UCRL-JRNL-202231

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February 6, 2004

Molecular and Cellular Probes

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A Multiplexed PCR-coupled Liquid Bead Array for the Simultaneous Detection of Four Biothreat Agents

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1 **ABSTRACT**

2 We have developed a 10-plexed PCR assay coupled to a 12-plexed liquid bead array to
3 rapidly screen environmental samples for *B. anthracis, Y. pestis, F. tularensis, and B.*
4 *melitensis.* Highly validated species -specific primer sets were used to simultaneously
5 amplify multiple diagnostic regions unique to each individual pathogen. Resolution of the
6 mix of amplified products was achieved by PCR product hybridization to corresponding
7 probe sequences, attached to unique sets of fluorescent beads. The hybridized beads were
8 processed through a flow cytometer, which detected presence and quantity of each PCR
9 product. The assay was optimized to allow for maximum sensitivity in a multiplexed
10 format. A high throughput demonstration was performed where 384 simulated
11 environmental samples were spiked with different amounts of *B. thuringensis* spores and
12 pathogen DNA. The samples were robotically processed to extract DNA and arrayed for
13 multiplexed PCR-liquid bead detection. The assay correctly identified the presence or
14 absence of each pathogen and collected over 3,000 individual data points within a single
15 8-hour shift for approximately $1.20 per sample in a 10-plexed assay.
16

17 **INTRODUCTION**

18 Biological weapons (BW) of mass destruction need not be disseminated in large
19 quantities in order to create terror and massive response as evidenced by the anthrax letter
20 attacks of October 2001, where five deaths occurred but 10,000 individuals were offered
21 prophylaxis treatment (8). Because *Bacillus anthracis* contamination was detected at the
22 Brentwood Mail Processing and Distribution Center in Washington DC (1), many other
23 mailrooms receiving mail from Brentwood were also evaluated for contamination. The
24 U.S. Department of Agriculture (USDA) screened over 18,000 samples using standard

1 plating techniques and over 4,600 samples using a real-time PCR assay from mailrooms
2 at 30 government buildings (16). As seen from this experience, the volume of
3 environmental samples needed for evaluation of contamination or decontamination
4 efficacy can quickly overcome the diagnostic capacity of most laboratories, becoming
5 more overwhelming if it is necessary to screen for the presence of multiple BW
6 organisms. The benefit of a high throughput multiplexed assay where multiple assays are
7 performed in a single tube is obvious when factoring in the economic cost of keeping
8 buildings off-line for long periods of time.

9 Currently, highly validated real-time 5' nuclease PCR assays (TaqMan® assays,
10 for example) are available through various federal agencies and each state's department
11 of health to screen environmental samples for pathogens (17). The existing technology,
12 although highly specific and sensitive, is limited by the optic capacity of the current real-
13 time PCR instrumentation to detection of 4 fluorophores per assay. This limitation
14 severely restricts the multiplexing capability of the 5′ nuclease assay.

15 Alternatively, many PCR reactions can be performed in a single tube, and then
16 resolved using a flow cytometer based liquid array; this approach combines the
17 specificity and sensitivity of a TaqMan® PCR assay with the multiplexed and high
18 throughput detection capabilities of flow cytometry. Fig. 1 describes the liquid array
19 assay. The liquid array system has the capability to detect 100 different PCR products by
20 differentiation of the 100 spectrally addressed bead sets.

21 We describe here the development of a 10-plexed PCR coupled liquid bead array
22 assay that targets the simultaneous detection of 9 multiple loci (number representing each
23 organism in parentheses) of *Bacillus anthracis* (2), *Yersinia pestis* (3), *Francisella*

1 *tularensis* (2), and *Brucella melitensis* (2) and one positive PCR control. The advantages
2 to this system are that 10 assays can be performed in a single tube to decrease sample
3 processing time, amount of labor required, and consumable costs while yielding an
4 increase in diagnostic power. Screening for multiple loci in each pathogen also increases
5 the confidence of detection by decreasing the chances of a false positive result. To
6 demonstrate the power of the liquid array in a high-throughput format, we performed the
7 multiplexed PCR-coupled liquid bead array assay in conjunction with an automated DNA
8 extraction protocol. 384 samples were processed and correctly identified for the presence
9 or absence of pathogen DNA in varying *B. thuringensi*s spore backgrounds in an 8-hour
10 shift.

