrum provides information about the background noise, thereby allowing it to be eliminated from data analysis.

To achieve maximum sensitivity, the signal to noise ratio has to be as high as possible. The signal is increased in two ways. First, the cuvette is efficient in gathering and transmitting the emitted light to the detector. Numerical simulation shows that about 13 percent of the emission is collected by the cuvette. This percentage is much larger than other standard collection methods such as the use of a lens or optical fiber. Secondly, the duration of time the spectrometer can collect the light can be adjusted over four orders of magnitude with the maximum of 65 seconds. This integration of the signal is important for low fluorescence.

The Signalyte™-II has been designed to minimize noise from the following sources:

- **Excitation source.** Background noise from the excitation light is minimized, because illumination is perpendicular to the waveguide and the majority of the excitation passes through the cuvette. Only small amount of scattered light is trapped by the cuvette.
- **Electronic noise.** The spectrometer is cooled providing low electronic noise. Thus allowing integration of the signal in time without raising the noise and long signal collection time is good for low concentrations.
- **Other instrument related noises.** Other noises in the instrument are primarily associated with optics. Appropriate optics and optical filters reduces this noise.
- **Non-specific binding of fluorescent dyes in sample.** Non-specific binding comes from fluorescent dyes that are not captured on purpose. This can be reduced by optimizing the assay.

Sample size is up to 35 microliters. The instrument simultaneously tests up to eight samples that are loaded using a standard multi-channel pipetter, plus an additional control to measure background Raman and Raleigh scattering. The sample holder including a reference is shown in Figure 3.

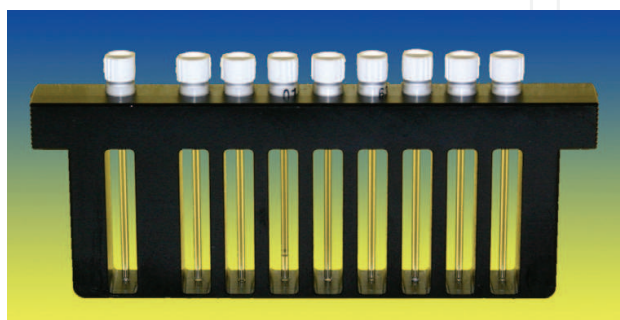


Fig. 3. Cuvette holder allows testing of 8 samples and a reference.

“Signalyte-II Control” is the software that operates Signalyte™-II. The interface of the software is shown in Figure 4.

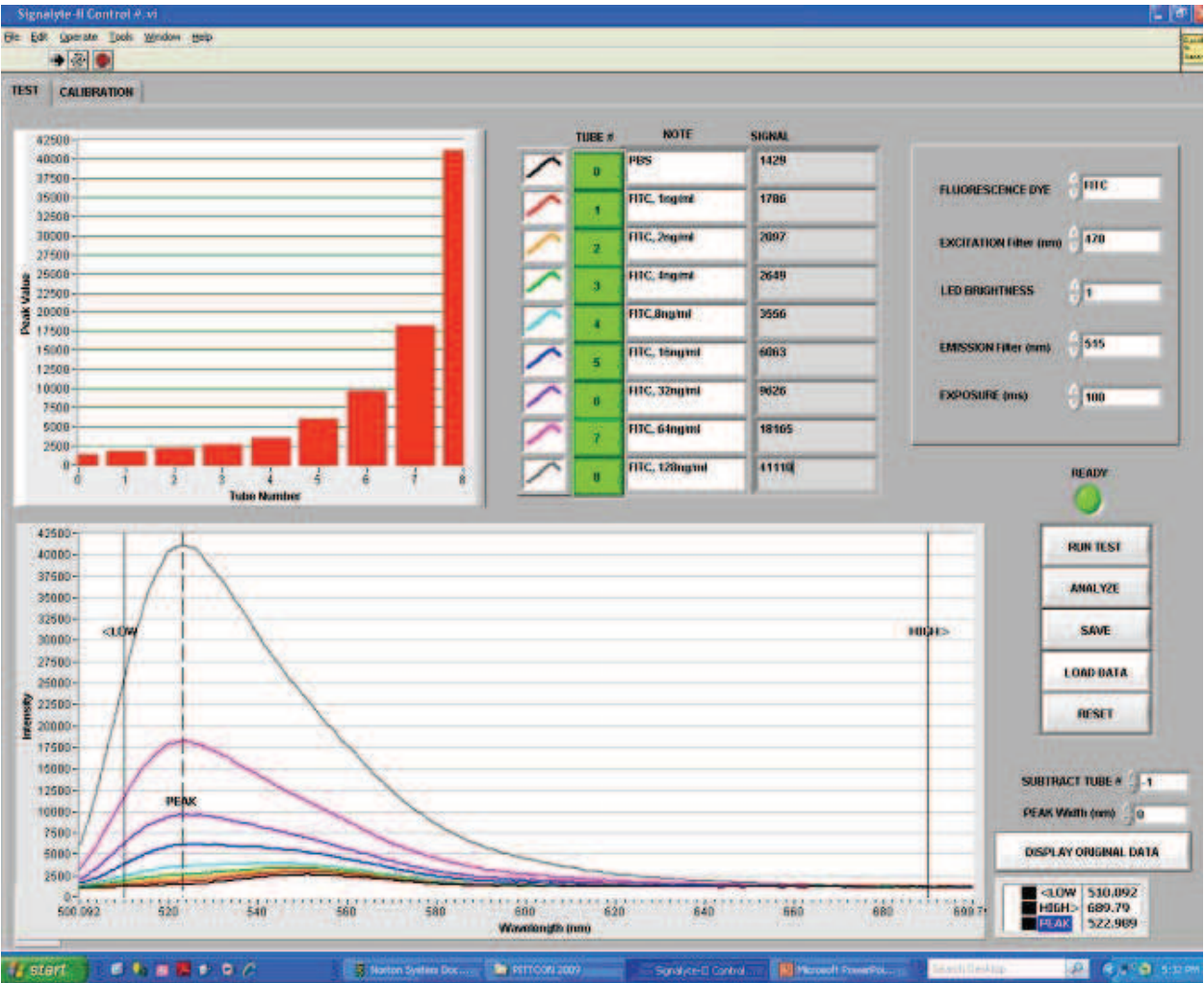


Fig. 4. Signalyte™-II user software interface

Signalyte™-II can test 8 samples plus a reference (tube #0). When testing the samples, the exposure time can be set from 5 milliseconds to 65 seconds. With long exposure time, weak signal can be integrated over a longer period of time, but background noise does not increase as much as the signal does because of the integrating waveguide technology and the fact that the detector is a cooled CCD. A typical exposure time for weak signal is 1 to 3 seconds.

