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ORIGINAL PAPER

Development of a Fluorescent Enzyme-Linked DNA Aptamer-Magnetic Bead Sandwich Assay and Portable Fluorometer for Sensitive and Rapid *Listeria* Detection

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Abstract A fluorescent DNA aptamer-magnetic bead sandwich assay was developed to detect listeriolysin O (LLO) protein from pathogenic *Listeria* bacteria using a peroxidase-linked system, Amplex Ultra Red (AUR; derivatized resazurin) substrate, and a custom-designed handheld fluorometer. The assay is highly sensitive with demonstrated limits of detection (LODs) in the range of 4 to 61 *L. monocytogenes* cells or the equivalent LLO produced by 4 to 61 cells on average in separate titration trials. Total assay processing and analysis time was approximately 30 mins. The assay has demonstrated the ability to detect 6 species of *Listeria* as desired by the USDA's Food Safety Inspection Service (FSIS). The portable system was designed to be used primarily with surface swab samples from fomites, but it can also be used to assess enrichment cultures. The minimal time to detect a positive enrichment culture in our hands from an initial 10 cell inoculum in 200 ml of broth has been 8 h post-incubation at 37 °C in shaker flask cultures. An optional automated magnetic bead assay processing and wash device capable of simultaneously processing 6 samples with low and consistent fluorescence background for higher volume central laboratories is also described.

Keywords Amplex red · Aptamer · Assay · *Listeria* · Listeriolysin · Magnetic bead · Portable · Resazurin · SELEX

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Introduction

The U.S. Dept. of Agriculture's Food Safety Inspection Service (USDA FSIS) has a zero tolerance policy for several pathogenic bacterial species in foods and therefore requires extreme sensitivity in order to detect *Listeria monocytogenes* in foods or on fomites and to confidently clear foods as quickly as possible for sale to the public [3, 6, 11, 15, 25–27, 32]. The genus *Listeria* currently contains at least 10 officially recognized species of which 6 species are the focus of food safety testing (*L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*) because their presence could mean that *L. monocytogenes* is present. Of these 6 Gram positive bacillus species only two species are known pathogens. *L. monocytogenes* is a dangerous human pathogen with mortality in the range of 20–30% [2, 10, 11, 31] and *L. ivanovii* is a non-human animal pathogen. While most serious cases of human listeriosis involve pregnant women, newborns, elderly and immunocompromised patients, the threat of listeriosis from the psychrophile *L. monocytogenes* induced the USDA to add a specific bacteriophage to refrigerated "meats ready to eat" (MREs) in 2000 to reduce the risk of listeriosis [16]. However, *Listeria* bacteria can contaminate other foods such as dairy products [2], frozen foods [2] and fresh produce with complex surfaces such as cantaloupe rinds [10] which are difficult or impossible to wash effectively.

One of the main mechanisms by which *Listeria* bacteria can enter the human food supply is via food processing surfaces and other fomites [3, 15, 26, 33], making surface hygiene critical to a safe food supply. Even well-cleaned stainless steel and other hard flat surfaces, which are seemingly easy to clean, can be susceptible to contamination by *Listeria* bacteria which can deposit in microscopic scratches

and grooves in the surface and later be transferred into or onto foods [3, 15]. Thus, establishing that a surface is truly clean and free of *Listeria* is desirable in the food safety testing industry. And while *L. monocytogenes* is the human pathogen in the *Listeria* genus, the food safety industry tests at the genus level to ensure that no *Listeria* species are detected [6, 26].

As such, we set out to develop a rapid and highly portable system based on our previous capture aptamer-magnetic bead (MB) and fluorescent reporter aptamer sandwich assay methods for *Campylobacter* [8] and *Leishmania* parasites [9]. We surmised that these former technologies could be adapted to the use of surface swabs or "spongicles" which can be squeezed to yield 2 ml of buffer which has interacted with the food processing surface. We also envisioned our portable system being able to detect *Listeria* bacteria in 1–2 ml samples drawn from enrichment broth cultures over time to detect *Listeria* or other foodborne pathogens in a central laboratory environment.

For this particular project, we developed aptamers against listeriolysin O (LLO) which is a well-characterized cytolysin expressed in the pathogenic species of *Listeria* (*L. monocytogenes* and *L. ivanovii*). LLO is attributed with the primary role in *Listeria*'s virulence, because its pore-forming ability enables phagosome lysis and escape of *Listeria* bacteria into the phagocyte's cytoplasm with subsequent spread to other cells [4, 5, 18]. Although LLO is an inducible secreted protein, especially while *Listeria* bacteria exist in phagosomes, it has been used as a target for immunoassay of *L. monocytogenes* in broth cultures [11, 25] and there is evidence that LLO exists in at least small amounts adhered to the surface of *Listeria* bacteria [4], thereby making it an excellent target for detection of *Listeria* pathogens in general.

In recent years, aptamers have increasingly been investigated as potential replacements for antibodies to detect foodborne and waterborne pathogens in various assay formats [7, 8, 12–14, 19–21, 23, 24, 27–32, 34] for their various advantages versus antibodies including lower overall costs, obviating host animals, and greater reproducibility by chemical synthesis [22]. As such, a number of important aptamer DNA sequences have been reported in the open literature. However, due to the proprietary nature of the highest affinity patent-pending LLO aptamer sequence (hereby designated LLO-3) used in our sandwich assay and its potential commercial value, that DNA aptamer sequence cannot be revealed at present. Instead, the focus of the present report is on the aptamer-based fluorescence assay's performance and characterization especially because it enables a platform sensor and assay technology for extremely sensitive detection of many foodborne, waterborne, and perhaps bloodborne pathogens.

Materials and Methods

DNA Aptamer Development, Cloning and Sequencing

Listeriolysin O (LLO, Cat. No. L2650-50) was purchased from United States Biological, Inc. Ten μg of LLO protein was immobilized onto~2×10^7 Dynal® M280 (2.8 μm diameter) tosyl-coated magnetic beads (MBs, Life Technologies Inc., Carlsbad, CA) for 1 h at room temperature on a rotating mixer. LLO-conjugated MBs (LLO-MBs) were collected using a Dynal® DynaMag-2 magnetic rack and washed in 1 ml of sterile phosphate buffered saline (PBS) three times before storage at 4 °C and use in Systematic Evolution of Ligands by EXponential enrichment (SELEX) aptamer development.