11 METHODS

12 ***B. thuringensis* spore purification.** *B. thuringensis* cultures were grown in brain heart
13 infusion (BHI) media until sporulation was complete, as judged by phase contrast
14 microscopy. Spores were harvested by centrifugation for 15 min at 3,220 × g and washed
15 four times with 10 ml dd H_2O. The cell pellet was resuspended in 10 ml of lysozyme
16 buffer (100 mM EDTA, 50 mM NaCl, 1 mg/ml lysozyme (Sigma-Aldrich, St. Louis,
17 MO) and incubated at 30ºC for 90 minutes. The spores were then washed 4 more times
18 with ddH_2O, resuspended in 4 ml of 5% Triton X-100 (T-8787, Sigma-Aldrich) and
19 divided into 1 ml aliquots. Each aliquot was sonicated for 2 minutes using a Branson
20 1500 tank sonicator (Branson Ultrasonics, Danbury, CT) on a setting of two and then
21 centrifuged at 770 × g for 15 min over a 20%-40%-60% Percoll (Sigma-Aldrich)
22 gradient. Spores were collected from the 60% portion of the Percoll gradient, washed
23 twice with ddH_2O and stored at 4°C. The viable spore concentration was determined by
24 serial dilution in BHI broth and plating onto BHI agar plates.

Sample preparation. 1×10^9 purified *B. thuringensis* spores were added to 600 µl lysis buffer (100 mM sodium phosphate, 10 mM EDTA, 0.01% Tween-20, pH 7.4) in a 2 ml screw-cap tube containing 45 µl each of <100 µm and 400-600 µm glass beads (Sigma Aldrich). The tubes were bead-beaten (Biospec Products, Bartlesville, OK) at maximum power for 3 min, centrifuged 1 min, and cooled on ice 3 min. The lysate was removed from the beads, vortexed and serially diluted in lysis buffer (10^4-10^7 spores/ml). DNA was also isolated from *Bacillus anthracis* Ames strain*, Brucella melletensis, Franciscella tularensis*and *Yersinia pestis* vegetative cells using the Master Pure DNA extraction kit (Epicenter, Madison, WI). The purified DNA was re-suspended in molecular grade water, quantified by a spectrophotometer, and used as background DNA to spike into samples containing pathogen DNA.

Multiplexed PCR amplification. Each amplification reaction (multiplexed or singleplexed) was performed in a total volume of 25 µl on a Tetrad thermal cycler (MJ Research, Waltham, MA). The reaction mix consisted of 2.5 U of Platinum Taq polymerase (Life Technologies, Rockville, MD), 1X Platinum Taq PCR buffer (20mM Tris-HCl pH 8.4, 50mM KCl), 4.5mM $MgCl_2$, 0.2µM each of dCTP, dATP, and dGTP, 0.4 µM of dUTP, 400 nM of each primer, with the forward primer of each set containing 2 internal biotinylated dTTPs as well as a 5′ biotin label (primer sets were obtained from Biosearch Technologies, Mountain View, CA). Sample template consisting of 5 µl of spore preparation, purified DNA, or environmental air sample was added to each tube. PCR conditions of all primer pairs were optimized to be the same (used in both multiplexed and singleplexed formats), with the following cycling conditions: 1 cycle for 2 min at 95ºC, 35 cycles of 30 s at 95ºC, 30 s at 55ºC, and 1 min at 72ºC, and a final

1 extension of 2 min at 72ºC. Sensitivity was assessed using template concentrations of 0,
2 10 fg, 100 fg, 1 pg, 10 pg, 100 pg, 1 ng and 5 ng.
3 Primers and internal probe sequences were obtained for *B. anthracis, B. melitensis, Y.*
4 *pestis* and *F. tularensis* from the Biological Aerosol Sentry and Information System
5 (BASIS) group at Lawrence Livermore National Laboratory (Livermore, CA) and the
6 Center for Disease Control and Prevention (CDC, Atlanta, GA). Both primers and probes
7 went through rigorous computational and wet laboratory screening processes to yield
8 highly specific pathogen signatures[5]. Primer and probe sequences for *B. thuringensis* and
9 *E. herbicola* were obtained from the Naval Medical Research Center (Bethesda, MD).