High-power light-emitting diode (LED) is used as illumination light source in Signaly™-II. Comparing to broad spectrum lamps, such as mercury or xenon arc lamps, which are widely used in fluorescence detection instruments, LEDs possess all of the desirable features they lack. Although arc lamps can generate a broad spectrum, only a small percentage of the projected light is utilized for fluorescence detection. Another problem for arc lampd is the excessive heat they generate. In contrast, LEDs are cooler, smaller, and provide a far more convenient mechanism to cycle the source on and off.

Compared to laser light, the wider bandwidth featured by LEDs is more useful for exciting a variety of fluorescent probes. The diverse spectral output afforded by LEDs can supply the

optimum excitation wavelength band for fluorophores spanning the ultraviolet, visible, and near-infrared regions. Furthermore, high-power LEDs generate sufficient intensity to provide a useful illumination source for a wide spectrum for fluorescence detection. Up to four LEDs can be installed in Signalyte™-II. In the following example, there are four LEDs, including red, amber, green and blue LEDs. The fluorescence dyes for the corresponding LEDs are listed accordingly as Cy5, ROX, Cy3, and FITC. When choosing one of these dyes, the excitation filter and emission filter are set at predetermined wavelengths that optimal for the chosen dye. excitation filter shows the center wavelength of the excitation light, and emission filter shows the cut-on wavelength of the long pass emission filter. Table 1 shows the instrument settings for different dyes.

Fluorescence Name	Excitation Wavelength	Long Pass Filter
Cy5	635 nm	LP665
ROX	590 nm	LP630
Cy3	530 nm	LP570
FITC	470 nm	LP515

Table 1. An example of Signalyte™-II setting for fluorescence dyes testing

After the samples are tested, the software automatically measures peak intensity of the signal spectrum, integrates signal strength into a bar chart display, and displays the relevant spectrum.

The user software interface shows three graphs of the testing result. The one on the lower left corner of the user software interface, Figure 4, is SPECTRUM. The one on the upper left corner is BAR CHART. On the upper right side above the SPECTRUM is SIGNAL. After RUN TEST, the software automatically finds the peak intensity of the signal spectrum. The full spectrum (350-800 nm) of each tested sample is displayed in SPECTRUM DISPLAY with colors assigned by SPECTRUM COLOR. The integrated signal strength is displayed in BAR CHART, and is displayed as integer values in BAR CHART.

The original data can be analyzed by dragging the three cursors, including LOW, HIGH, and PEAK, on the SPECTRUM graph. For example, you can expand and investigate a sub spectrum between wavelength range of LOW to HIGH by dragging the two cursors. You can also choose your own PEAK wavelength by changing the PEAK cursor location.

The background noise can be illeminated from the emission spectrum by subtracting tube # 0 (reference) to provide a net fluorescence signal. This feature allows the Signalyte™-II instrument to provide both highly sensitive and precise quantitative data.

Data can be saved as an Excel spreadsheet on a computer connected by USB cable. You can also upload previously saved data to review it.

The limits of detection for three fluorescent dyes and three fluorescent particles using Signalyte™-II are shown in Table 2.

The Signalyte™-II enables numerous assay applications, such as multiplex immunoassays, FRET- based assays, chemiluminescence detection, end point PCR, and DNA and RNA detections without using thermal cycling amplification, multiplex quantum dots assays, polarization assays, etc.

Number of Detected Dye Molecules	Number of Detected Particles
Cy TM 5: 2.5 pM (5×10 ⁷ dyes)	Purple: 10 nanoparticles
FITC: 25 pM (5×10 ⁸ dyes)	Sky Blue:12 nanoparticles
ROX: 25 pM (5×10 ⁸ dyes)	FluoSphere: 1.3×10 ⁴ particles

Table 2. SignalyteTM-II is typically a factor of 100 to 1000 times more sensitive than plate readers.

3. Ultra-sensitive immunoassays using SignalyteTM-II

Escherichia coli O157:H7 Immunoassay. *E. coli* O157:H7, the most common serotype of enterohemorrhagic *E. coli* (EHEC), is responsible for numerous food-borne and water-borne infections worldwide. Consequently, rapid and sensitive assays for detection of *E. coli* O157 are highly desirable. We have investigated two approaches for the detection of *E. coli* O157 based immunomagnetic separation and on filtration.

Immunomagnetic separation (IMS) techniques are routinely used for isolation, and detection, of EHEC O157:H7 from enriched food and water samples. The assay protocol is as follows. Antibody-coated magnetic beads were first added to the test samples to specifically capture *E. coli* O157:H7 bacteria. Other bacteria and contaminants in the water samples were removed by subsequent washes. The bead-bound *E. coli* O157:H7 were recognized by a Cy5-conjugated anti-O157:H7 polyclonal antibody to form an immunosandwich complex. After washing to remove the excess antibodies, the antibody/cell complex was dissociated from the beads into the liquid phase. The Cy5 fluorescence intensity of the supernatant, which was related to the target concentration, was measured using the SignalyteTM-II.