All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT; Coralville, IA). MB-based SELEX was performed using 160 nmoles of 72 base SELEX template library sequence: 5′-ATCCGTCACACCTGCTCT-N_{36}-TGGTGTTGGCTCCCGTAT-3', where N_{36} represents the randomized 36-base region of the DNA library. Primer sequences were: 5′-ATACGGGAGCCAACACCA-3′ (designated forward or F) and 5′-ATCCGTCACACCTGCTCT-3′ (designated reverse or R) to prime the template and nascent strands, respectively. The lyophilized 160 nmole random library template was rehydrated in 500 μl of sterile TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0), mixed with 500 μl of PBS and heated to 95 °C for 5 min to ensure that the DNA library was completely single-stranded and linearized. After brief cooling, the 1 ml of DNA template solution was added to~2×10^7 LLO-coated MBs and mixed at room temperature for 1 h. Following interaction with the randomized DNA library template, DNA-LLO-MB complexes were separated from unbound DNA by collection on the DynaMag-2 magnetic rack and the supernatant was aspirated and discarded. DNA-LLO-MBs were then washed three times in 100 μl of PBS with vigorous vortex mixing followed by magnetic collection. Following the third wash, DNA aptamer-LLO-MBs were resuspended in 150 μl of sterile, nuclease-free water and heated at 95 °C for 5 min to release bound DNA aptamers. The hot supernatant was collected and 5 μl aliquots of eluted DNA were PCR-amplified in 100 μl reaction volumes using a SpeedStar® (hot start) PCR kit (Takara Bio Inc., Shiga, Japan). PCR was conducted as follows: an initial 94 °C phase for 5 min, followed by at least 20 cycles of 30 s at 94 °C, 30 s at 60 °C, and 15 s at 72 °C followed by a 72 °C completion stage for 5 min, and refrigeration at 4 °C.

PCR amplicon bands were verified to be 72 bp after each round of SELEX by electrophoresis in 2% TAE (Tris-Acetate EDTA) agarose gels with ethidium bromide staining. If more than one band emerged, the 72 bp band was excised on a UV transilluminator with a sterile razor blade and aptamers from

the gel slice were eluted into 50 μl of Qiagen elution buffer using a Qiagen Gel Purification spin column (Germantown, MD). If the aptamer amplicon was faint or not visible in the gel, the amplicon was PCR amplified for several more rounds. Negative control PCR reactions without the SELEX template were run to ensure that nonspecific DNA was not amplified. For the second and subsequent rounds of SELEX, the 50 μl of elution buffer were diluted in 50 μl of PBS, followed by dilution in 800 μl of PBS and addition of 100 μl of fresh LLO-MB conjugate (~2×10^7 MBs). This constituted the first of 8 rounds of LLO-MB-SELEX. Following round 8, aptamers were cloned into chemically competent *E. coli* using a Lucigen GC cloning kit (Middleton, WI) according to the manufacturer's protocol and clones were sent to Sequetech, Inc. (Mountain View, CA) for proprietary GC-rich DNA sequencing.

ELISA-like ("ELASA") Aptamer Microplate Affinity Ranking

L. monocytogenes ATCC No. 19115 was grown on blood agar plates at 37 °C overnight and diluted in sterile PBS to prepare stock cultures which were stored at 4 °C until used and quantified by spread plate counts performed in triplicate and averaged. To evaluate relative affinity rankings for each of the candidate LLO aptamers, an enzyme-linked aptamer sorbent assay (ELASA) was conducted as previously reported [7, 9] by first immobilizing 1000 live *L. monocytogenes* cells per well in 100 μl of 0.1 M $NaHCO_3$ (pH 8.5) overnight at 4 °C in covered flat-bottom polystyrene 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). The plates were decanted and washed 3 times in 200 μl of PBS. Wells were then blocked with 150 μl of 10% ethanolamine in 0.1 M $NaHCO_3$ for 1 h at 37 °C followed by 3 more washes with 200 μl of PBS as before. A total of 20 different 5′-biotinylated LLO aptamers were synthesized in 96 well plates by IDT and rehydrated in 100 μl of PBS for 1 h with gentle mixing on an rotary mixer and applied to their corresponding microplate wells at 1 nanomole per well for 1 h at room temperature with gentle mixing. The plates were decanted and washed 3 times in 200 μl of PBS for at least 5 min per wash with gentle mixing. One hundred μl of a 1:5,000 dilution of streptavidin-peroxidase from a 1 mg/ml stock solution (Thermo Fisher Scientific, Product No. 21126) in PBS was added per well for 30 min at room temperature with gentle mixing. The plates were decanted and washed 3 times with 200 μl of PBS per well as before. One hundred μl of One-Component® ABTS substrate (Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD) which had been equilibrated to room temperature was added to each well and incubated for 15 min at room temperature. Reactions were halted by addition of 100 μl of 1% SDS as the strongest reactions approached an absorbance of 2.0 at 405 nm using a Thermo Electron MultiSkan™ microplate reader (Thermo Fisher Scientific; Waltham, MA).

Portable Fluorescence (FLASH) Reader and Automated Magnetic Assay Processor

A custom handheld fluorometer referred to as the FLASH (FLuorescence Assay System Handheld) reader was designed and built by Nanohmics, Inc. (Austin, TX) as previously described [9] to provide very sensitive and quantitative fluorescence measurements. The core functionality of the FLASH reader was provided by a computerized numerical control (CNC)-machined aluminum optomechanical assembly, featuring an integral epifluorescence optical configuration. The instrument features a sample receptacle enabling the measurement within standard cuvettes, with a primary focus within the bulk solution. The FLASH reader system was optimized to coincide as closely as possible to the excitation and emission peaks of Amplex Ultra Red (AUR, ex/em 568/581 nm). Thus, fluorescence excitation was provided by a 2.6 mW epoxy-encased light emitting diode (LED525E, Thorlabs Inc.) with center wavelength of 525 nm. The excitation source was passed through a single bandpass filter (FF01-525-15, Semrock Inc.), centered at 525 nm with 15 nm bandwidth FWHM. A 45-degree dichroic beam-splitter with a cut-on frequency of 565 nm (565DRLPXR, Omega Optical) which enabled separation of the excitation and emission bands, while a 575 nm cut-on longpass filter (575ALP, Omega Optical Inc.) provided rejection of out-of-band light within the collection optics chain. A photomultiplier tube (PMT; Cat. No. H10722-01, Hamamatsu Corp.) provided signal transduction of the collected optical signal.