10 **Fluorescent bead preparation.** Each 5′-amine-labeled nucleotide probe (Integrated
11 DNA Technologies, Coralville, IA) representing a pathogen's internal PCR amplicon
12 sequence was designed to contain an 18-C spacer between the reactive group and the 5′
13 end of the oligonucleotide for optimum hybridization. The probe was coupled to
14 carboxylated beads internally dyed with a unique spectral address (Luminex) using a
15 modification of the carbodiimide coupling method (14). Briefly, 25 µg of 1-ethyl-3- [3-
16 dimethylaminopropyl]-carbodiimide hydrochloride (EDC) (Pierce Biotechnology,
17 Rockford, IL) was added to a mix of one nmole probe and 1×10^6 beads in 0.1M 2-[N-
18 morpholino] ethanesulfonic acid (MES) buffer, pH 4.5 and incubated at RT for 30 min. A
19 second 25µg aliquot of EDC was added and incubated as before. The beads were rinsed
20 in 1 ml phosphate buffered saline (PBS) containing 0.02% Tween-20 (Sigma),
21 centrifuged at 13,000 rpm for 2 min, rinsed in PBS containing 0.1% sodium dodocyl
22 sulfate (SDS), centrifuged as before, and stored in 0.1 M MES buffer in the dark at 4ºC.
23 Each probe/bead conjugate was stored separately, and a fresh bead set containing all

conjugates was prepared for each liquid bead array assay. Streptavidin coated (LumAvidin) fluorescent beads were supplied by Luminex.

Hybridization. A bead set was prepared, consisting of 3×10^6 beads of each conjugate, 3×10^6 Lumavidin Beads (Luminex) and 3×10^6 BSA-coated beads (19) in 1X TMAC buffer (4.5 M tetramethyl ammonium chloride, 0.15% SDS, 75 mM Tris pH 8.0, 3.0 mM EDTA pH 8.0) to a final volume of 1ml. One µl of the amplified PCR reaction was added to 22 µl of the bead mix. PCR products and bead mix were denatured at 95ºC for 2 minutes and allowed to hybridize at 55ºC for 5 minutes. The mix was transferred to a 96 well filter plate (Millipore, Bedford, MA). The beads were washed once in 500 ml 1X TMAC and incubated with 60 µl of 60pg/µl Streptavidin- phycoerythrin (SAPE) (Caltag Laboratories, Burlingame, CA) for 5 minutes. The hybridized beads were washed again with 1X TMAC buffer and re-suspended in 100 µl 1X TMAC buffer.

Bead analysis. Data for each sample was acquired using a flow cytometer with a high speed digital signal processor (IS100, Luminex): the classification laser (635 nm) examined the dye molecules embedded in the beads and the reporter laser (532 nm) examined the SAPE fluorescent molecules attached to hybridized PCR product. At least 100 beads were interrogated from each bead set in the assay, and the mean fluorescence intensity (MFI) was calculated and recorded for each set; analysis was completed in 60 s for each sample.

High-throughput exercise. Template purification was automated using the Biomek® FX Laboratory Workstation (Beckman Coulter, Fullerton, CA) configured with a 200 µl 96-channel pipette head and an 8-channel pipette head on a dual bridge system. The workstation deck was equipped with a vacuum manifold (MAVM0960R, Millipore, Bedford, MA). A 250 µl aliquot of sample was transferred from each well of a 96-well deep well plate to a 96-well 0.22 micron filter plate (MAGVN22050, Millipore) followed

1 by a 3 minute vacuum interval; the filtrate was captured in a 96-well filter plate
2 (MANU030050, Millipore). The manifold was subjected to 18 inches Hg vacuum
3 pressure for 6 minutes, followed by one wash cycle of 150 µl TE and 2 wash cycles of
4 150 µl deionized water, with 6 minute vacuum intervals between wash cycles. The
5 template was eluted into the filter plate using 100 µl deionized water; 5 µl aliquots of the
6 elution were transferred to each of two 96-well PCR plates, and a 75 µl aliquot of the
7 elution was transferred to a 96-well microtiter plate for archiving. Vacuum pressure was
8 generated using a rotary vane pump (GAST Manufactering, Benton Harbor, MI).
9

10 **RESULTS**

11 **Optimization of PCR and Bead detection.** Specific amplification products were
12 successfully detected from all respective organism/primer pair combinations, when
13 assayed in the singleplexed or in the multiplexed format. Table 1 summarizes the primer
14 and bead assay probe length, GC content, Tm, expected amplicon size and limit of
15 detection for each pathogen target. Fig. 2A shows results of typical multiplexed reactions
16 starting with 10 fg of DNA template for each pathogen. The dot plot in Fig. 2B illustrates
17 the ability to resolve 10 amplicons generated from the multiplexed reaction and sort each
18 bead's spectral address, compared to the incomplete resolution achieved by gel
19 electrophoresis on 4% agarose of the same 10 amplicons (Fig. 2C). In complex
20 multiplexed formats such as this, one can see the discriminatory power of the unique
21 fluorescent bead sets to specifically detect by sequence hybridization the presence of a
22 PCR product, and therefore of the target organism.
23 A positive PCR signal, as detected by the liquid bead array assay, was defined for each
24 primer set as a mean fluorescence intensity (MFI) of twice the background fluorescence