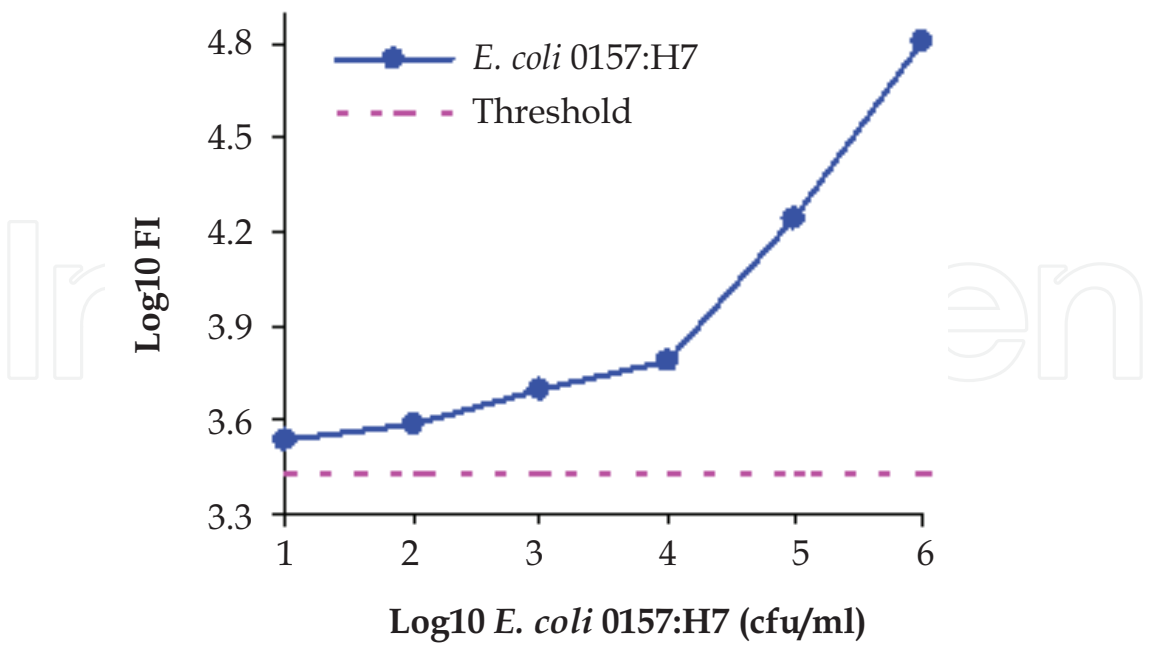


Fig. 5. Results from Creatv’s immunoassay kit for detection of 10-fold serial dilutions of *E. coli* O157:H7 read with the SignalyteTM-II. For IMS detection format, the limit of detection is 10 cfu/ml.

Our data demonstrate that it is possible to detect as few as 10 cfu/ml of *E. coli* in a 1 ml sample using an IMS format (Figure 5), thus allowing for rapid detection of low concentrations of bacterial cells. The same assay read on a 96-well fluorescent plate reader BMG FLUOstar Omega (BMG Labtech) (Figure 6) had limit of detection of 10⁵ cfu/ml.

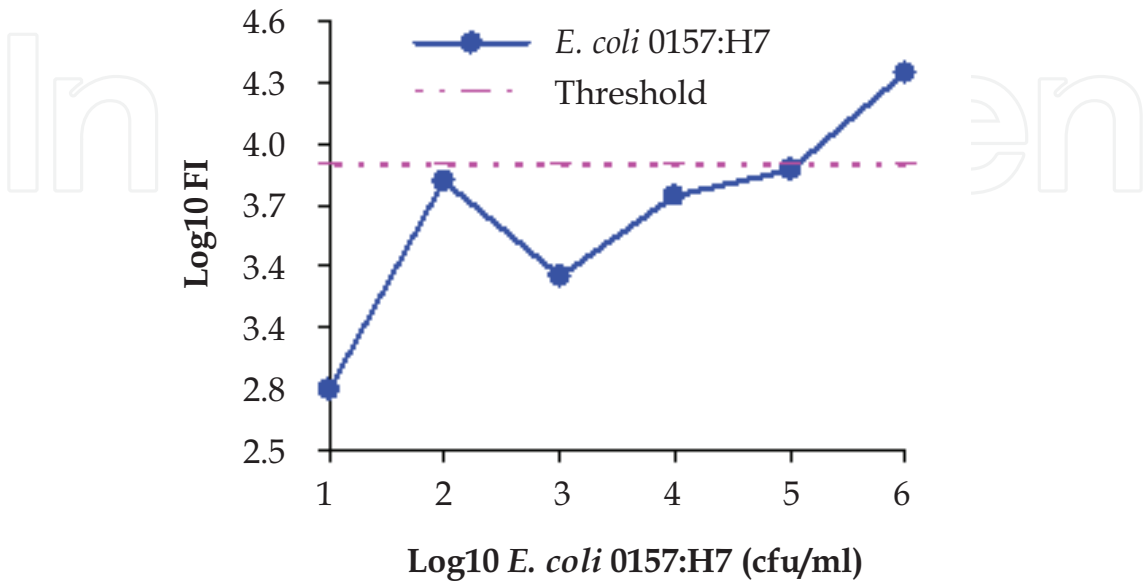


Fig. 6. Results from Creatv’s fluorescence immunoassay kit for detection of 10-fold serial dilutions of *E. coli* O157:H7 read with the BMG FLUOstar Omega. The limit of detection is 100,000 cfu/ml.