A custom fluorescence measurement printed circuit board was designed to provide control of the excitation source (LED), the PMT gain, and 16-bit ADC conversion of the PMT signal. The LED source was modulated with a 5 kHz sinusoidal signal, and the resulting PMT signal was passed through a 10 kHz anti-aliasing filter. This signal was deconvolved via a Fast-Fourier Transform (FFT), which provided enhanced signal-to-noise ratio via synchronous detection of the signal component at the modulation frequency. Control of these electrical subsystems has been achieved through use of an integrated single board computer, running custom written software that enables the adjustment of both PMT and excitation sinusoidal gain settings.

A proprietary automated magnetic assay processor instrument was also engineered and developed by Nanohmics, Inc. for this project to perform all of the sample mixing, MB collection, washing, and other assay steps for up to 6 assays conducted simultaneously. Within the instrument, a network of tubing (Pharmed BPT, Saint-Gobain) provided fluid paths for protocol execution, while peristaltic pumps (P625, Instech) and solenoid-actuated pinch valves (EW-98302-02, Cole

Parmer) provided fluid pumping and control. Each instrument module featured a network of tubing, valves, a single peristaltic pump (Instech P625), and a magnetic collection assembly consisting of a neodymium magnet array that is actuated using a solenoid linear actuator (McMaster, No. 70155 K78). Electronic control was facilitated via use of an embedded programmable logic controller (PLC, DirectLogic 205, Automation Direct). This PLC featured custom-written firmware that accepted commands from the desktop-based user software over a USB connection which actuated the corresponding electro-fluidic hardware.

Enzyme-linked Fluorescent Aptamer-Magnetic Bead LLO Sandwich Assays (Manual, Automated and Timed Enrichment Culture Studies)

The manually processed assay limit of detection (LOD) and cross-reactivity studies were conducted at Metrix360 Laboratories (San Antonio, TX) for third party validation. The basic assay protocol has been previously described [9]. At Metrix360 Laboratories, live *L. monocytogenes* ATCC 19115 bacterial subcultures were streaked onto Tryptic Soy Agar (TSA) plates and cultured at 36 °C±1 °C for 24 h±2 h. Stock cultures were lifted from a TSA plate in 1 ml of sterile PBS and cell density was adjusted (diluted) to reach an absorbance of 0.45 to 0.50 at 600 nm which equated to ~10^9 bacteria per ml for further dilution in PBS to the desired cell concentrations (target ranges; 0, 10, 100 and 1000 cfu/100 μl). LOD studies were conducted as 3 independent titration experiments or trials in which 9×1 ml samples per cell concentration were drawn after thorough mixing of the bacterial suspension. Samples were processed and assessed for fluorescence intensity by the FLASH reader and immediately after that by a Quantifluor™ handheld fluorometer (Promega, Inc.)in most cases as described below. All other studies including the autoprocessor background variation and timed enrichment culture studies were conducted at Pronucleotein Biotechnologies, Inc.

For manual assay processing, 20 μl of fresh 5′-biotinylated LLO-3 capture aptamer-streptavidin-Dynal/Life Technologies M280 (2.8 micron) magnetic beads (~4×10^7 aptamer-MBs) were added to 1 ml of sterile PBS containing live bacteria at the concentrations indicated in the figures or figure legends. Tubes were mixed gently on a rotating mixer for 15 min at RT. A Dynal MPC-S® or comparable magnetic rack was used to collect MBs in microcentrifuge tubes for 1 min. The supernatant devoid of MBs was carefully aspirated with a 1 ml pipette tip and discarded in 5% bleach water solution. Five hundred picomoles of 5′-biotinylated reporter LLO-3 aptamer in PBS was added to each tube and tubes were gently mixed again for 10 min at RT. MBs were again collected on the magnetic rack for 1 min. MBs were washed 3 times for 1 min per wash in 1 ml of PBS and resuspended by gentle pipetting 3 times with magnetic collection for 1 min between each wash. The supernatant was removed and the MBs with aptamer-captured soluble LLO and potentially some whole bacteria having LLO on their surface [4] were resupended in 500 μl of 0.25 μg/ml of streptavidin-horseradish peroxidase (SAv-POx) in PBS per sample for 10 min at RT with gentle mixing. MBs were again collected using the magnetic rack for 1 min per sample and washed 3 times in PBS with resuspension in fresh 1 ml volumes of PBS and magnetic collection as before. Amplex® Ultra Red (AUR; 1 mg, Life Technologies Inc.) was stored at −20 °C, thawed just prior to use and dissolved in 100 μl of pure DMSO by brief vortex mixing. Stock AUR solution was diluted 1:1000 in PBS prior to use along with 25 μl of 3% H_2O_2 per ml of diluted AUR. MBs were collected using the magnetic rack and resuspended in 1 ml of diluted AUR solution with 0.075% H_2O_2, vortex mixed on the lowest mixer setting for 5 s and transferred to polystyrene cuvettes (Thermo Fisher Scientific No. 14-955-129) containing an additional 1 ml of diluted AUR plus 0.075% H_2O_2 solution. Fluorescence was assessed within the first 1 min of development using the FLASH reader with PMT settings between 20 and 30% of maximum voltage. Metrix360 Laboratories also validated the cell concentrations by subsequent spread counts which were assessed at 48 h±4 h after growth at 36±1 °C. The identity of each bacterial species was confirmed by modified USDA FSIS methods (e.g., for *Listeria monocytogenes* catalase, oxidase and other biochemical testing using the Remel MICRO-ID *Listeria* Microbiological Identification kit). Statistical analysis of the fluorescence values and colony counts from the various bacterial concentration groups was conducted by one-way ANOVA followed by a Dunnett's post-hoc test.

For the timed enrichment culture studies, an average of 10 *L. monocytogenes* ATCC 19115 cells were inoculated (spiked) into 200 ml of sterile Tryptic Soy Broth (TSB) in sterile 400 ml Erlenmeyer flasks plugged with sterile cotton and cultured at 37 °C with gentle shaking for up to 9 h. Three x 1 ml samples were drawn from two separate spiked and control (blank) shaker flasks each hour for 9 h for the LLO sandwich assay and fluorescence assessment using the FLASH reader. In addition, 100 μl samples were drawn hourly from the flasks for spread plate counts on blood agar plates cultured at 37 °C overnight to verify that the *Listeria* culture was growing and to determine approximately how many cells were detected each hour.

For automated assay processing, the Nanohmics, Inc. 6-module automated assay processor rehydrated two separate tubes of capture aptamer (LLO-3)-MBs and reporter aptamer (also LLO-3)-5′- biotin-streptavidin-peroxidase conjugate and performed most of the manual assay steps described above. The only steps not performed by the automated assay device were addition of the test sample containing *Listeria* bacteria to the lyophilized capture aptamer-MB reagent and the addition

of AUR just prior to reading fluorescence in the FLASH reader. All capture and reporter assay reagents were lyophilized overnight in 5% trehalose and sterile PBS at Quality Bioresources, Inc. (Seguin, TX).