1 intensity (BFI) of that primer set. The BFI for the singleplexed assay format was
2 calculated from the average of 80 reactions on a 96-well plate where no template existed
3 for a primer set. BFI for multiplexed assay format was calculated from the triplicate no-
4 template control reactions performed. Since routine use of this multiplexed assay for
5 environmental monitoring will most likely result in negative results, a human myoglobin
6 gene not found in bacteria was developed for use as a positive PCR control when testing
7 unknown environmental air samples. Five ng of human DNA was added to the
8 multiplexed reaction, which corresponds to 1000 copies of the original myoglobin gene.
9 Negative controls were also included, consisting of all reagents and replacing dH_20 for
10 pathogen DNA.
11 The individual primer/probe sets used in the multiplexed PCR described in this paper
12 were validated previously for use in TaqMan® PCR analysis, and found to be specific for
13 the pathogens of interest[5]. To validate the multiplexed PCR-coupled liquid bead array
14 assay, the effects of PCR product amplification and the subsequent bead assay detection
15 were both considered; each parameter contributes to assay sensitivity and specificity.
16 PCR results were assessed by liquid bead array detection and gel electrophoresis. PCR
17 reactions were performed first with each single primer pair and their matching bead sets,
18 then all primer sets for a particular pathogen were tested together. For bead assay
19 validation, single primer set PCR reactions were tested with all bead sets for a particular
20 pathogen to see if they cross-reacted. Primer sets passing all these criteria were at last
21 multiplexed together. Several controls were used for the liquid array assay. The
22 LumAvidin fluorescent label control bead set contained an outer layer of Avidin, for near
23 covalent binding of phycoerythrin; this assured the reporter dye was functioning properly.
24 Another bead set contained an outer layer of Bovine Serum Albumin (BSA) and served

1 as a nonspecific hybridization control. Finally, the PCR control amplicon continued to
2 serve as a positive control, because if it was detected both the PCR and liquid array steps
3 were performed correctly.

4 Tetramethyl ammonium chloride (TMAC) was added to the bead hybridization reaction
5 to minimize Tm differences that favor some PCR products hybridizing over others (18).
6 The concentration of the streptavidin phycoerythrin (SAPE) fluorescent dye used for
7 detecting PCR product hybridization to the beads and PCR product to bead hybridization
8 time was optimized. It was empirically determined that a wash step was necessary after
9 the hybridization of the PCR product onto the beads to significantly reduce the beads
10 nonspecific background fluorescence (data not shown).

11 **Sensitivity of singleplex vs. multiplex PCR.** Table 1 shows the limits of detection for
12 each primer set when tested singly (duplicate reactions) or multiplexed (triplicate
13 reactions). Detection limits ranged from 10 fg DNA detected (~1 DNA copy) to 10 pg
14 (~10^4 DNA copies). We observed that fluorescence detection followed a 2-5 log dynamic
15 range amplification (Fig. 2A) and results were semi-quantitative within those DNA
16 concentration ranges. Not without precedent, the limits of detection changed when primer
17 sets were multiplexed; generally a decreased sensitivity was observed (Table 1). Exeter
18 and Lewinski (11) showed that Taq polymerase was especially limiting to multiplex
19 sensitivity; by doubling the amount of Taq enzyme used, we also increased sensitivity
20 (data not shown). A comparison of the two *B. anthracis* primer sets in single and
21 multiplex formats (Figure 3) indicates that the sensitivity in the multiplex reaction
22 decreased 10-fold from the singleplex reaction, but was sensitivity remained high at 100
23 fg of pathogen DNA, equivalent to ~20 DNA copies.