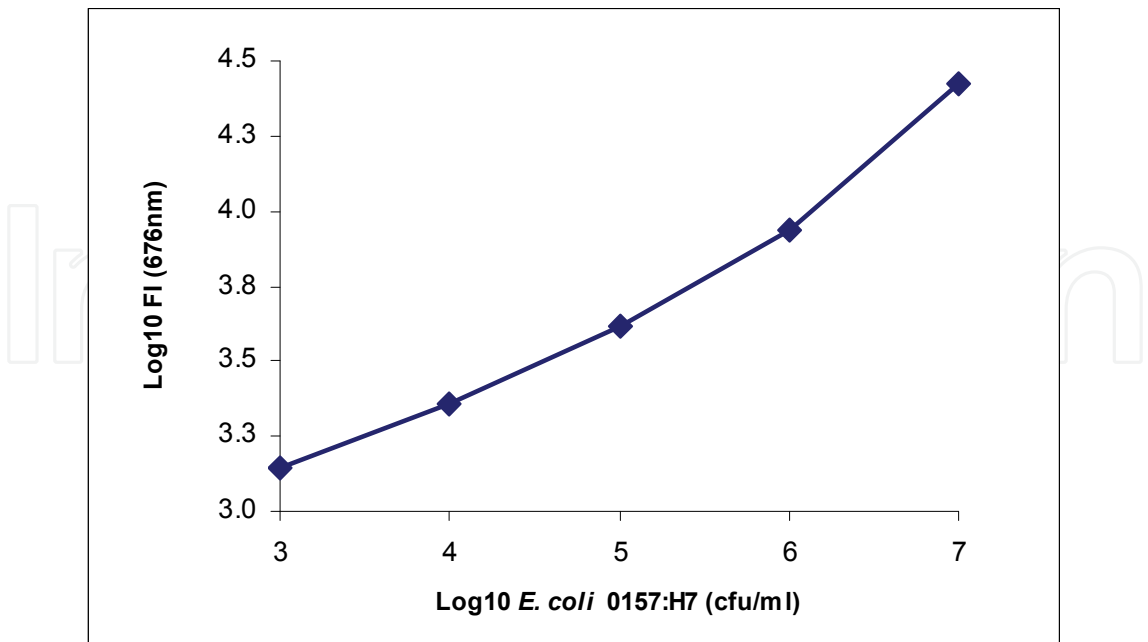


Fig. 7. Results from polyclonal antibody fluorescence immunoassay for detection of 10-fold serial dilutions of *E. coli* O157:H7 demonstrating 1000 cfu/ml sensitivity. For filtration-based format, the limit of detection was 1000 cfu/ml.

Alternatively, *Escherichia coli* O157:H7 may be detected using a filtration-based format. The assay protocol is as follows. *E. coli* O157:H7 cells were grown to an approximate cell density of 10^9 cfu/ml. Cells were serially diluted by 10-fold increments in phosphate buffered saline and contacted for 1 hour by tumbling at 37° C with Dylight 649 conjugated *E. coli* O157:H7 polyclonal antibody. Cell suspensions were filtered through Durapore PDVF (0.1 μ m pore size) spin filters and the filters exhaustively washed to remove excess antibody. The antibody/cell complex trapped on spin filters was then dissociated with a low pH buffer and the fluorescent antibodies quantified with the Signalyte™-II.

As shown in Figure 7, there was an approximate linear relationship between signal versus log cell concentration; the limit of detection was 1000 cfu *E. coli* O157:H7 in a 1 ml suspension with polyclonal antibody.

The sensitivity using this format is lower due to non-specific binding of antibodies to the filter polymer, which was eluted with the low pH buffer. We are currently evaluating alternative filter polymers to eliminate this non-specific binding, which will result in lower detection limits. Note that since larger volumes of sample can be incubated with antibody and filtered, the ultimate detection limit is dependent on sample size.

Further, this technology is applicable to any pathogen for which suitable antibodies are available.

4. Integrating waveguide technology in flow through format

IWT is also applicable to the analysis of multiple samples in a flow through format that would be suitable for continuous monitoring. A schematic of the concept is shown in Figure 8, where the sample enters from one end of the capillary and exits from the other end. Again, the capillary along with the sample enclosed by the capillary together acts as a waveguide. The excitation light is incident perpendicular to the capillary, while the fluorescent emission is gathered by a lens, passing through filters and lenses to a detector. The advantage of a flow-through format is the ability to couple high analyte sensitivity with automated detection. A prototype system has been fabricated and is currently in development.

4.1 Flow through format demonstration by using Cy5 fluorescence solution

Proof-of-concept has been demonstrated by passing through a serial dilution of Cy5 fluorescence dye ranging from 1 picomolar to 100 picomolar using a flow through format. Different concentrations of Cy5 were placed in vials respectively. The sample was pumped into the top of the capillary by a parastatic pump at a flow rate of 0.5 ml/min. The parastatic pump had two tubings, input and output. The input tubing was immersed into a vial with Cy5 while the output tubing was connected to the top of the capillary so that the sample circulated through the capillary. The sample and the capillary formed a waveguide which was illuminated by a 635 nm laser, and signal was detected by a PMT.

The tubing was initially immersed into PBS buffer, followed sequentially by Cy5 solutions of 1 picomolar, 10 picomolar, and 100 picomolar. Finally, the input tubing was left exposed to the air. A software was used to monitor the signal on a real time basis. Figure 9 shows the PMT signal changing over time, which correlated with the Cy5 sample concentration. The signal dropped to background level when there was no liquid (air only) in the flow cell.