Results

Figure 1 illustrates the results of ELASA rankings for relative aptamer binding affinities of the 20 LLO aptamer candidates versus 1000 immobilized *L. monocytogenes* cells per well. From this analysis, LLO-3 emerged as the marginal top candidate, although LLO-15 and several other aptamers appeared very attractive as well. While ELASA is a good initial screening tool, in our experience, ELASA alone cannot be used to predict how all aptamer candidates will perform in the MB sandwich assay. As a result of extensive empirical evaluations of various pairings of the top 10 aptamers from Fig. 1, which is too extensive to report here, LLO-3 paired with itself in both the capture aptamer-MB and reporter aptamer roles emerged as the best overall aptamer combination to proceed into full assay development and characterization.

Figure 2 illustrates the appearance of the FLASH reader and some of its physical features as previously reported in this journal [9] along with two of the control screens accessible via the on board computer. The FLASH reader was used to quantitate fluorescence of the *Listeria* LLO sandwich assay tests reported herein. When all of the *Listeria* assay data across a broad range of low to high fluorescence values (total of 306 samples) was compiled and compared to readings taken with a commercially available handheld fluorometer called the Quantifluor™ (Promega Corp.) taken immediately after the FLASH reader readings, it became apparent that the instruments were quite comparable (Fig. 3). Data in Fig. 3 demonstrate that the custom FLASH reader and commercial Quantifluor™ instrument both gave linear responses over a broad range of fluorescence values from 0 to~35,000 relative fluorescence units when the two instruments were calibrated to read with approximately the same sensitivity based on an AUR fluorescence standard sample. The R^2 correlation coefficient of 0.9792 solidified confidence in the FLASH reader for accurate quantitation.

Once confidence in the FLASH reader's quantitation was established, it and the Quantifluor™ were used to assess fluorescence titrations and LODs at Metrix360 Laboratories for manually processed assays in PBS as revealed in Fig. 4. It should be noted that while the results are expressed in terms of cells or cfu per 100 μl, since LLO is the actual target protein which is mostly secreted but can also be cell wall-associated [4], detection is really based on the number of cell equivalents which produced the amount of LLO detected. However, some actual whole *Listeria* cells may also be captured by the aptamer-MB conjugates via LLO on the cell surface. Whole *Listeria* cell capture was also confirmed by several experiments (not presented herein) in which the collected magnetic beads were plated onto blood agar and produced colonies after overnight incubation at 37 °C. Regardless of what is actually captured (soluble LLO proteins or whole bacterial cells), Figure 4 reveals a highly sensitive assay which demonstrated some variations in the LODs.

Table 1 reveals that when analyzed statistically, the assay was able to detect as low as 4±2*L. monocytogenes* cells or LLO from an average of 4 cell equivalents above background ($p<0.001$ by ANOVA) in the second trial, but only an average of 19 and 61 cells or cell equivalents in the other two trials. Hence, it is difficult to pin down the assay to one specific LOD value, but the range appears to be about 4 to roughly 61 cells or LLO-producing cell equivalents on average. When the

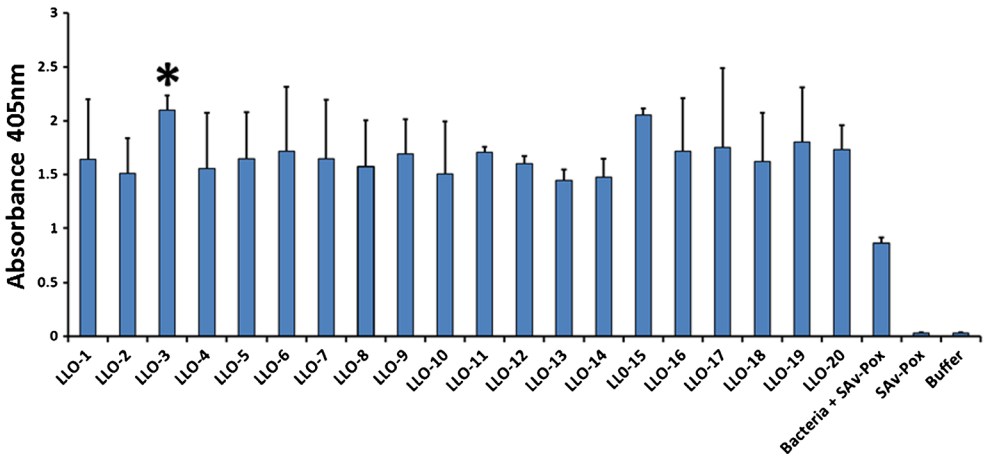

Fig. 1 Results of ELASA rankings for relative aptamer binding affinity of the 20 LLO aptamers versus 1000*L. monocytogenes* ATCC 19115 cells immobilized per well. *Bar heights* represent the average absorbance at 405 nm of 2 independent trials and error bars represent the range of the absorbance values for the 2 trials. Results of negative controls consisting of PBS buffer, streptavidin-peroxidase (SAv-Pox) and SAv-Pox plus 1000*L. monocytogenes* bacteria per well without aptamers are also shown. The *asterisk* indicates the highest overall affinity and most consistent (LLO-3) aptamer which eventually served as both the capture and reporter aptamer in the sandwich assay