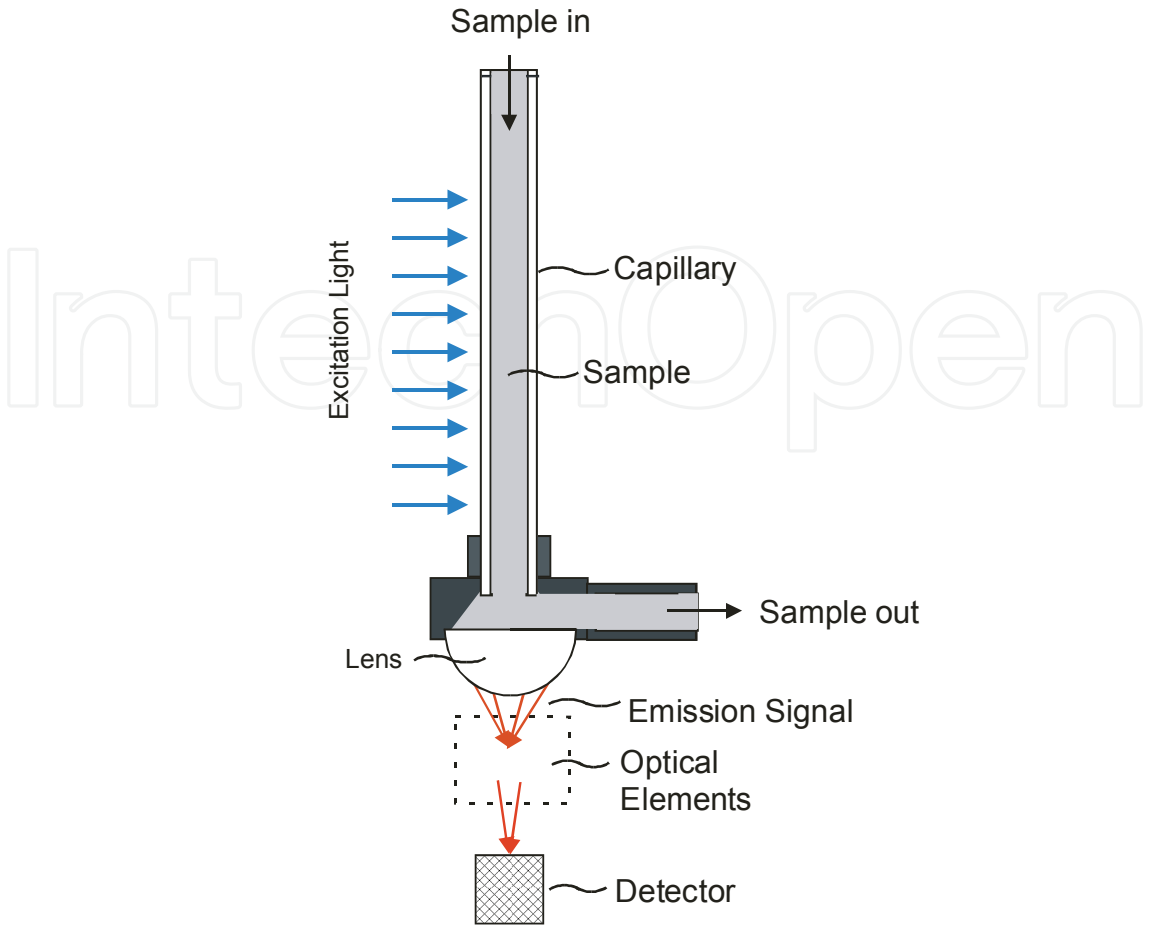


Fig. 8. Integrating Waveguide Technology in a flow through format.

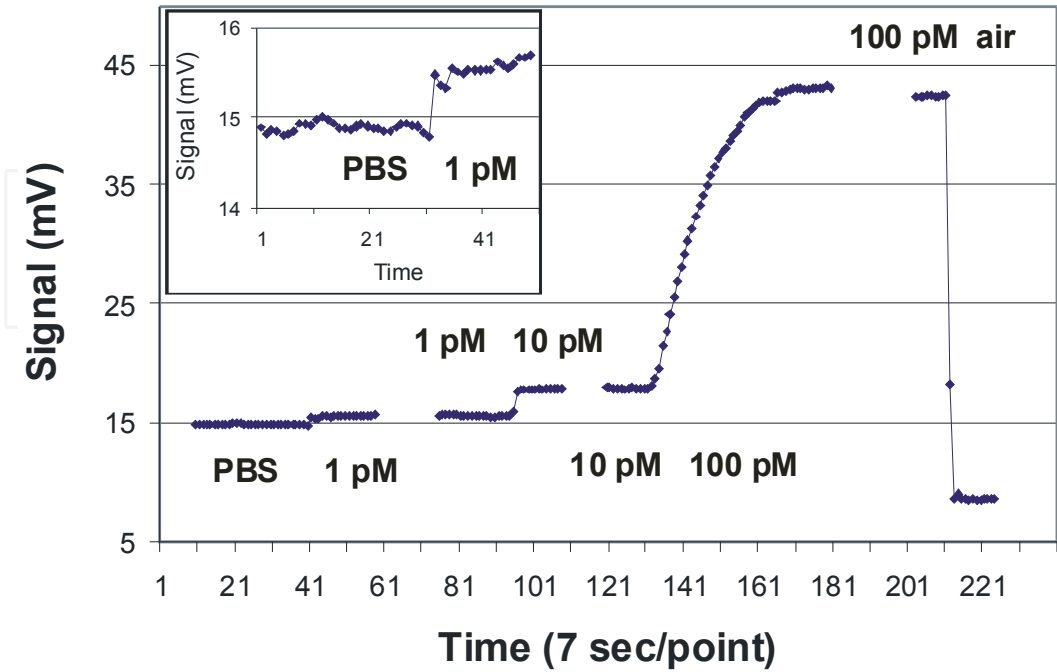


Fig. 9. Cy5 flow through data with a flow rate of 0.5 ml/min.

4.2 In-line detection of treated waste water using ATP assay

As fresh water supplies dwindle, it has become apparent that all water sources must be available for supply to both irrigation and consumption uses. Treating and supplying municipal water is well established throughout the world. However, the reclaiming of water from human and animal waste, most notably for reuse in crop irrigation, is coming to a front as water supplies become scarce.