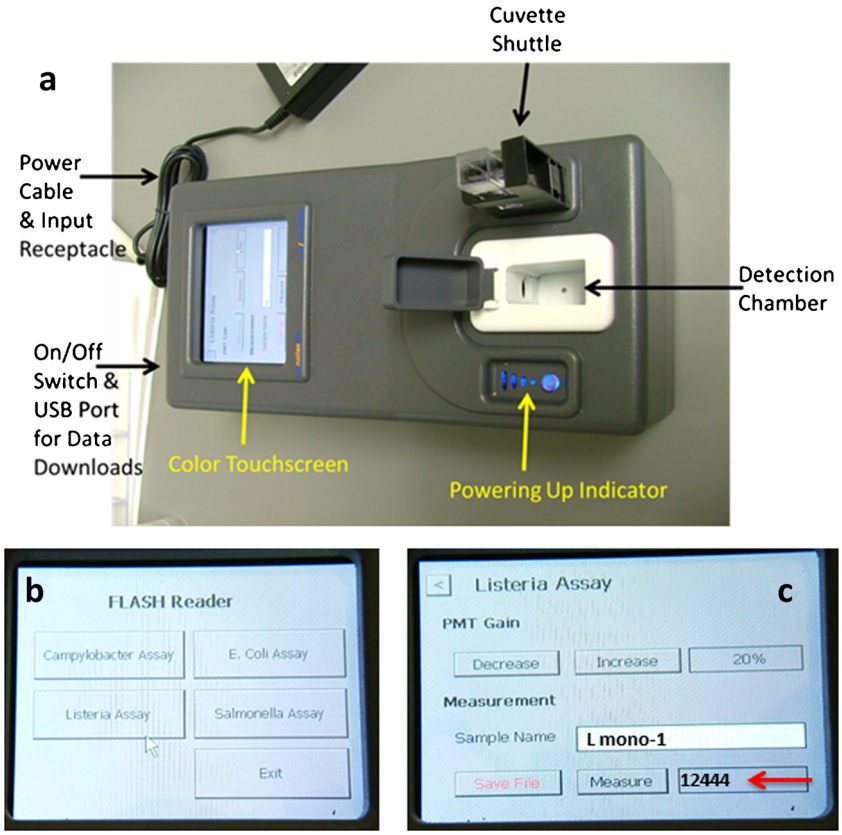

Fig. 2 **a.** Top view of the FLuorescence Assay System Handheld or "FLASH" reader showing major features as indicated. **b**. Initial foodborne pathogen assay selection menu. **c**. *Listeria* assay control screen showing buttons to control the photomultiplier (PMT) voltage or sensitivity, sample and file name boxes, measurement actuating button and fluorescence output value (indicated by *red arrow*)

three separate trials shown in Table 1 are averaged together as shown in the lower right quadrant of Fig. 4, it is actually possible to build a statistical case that the LOD is 5 cfu per 1 ml sample since the ANOVA indicates that the fluorescence for 5 cfus was statistically above background with a p value< 0.001 (data not shown).

Clearly, the LLO sandwich aptamer-MB assay can be quite sensitive, but just as important as sensitivity for such an assay is the quality of specificity. Extensive inclusivity and exclusivity (cross-reactivity) data presented in Fig. 5 indicate that the LLO assay has a strong preference for the six *Listeria* species which were assayed, but not the other species used as samples. It is important to note that the fluorescence values were not normalized by cell concentration (cfu plate counts), but the fluorescence data resulted from a fairly narrow average cell concentration range across all species of 26 to 85 cfu per

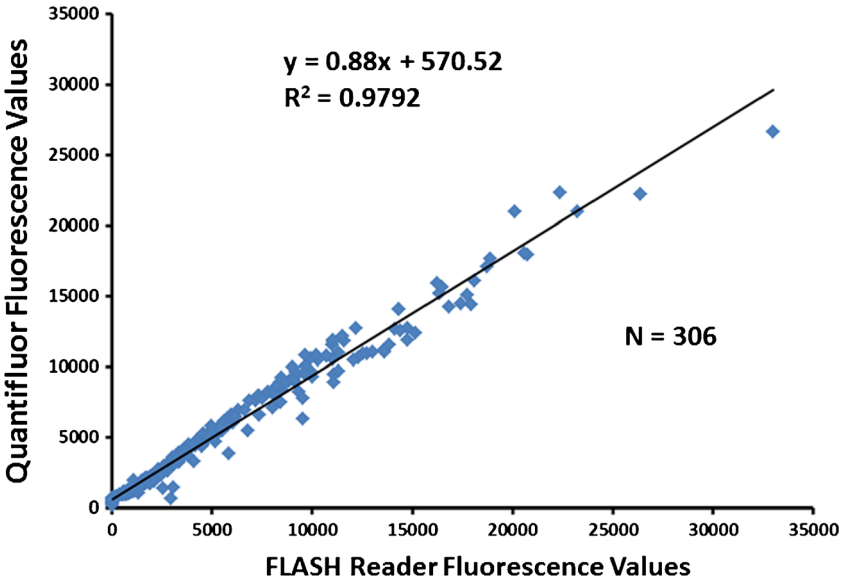

Fig. 3 Comparison of fluorescence values acquired by the FLASH reader immediately prior to the commercially available Quantifluor™ handheld fluorometer for 306 *Listeria* and other bacterial assays conducted with the LLO-3 aptamer-MB AUR fluorescent sandwich assay. The high correlation coefficient (R^2=0.9792) confirmed that the FLASH reader was performing as needed against a standard instrument. Both instruments were calibrated against the same fluorescent AUR sample exposed to SAv-POx and H_2O_2