A number of companies are studying the possibility of treating animal waste to remove chemical and bacterial contaminants, while retaining the water to use on crops. This water quality should conform with applicable national and international regulations and at a minimum follow the World Health Organization's guideline of 1000 coliforms/100 mL for irrigation water. This presents a problem for most water specialists, in that coliform determination is usually done off site and by specialized technicians using testing that requires several days for verification. Here is the point at which Creatv's Integrating Waveguide Technology flow-through biosensor will simplify and streamline the process. Combined with Creatv's buoyant silica microbubbles which are capable of capturing and concentrating various coliforms with high binding efficiency, this technologies can provide the means to concentrate bacteria in real time while providing an automated detection platform to determine coliform contamination. Creatv is collaborating with a developer of a waste treatment instrument, Spiralcat of Maryland, which has the need to test water reclaimed from animal waste using an in-line system.

Creatv has developed poly-L-Lysine coated buoyant silica microbubbles which can bind and retain gram negative and gram positive bacteria cells. The binding efficiencies of Poly-L-Lysine coated microbubbles are 90% for *E. coli* O111, 60% for *E. coli* O26, 58% for *Enterobacter* and 48% for *Salmonella*. To evaluate the use of microbubbles in a flow-through in-line testing environment, they used a cartridge system, as shown in Figure 10. The sample flows in from the top and leaves from the bottom. After the coliform are captured on the surface of microbubbles, an adenosine 5'-triphosphate (ATP) assay using Promega Bactiter-Glo™ reagents has been adapted to generate luminescence signal. There are two steps in the process, reaction and detection. After all input sample was flowed through the system, the cartridge was washed by PBS. The "capping" frit was then removed and an ATP Luminescence solution was added to the cartridge, the solution was incubated for 5 minutes at room temperature. After incubation, the samples are transferred to the in-line biosensor, where luminescence signal was tested and coliform concentration was determined.

The in-line biosensor shown schematically in Figure 11 is a portable instrument that can be used for onsite testing. After the ATP incubation was finished, the sample was injected into the testing cartridge, in this prototype a syringe was used to inject the sample. The glass tube and the sample in the testing cartridge act as a waveguide for gathering and guiding the luminescence signal to the PMT detector. Computer software controls the PMT, reads out the signal, and displays the result. When testing is done, sample is removed from the outlet by a syringe. The syringes are used to demonstrate the feasibility of the in-line testing concept, and are to be replaced by an automated pumping system, in future designs.

Creatv made several different testing cartridges, and tested them with ATP assays. The optimized cartridge used in the final design was built with a flat glass window at the exit. The testing cartridge consists of a sample chamber, a glass tube that holds the sample, an inlet, an outlet, and a flat glass exit window, as shown in Figure 12. The sample is injected into the chamber from the inlet located at the bottom of the testing cartridge. Sample and the

glass tube act as a waveguide for gathering and guiding luminescence to the exit window. The exit window is mounted on top of the input window of the PMT. Computer software controls the PMT, reads out the signal, and displays the result. When testing is done, sample is sucked up from the outlet through a capillary tube. The testing cartridge is reusable by washing the chamber several times with buffer. Buffer can be injected from the inlet and removed from the outlet.

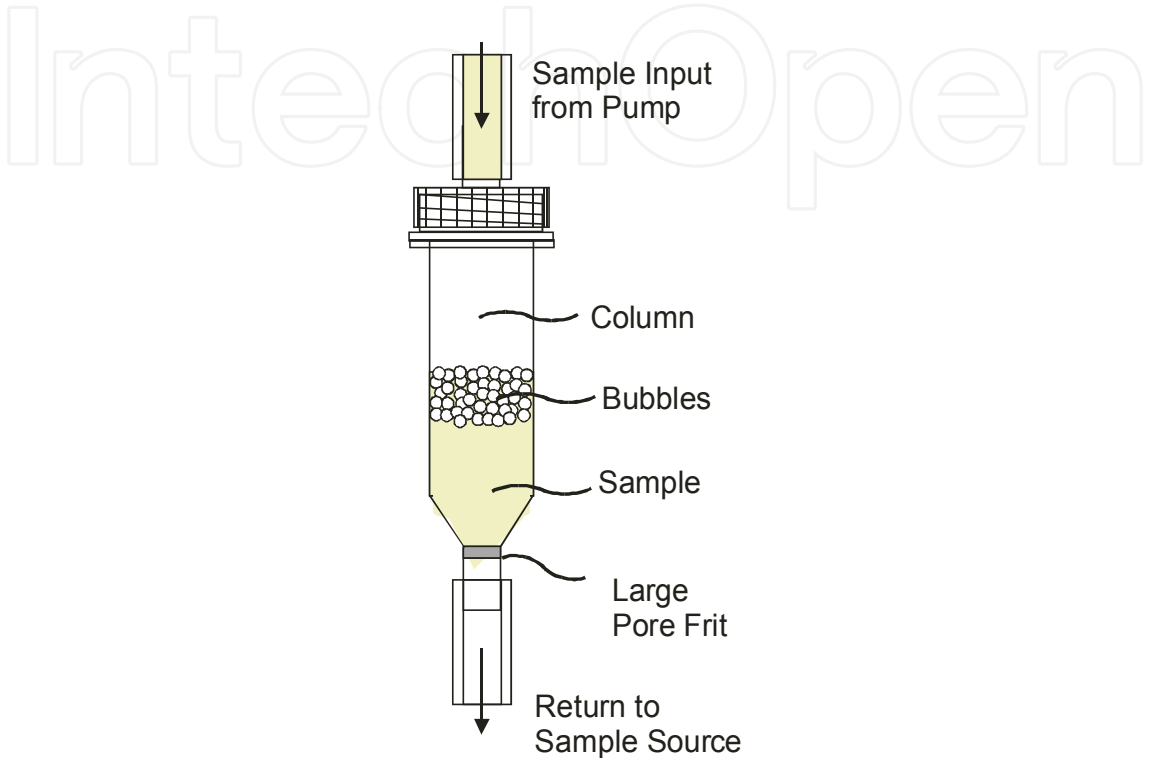


Fig. 10. Flow through cartridge with bubbles.

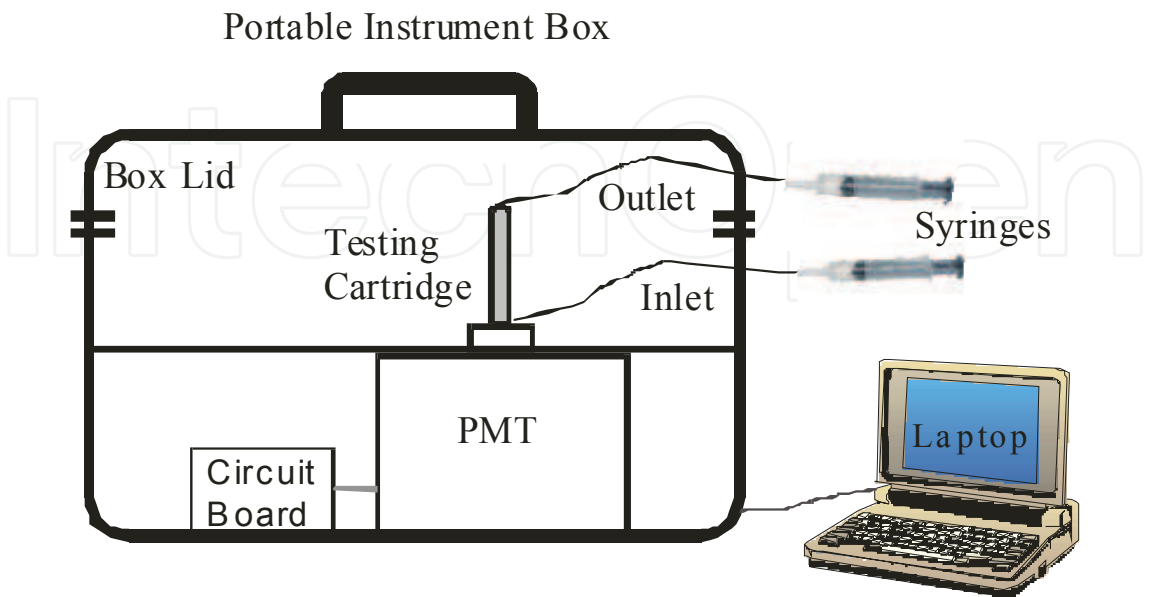


Fig. 11. Schematic of the detection instrument

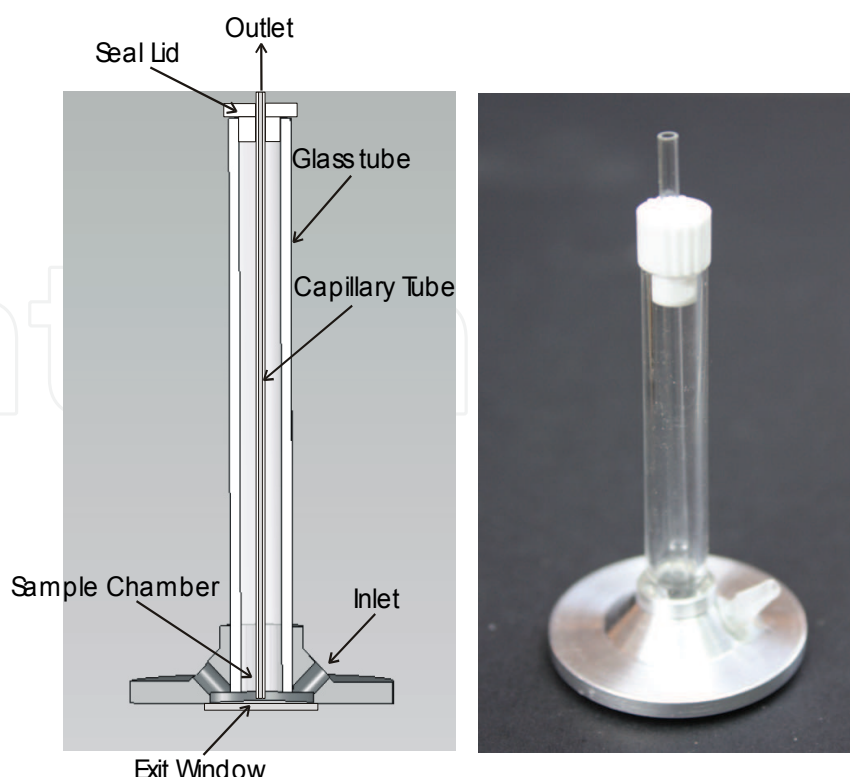


Fig. 12. Illustration of disposable cartridge construction and the photograph of the cartridge.

The full bubble and luminescence assay was run using sterile water to obtain a positive control. A summary of the developed protocol is:

1. Add 250 μ L microbubbles to a column with a large pore frit and a capping frit (Cartridge picture) and attach to a peristaltic pump.
2. Flow 100 mL sample at 2 mL/min through the column.
3. Wash the column with 5 mL PBS.
4. Add 100 μ L PBS and 100 μ L Luminescent Reagent to column, Incubate 5 minutes.
5. Remove 200 μ L of solution to a 96 well plate for detection.

The positive controls were run in 100 mL sample inputs, with cultured *E. coli* O157 serial diluted from 1:10 concentrations of 1 cfu/mL to 10^6 cfu/mL.