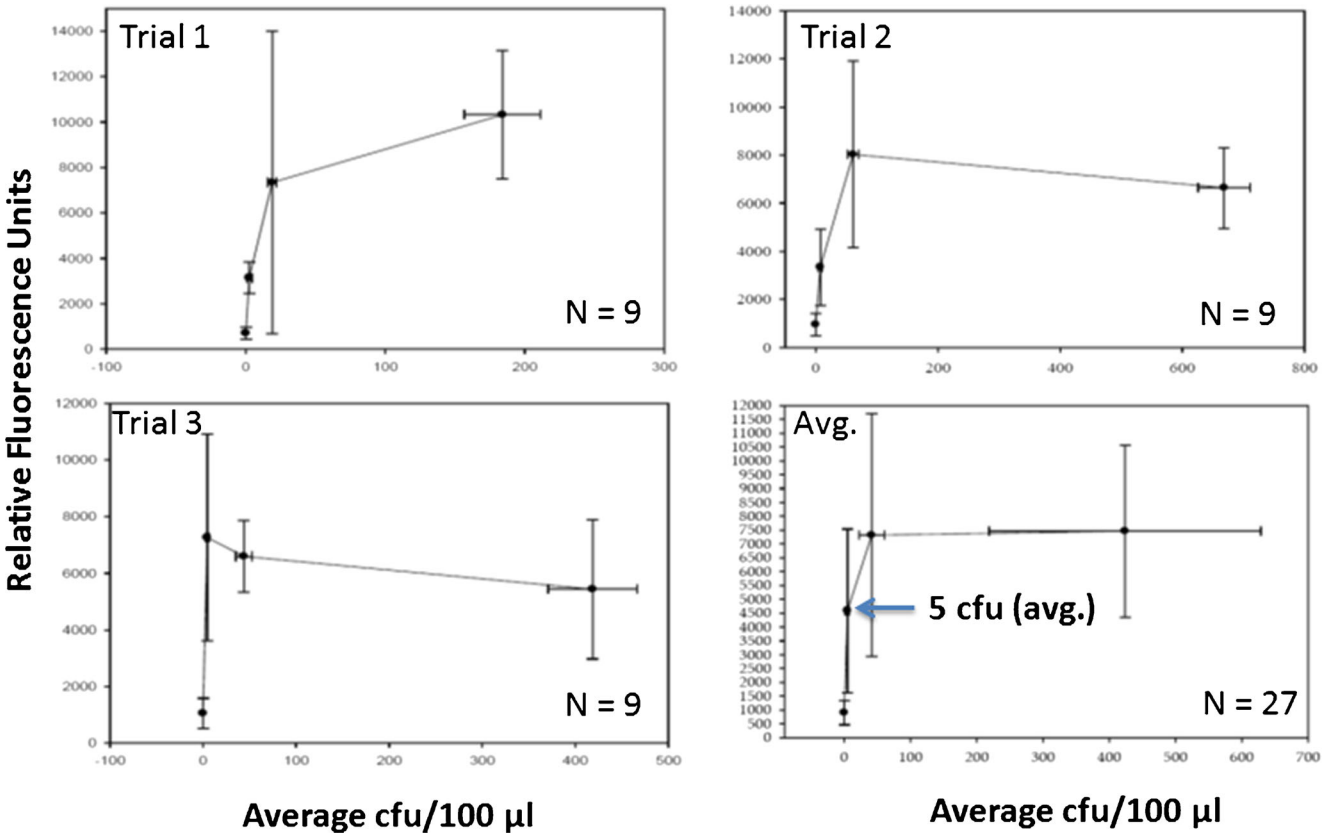

Fig. 4 Fluorescence versus colony counts (cfu/100 µl) for 3 separate trials of the LLO aptamer-MB AUR sandwich assay. Average colony counts are plotted on the x-axis versus average fluorescence for 9 samples (N=9) drawn per level of *L. monocytogenes* bacteria. The *graph in the lower left quadrant* resulted from compiling and averaging results from the 3 trials (N=27) and indicates that overall the LOD may be as low as 5 cfu/sample or the equivalent LLO produced by 5 cells. The 3 trials suggest that the LOD may be as low as 4 to 61 cells or the amount of LLO produced by that number of cells. Averages and standard deviation *error bars* are plotted

sample (data not shown). Figure 5 therefore clearly indicates that all of the *Listeria* species examined showed fluorescence intensities significantly above background fluorescence while the other non-*Listeria* species were not significantly above background. Therefore, the assay appears to be specific at the *Listeria* genus level as desired by the food safety testing industry.

One of the most difficult hurdles in this assay development project was to keep background fluorescence as low and consistent as possible and accurately quantify fluorescence on the low end of bacterial cell concentrations. The inconsistencies were thought to arise from three major sources: 1) human pipetting error, 2) inconsistent washes between different human users, and 3) variability in LLO production and the number of *Listeria* bacteria potentially captured on aptamer-MBs between different assay runs. To address these problems, we teamed with Nanohmics, Inc., designed and built the 6-module assay autoprocessor diagramed in Fig. 6 and illustrated in Fig. 7. Figure 6 gives a schematic of liquid buffer flow through one autoprocessor module from the reservoir through

Table 1 Summary of fluorescence and colony count data from *L. monocytogenes* LOD assay trials

Trial 1		Trial 2		Trial 3	
Avg. cfu±SD	Avg. Fl. ± SD	Avg. cfu±SD	Avg. Fl. ± SD	Avg. cfu±SD	Avg. Fl. ± SD
0±0	704±268	0±0	1,054±531	0±0	957±456
2±2	3,141±694	4±2	7,261±3,642*	8±3	3,347±1,589
19±3	7,335±6,665*	44±9	6,595±1,274*	61±9	8,027±3,877*
184±27	19,320±2,833*	419±48	5,433±2,456*	668±42	6,642±1,673*

Mean values for 9 samples are reported ± standard deviations of the means. *Asterisks* (*) denote average fluorescence values significantly above background at p=0.001 according to ANOVA

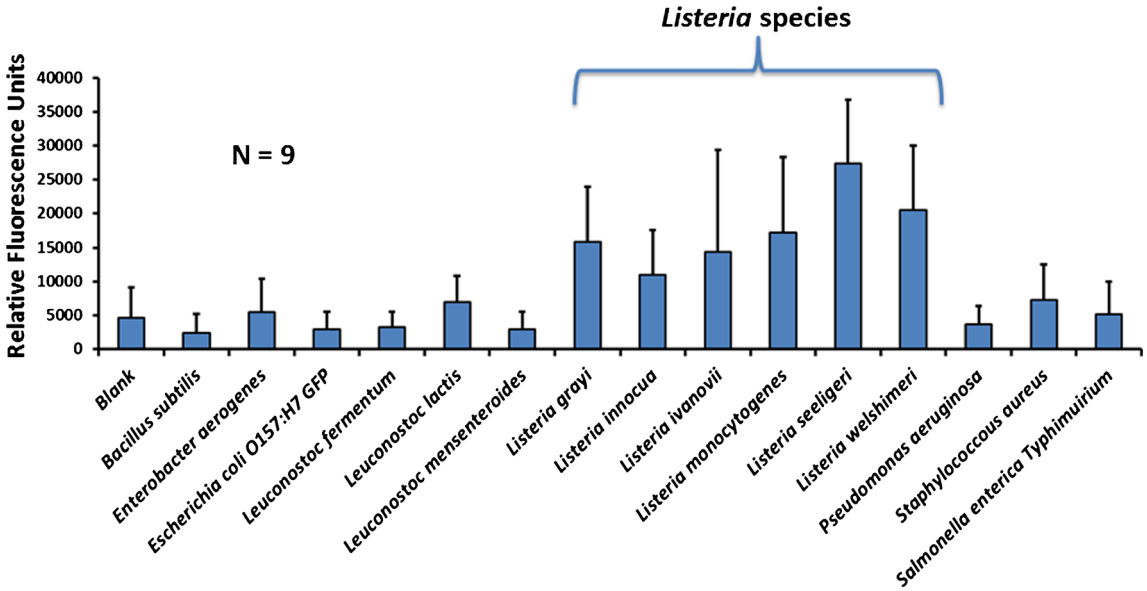

Fig. 5 Results of extensive cross-reactivity studies for the LLO assay using 26–85 cfu/sample of six different *Listeria* species and related or unrelated species as well as negative controls as assessed by the FLASH reader. *Bar heights* represent the fluorescence means of 9 samples (N=9) and *error bars* represent the standard deviations of the means for each species examined as well as blank controls without bacteria

a peristaltic pump to the various lyophilized reagent tubes, past the capture magnet assembly and finally to the cuvette for fluorescence development and assessment in the cuvette. Other fluid pathways for waste ejection and cleaning fluid intake, etc. are also shown along with a 3-D diagram of a single autoprocessor module.

Figure 7 supplies a photo of the overall computer-controlled 6-module assay autoprocessor including the command graphical user interface (GUI) with boxes for variable time and other parameter input. Figure 7 also illustrates how tubing is inserted into the lyophilized reagents for rehydration and to be siphoned into the system or finally ejected into the cuvette. The lower left quadrant of Fig. 7 provides actual fluorescence data from two separate trials of the *Listeria* LLO assay conducted 6 times (once in each of the modules) with 0 and 1000 *L. monocytogenes* cells per ml. The horizontal dotted line across the graph in Fig. 7 illustrates the consistent background obtained with the assay autoprocessor system across two trials with essentially the same average background fluorescence and very small standard deviation error bars for the background in each trial.

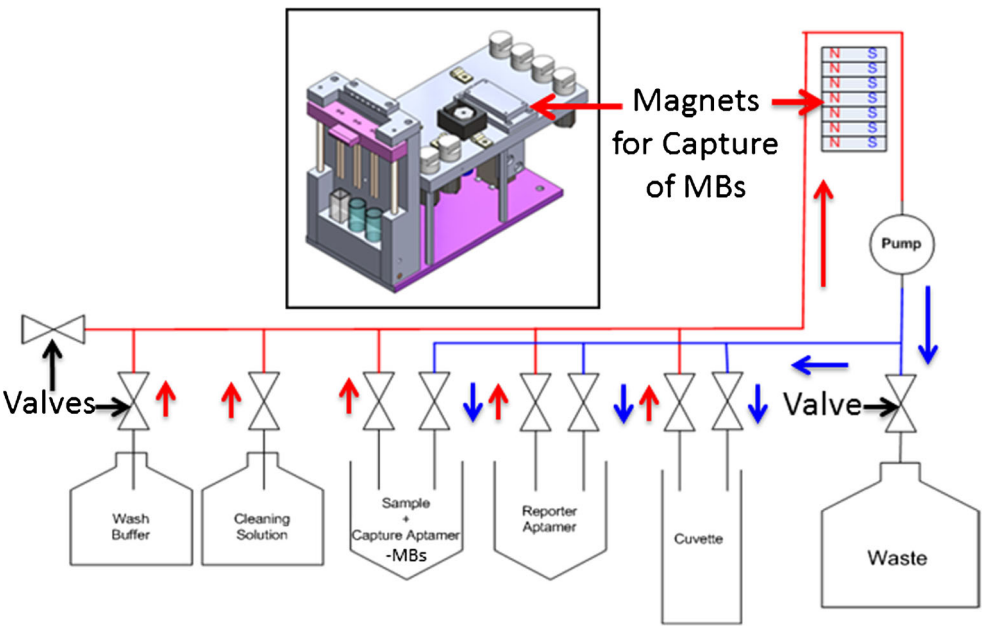

Fig. 6 A 3-D engineering drawing and schematic showing liquid flow into and out of a single module of the magnetic assay autoprocessor. The positions of the peristaltic pump, magnets, pinch valves, reagent tubes, cuvette, liquid reservoirs for wash buffer and cleaning solution and waste are indicated

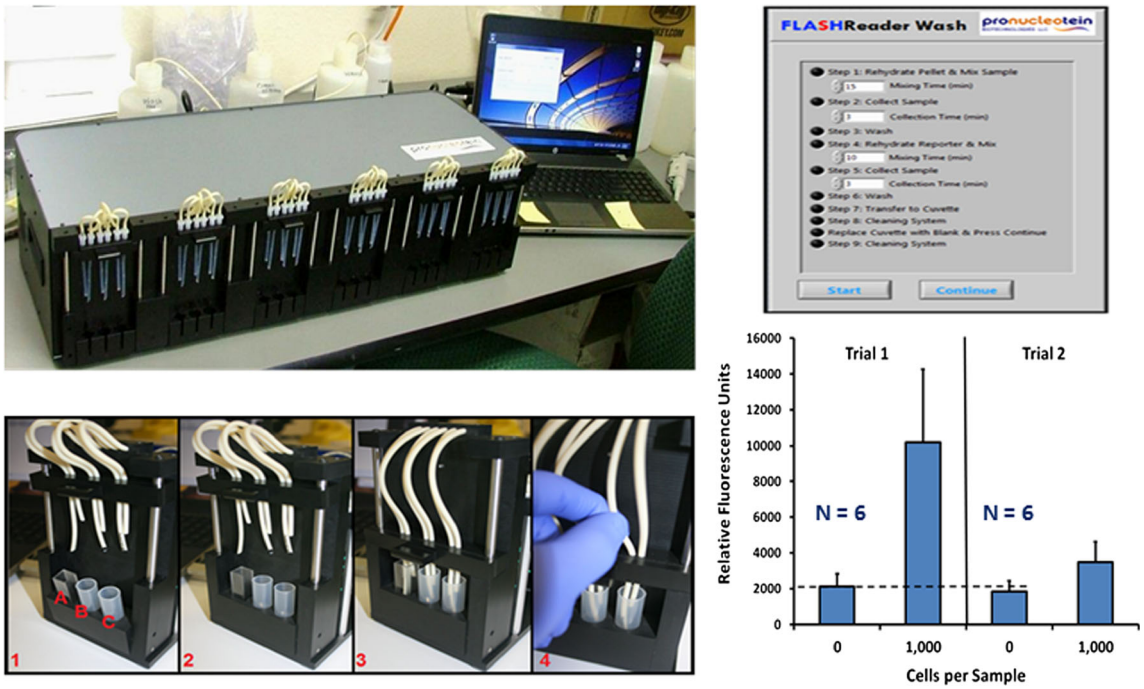

Fig. 7 Photos of the 6-module assay autoprocessor showing the positions of the cuvette (**a**) and lyophilized capture aptamer-MBs and reporter aptamer reagents (**b** and **c**) in the lower left region as well as the insertion of tubing (sequence 1 → 4). The *upper right quadrant* illustrates the graphical user interface (GUI). The *lower right quadrant graphs* show consistent background fluorescence across two separate trials (*dotted vertical line*) for the LLO assay as assessed by the FLASH reader after processing of 6 blank samples in the assay autoprocessor and 6 samples containing 1000 *L. monocytogenes* cells per sample. *Bar heights* represent the mean fluorescence and *error bars* represent standard deviations of the means

The consistent background and high signal at 1000 *L. monocytogenes* cells per sample in Fig. 7 indicated that the assay and system were ready for timed enrichment culture studies to determine the first point in time at which a positive could be detected. Due to the statistical difficulty in guaranteeing one cfu in each spiked shaker flask, the experiment was modified to allow spiking with an average of 10 cfu of *L. monocytogenes* per 200 ml of TSB to enable a more consistent inoculum. This led to detection of 70 colonies from a 100 μl sample by 8 h post-inoculation and incubation at 37 °C as shown in Fig. 8. Thus, if one interpolates from 10 cells to a 1 cell inoculum with a measured generation time of roughly 36 mins for *L. monocytogenes* (data not shown), this would add approximately 4 more generations to reach 10 cells per flask (assuming healthy uninjured cells in the inoculum) or about 2 more hours. This leads to the conclusion that we are probably at present able to detect *Listeria* in enrichment cultures in the 8–10 h timeframe.