Using the samples from the various batch runs from Spiralcatt, this experiment was repeated by spiking *E. coli* into the Spiralcatt reclaimed water sample. In Figure 12, the above protocol was used and every concentration was done in triplicate. The “zero” concentration was run 26 times in order to determine limit of detection (LOD). As seen in Figure 13, R values of the pure culture using the bioluminescent reagent was +99%, the R value for *E. coli* spiked in water was 98%, and the R value of the full assay using a “real” output sample from Spiralcatt was 91%. Importantly, the capture rate of *E. coli* did seem to be lower in the Spiralcatt samples, but additionally the negative control of the Spiralcatt sample had a 40% lower background signal with a lower deviation between runs. These results show us that the Luminescent Bubbles assay is highly reproducible over time using “real” samples sampled on different days. Further, the assay is in a format which is adaptable into the prototype instrument we designed with no technician input needed to run a liquid sample.

Test results in Figure 13 show that the in-line luminescent detection system can detect 100 cells of *E. coli* in a 100 mL sample of Spiralcatt reclaimed water.

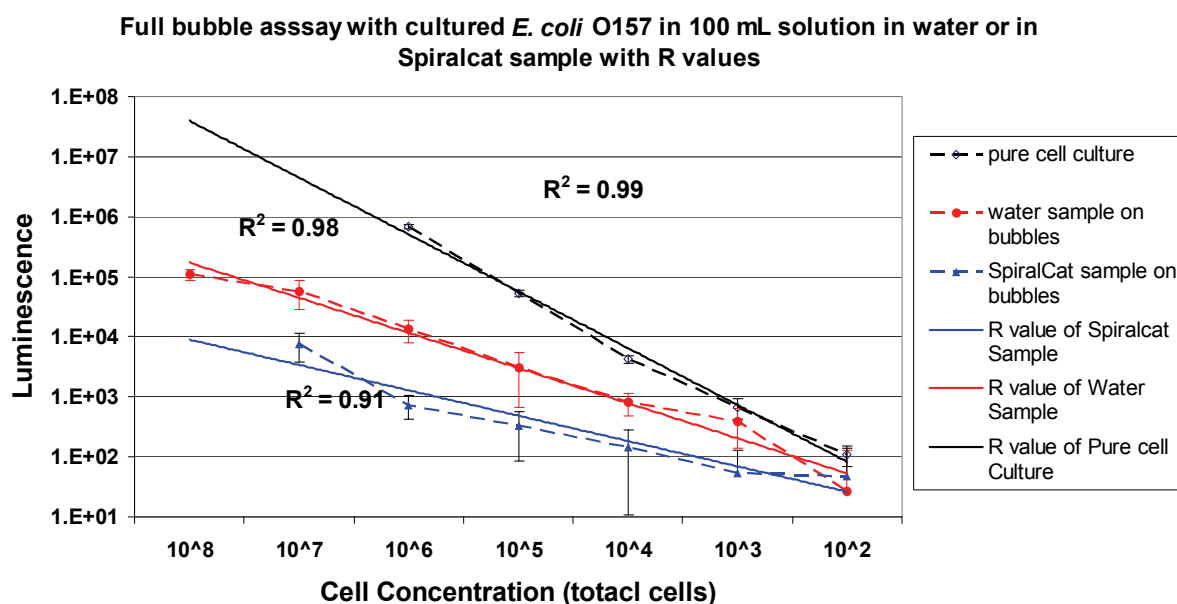


Fig. 13. Comparison of experiment results from the pure cell culture using the bioluminescent reagent, *E. coli* spiked in water using Creatv's microbubble assay, and the same assay using a reclaimed water sample from Spiralcat.

5. Conclusion

The ultra-sensitive fluorescence detection instrument, Signalyte™-II, is based on Integrating Waveguide Technology. The sensitivity is achieved by maximizing the signal while minimizing background noise. A very sensitive *E. coli* O157:H7 detection assay based on IMS techniques was developed and fluorescent signal was tested on Signalyte™-II. For IMS detection format, testing data demonstrate that as few as 10 cfu/ml of *E. coli* in a 1 ml sample is detectable. Another application of Integrating Waveguide Technology is in flow-through format. We demonstrate a real time fluorescence detection system using Cy5 dye. The flow through format can also be adapted to luminescence detection. We developed an in-line detection system of treated waste water using ATP assay. Testing result shows that 100 cells of *E. coli* in a 100 mL sample of reclaimed waste water is detectable.

6. Acknowledgement

The authors would like to thank our collaborators from US Department of Agriculture for their team work and using of their facilities. This work was supported by a grant from the National Institutes of Health Phase II SBIR Grant No. R44 CA094430, and a grant from National Science Foundation Phase I SBIR Grant No. IIP-0945747.

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Environmental Biosensors

Edited by Prof. Vernon Somerset

ISBN 978-953-307-486-3

Hard cover, 356 pages

Publisher InTech

Published online 18, July, 2011

Published in print edition July, 2011

This book is a collection of contributions from leading specialists on the topic of biosensors for health, environment and biosecurity. It is divided into three sections with headings of current trends and developments; materials design and developments; and detection and monitoring. In the section on current trends and developments, topics such as biosensor applications for environmental and water monitoring, agro-industry applications, and trends in the detection of nerve agents and pesticides are discussed. The section on materials design and developments deals with topics on new materials for biosensor construction, polymer-based microsystems, silicon and silicon-related surfaces for biosensor applications, including hybrid film biosensor systems. Finally, in the detection and monitoring section, the specific topics covered deal with enzyme-based biosensors for phenol detection, ultra-sensitive fluorescence sensors, the determination of biochemical oxygen demand, and sensors for pharmaceutical and environmental analysis.

How to reference

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Shelton, Shuhong Li, Peixuan Zhu, Daniel Adams, Platte Amstutz, Lynda Kiefer and Cha-Mei Tang (2011). Ultra-sensitive fluorescence detection using integrated waveguide technology, *Environmental Biosensors*, Prof. Vernon Somerset (Ed.), ISBN: 978-953-307-486-3, InTech, Available from: <http://www.intechopen.com/books/environmental-biosensors/ultra-sensitive-fluorescence-detection-using-integrated-waveguide-technology>

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