Discussion

The goals of the present assay and handheld reader development were to arrive at a system which is both highly sensitive and highly portable when needed to detect *Listeria* species on fomites and in enrichment cultures. The system is primarily intended to be able to provide preliminary screening for low levels of *Listeria* and eventually other pathogens on surfaces where foods are processed such as stainless steel tables, conveyor belts, meat grinders, etc. so as to avoid mass contamination of foods passing through these processing points.

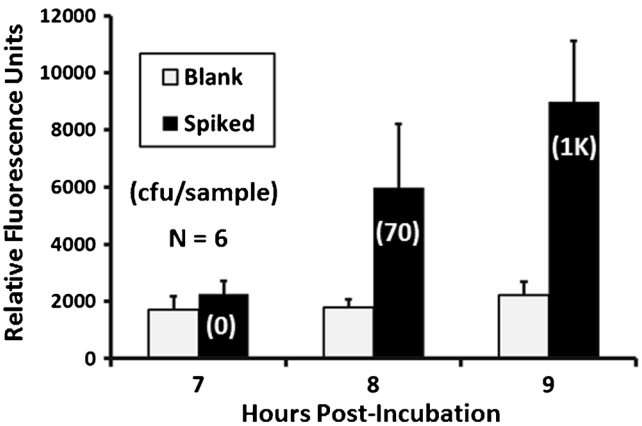

Fig. 8 Results of a timed shaker flask enrichment culture study performed by adding an average of 10 *L. monocytogenes* cells to 200 ml of TSB and drawing 1 ml samples at the times indicated and testing with the LLO assay and FLASH reader. Data suggest the first positive detection events at 8 h post-inoculation and incubation. Colony counts for 100 μl samples drawn from the shaker cultures at each time period indicated are given in parentheses inside the bars. *Bar heights* represent the fluorescence means and *error bars* represent the standard deviations of the means of 6 samples (3 samples x 2 different flasks)

While the system is intended to be mobile, it can also serve as a sensitive screening method in the central testing laboratory, especially when used in conjunction with the optional higher throughput 6-module magnetic assay autoprocessor described herein to process 6 samples simultaneously with low and consistent background. We have also developed aptamers against *Listeria* flagellins which are expressed below 28 °C (approximately room temperature [17]) for fomite swab testing, but the LLO aptamer appears to work best for detection of *Listeria* found on surfaces and from enrichment cultures incubated at 36–37 °C using our magnetic bead sandwich assay. In its role for testing of enrichment cultures, it appears that the present assay and FLASH reader system can detect *Listeria* contamination in as little as 8–10 h post-inoculation or incubation at 36–37 °C in shaker flask cultures. The whole assay from addition of the sample to acquisition of fluorescence data only requires~30 mins.

Real-time PCR-based detection systems for *Listeria* have proven to be both rapid and highly sensitive [6] but generally lack portability and are relatively expensive. Our handheld FLASH reader is projected to cost far less than a real-time or conventional thermal cycler and the aptamer-MB-based assays can be priced to compete very well with conventional PCR or immunoassays. In addition, PCR is inhibited by collagen and heme in foods [1], whereas our FLASH reader and AUR-based aptamer-MB fluorescence assays are not inhibited by any known components of foods which largely wash away during assay processing. PCR will continue to play a very important role in the food safety testing laboratory as a confirmatory technique along with culturing on selective media, biochemical and ribosomal RNA testing. Our aptamer-MB assay is actually meant to compete more with lateral flow immunoassay test strips and other rapid screening or presumptive tests, but will provide greater sensitivity.

Clearly, our current assay is highly sensitive and appears to be specific for six of the known *Listeria* species. The USDA's FSIS would be suspicious of any foods or surfaces testing positive for any *Listeria* species. Therefore, the genus-level specificity of our *Listeria* test is desirable for testing. Oddly, however, the aptamer used for this test was raised against LLO which is supposedly only present in the two known pathogenic species of *Listeria* (*L. monocytogenes* and *L. ivanovii*). The fact that our aptamer-based assay detects at least six species of *Listeria* is therefore somewhat surprising, but serendipitous, and probably indicates that an LLO-like protein or a common epitope is produced by the other 4 genetically related species of *Listeria*, thereby enabling us to detect them as well. Because LLO is secreted and inducible, our assay is probably mostly gleaning soluble LLO protein from surfaces or culture media, although some LLO associated with the cell walls [4] may enable actual *Listeria* cell capture on aptamer-MBs. *Listeria* and many other bacterial species are catalase positive and can contribute to the breakdown of H_2O_2 as does the peroxidase in our assay, thereby elevating AUR-derived fluorescence in a nonspecific manner. However, cross-reactivity data in Fig. 5 suggest that this is not a major problem (i.e., *Listeria* species gave higher fluorescence signals than other catalase positive species indicating that the fluorescence is not primarily emanating from bacterial catalase).

A final interesting observation about this LLO assay is the fact that the LLO-3 aptamer worked best when paired with itself in a sandwich assay. The fact that LLO-3 worked best in both the capture and reporter roles suggests that: 1) it bound with the highest affinity to an accessible epitope and 2) that epitope exists in at least two loci on LLO. LLO is a pore-forming protein which most likely contains multiple identical protein subunits with the same exposed epitopes, thereby explaining how a sandwich assay employing just one aptamer sequence for both capture and reporting is possible. Regardless of the molecular details, it is clear that a platform handheld fluorescence sensor technology and assay format have been developed which can be used to sensitively detect *Listeria* species and can probably be extended to detect many other pathogenic bacteria, viruses and associated proteins or biotoxins to provide a safer food supply.

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