1 **High-throughput demonstration of the bead array assay.** The main bottleneck to
2 PCR detection of environmental samples is the time and labor involved in sample
3 processing. We tested how our assay performed in an 8 hour period, when paired with a
4 high-throughput template purification protocol. Four replicate 96-well plates were spiked
5 with rows of either 1×10^7 or 1×10^5 bead beaten, or 1×10^7 unbroken *B. thuringensis*
6 spores to mimic extraneous DNA normally present in environmental samples. *B.*
7 *thuringensis* DNA was extracted and purified using a high throughput screening system.
8 Wells were then spiked with 10 pg of target DNA or left empty to serve as non-template
9 controls. Multiplex PCR reactions and detections were performed in quadruplicate with
10 all primer sets included in the PCR mix. Fig. 4 shows the MFI detection of the spiked
11 samples when tested in different *B.thuringensis* spore backgrounds in comparison to
12 control wells containing no *B.thuringensis* spores. Pathogen amplification products were
13 not detected from wells containing no template or those with *B. thuringenis* spores only.
14 Two-tailed t-tests (Excel, Microsoft Corp., Bellevue, WA) with a minimum of 10
15 repetitions were used to evaluate any differences in detection of the spiked pathogen
16 DNA template in the different background preparations; a significant test was defined as
17 $P < 0.01$. Primer sets BA-1 and BA-2 showed a significant difference in detection of *B.*
18 *anthracis* DNA when in a 10^7 *B. thuringensis* spore background ($P = 0.006$ and 0.001,
19 respectively). *B. anthracis* and *B. thuringensis* are closely related organisms, and it is
20 possible some primer hybridized to *B. thuringensis* DNA and therefore reduced the signal
21 for *B. anthracis*. All other primer sets reported no significant differences.
22

1 **DISCUSSION**

2 Multiplexed real-time PCR reactions of 2-4 primer sets have been demonstrated for the
3 simultaneous detection of multiple pathogens from a single sample, in a variety of sample
4 matrices including plant material (3, 21, 23, 25), blood (10), and cerebral spinal fluid (5).
5 The current 4-dye limit available for real-time multiplexed PCR assays is presently the
6 maximum instrumentation capability. By coupling multiplexed PCR technology with
7 hybridization of the resulting amplification products on liquid bead arrays, 100
8 fluorescent signatures become available to simultaneously detect 100 different PCR
9 products. We have not only demonstrated the feasibility of this approach, but have
10 streamlined sample processing by using a robotic instrument to extract environmental
11 DNA in a 96-well plate format. Our assay was designed to rapidly screen air samples for
12 9 diagnostic regions identifying the BW pathogens *B. anthracis, Y. pestis, F. tularensis,*
13 and *B. melitensis*. Individual PCR reactions within our multiplexed assay showed the
14 lower limit of sensitivity varied from 100 fg to 10 pg starting DNA concentrations
15 (equivalent to 20- 2000 organisms). A human myoglobin gene not found in bacteria
16 served as a positive PCR control. The positive PCR control also served to ensure that the
17 PCR products were added to the liquid array mix and correctly identified. Other liquid
18 array controls included lumavidin beads to monitor the correct reporting of the SAPE
19 fluorescent dye and BSA control beads to monitor nonspecific hybridization.

20 For any PCR reaction to be successful in screening environmental samples, primer sets
21 must go through rigorous testing to ensure their specificity to the desired pathogen and
22 their lack of cross-reactivity to closely related organisms. Cautionary tales of inadequate
23 screening processes can be found in the literature. For example, an IS900 insertion
24 element was used to develop specific PCR primers for *Mycobacterium avium* subsp.

1 *paratuberculosis* (4, 15), only to find that it cross reacted with environmental
2 *Mycobacteria* sp. in ruminant feces, which turned out to share 71 and 79% in sequence
3 homology (6).
4 To minimize false positive results, our primer sets were initially screened
5 computationally (12) and then further screened by laboratory analysis, which involved
6 extensive testing for detection of a wide range of a pathogen's isolates while excluding
7 closely related organisms, background DNA typically found in air and soil samples, and a
8 zoological panel of human, pig, rabbit, rat, bovine, chicken, dog and mouse DNA. Primer
9 sets developed in this fashion have been extremely successful in the Biological Aerosol
10 Sentry and Information System (BASIS) environmental monitoring program. BASIS,
11 originally designed for use at the 2002 Winter Olympics, was deployed in 2001 to
12 monitor air in U.S. cities. Over 400,000 diagnostic TaqMan assays of complex
13 environmental samples have been completed by BASIS and it's successors with no false
14 alarms when all diagnostic markers were reviewed (13).
15 Bacterial genomes are dynamic and the ability of organisms to acquire genetic
16 information from one another in the environment is well known (22). If by chance an
17 environmental organism recently acquires a diagnostic region we have assigned to detect
18 a pathogen, a false positive reaction could ensue. Increasing the number of diagnostic
19 genetic regions examined per pathogen increases the confidence of a correct
20 identification by decreasing the chances of a false positive. A multiplexed assay makes it
21 possible to simultaneously screen for multiple confirmatory diagnostic regions efficiently
22 and cost effectively.
23 The limit of detection of the template in our multiplexed assay was not always as
24 sensitive as in our singleplexed assay. This is most likely due to the increased complexity

1 of the multiplexed reaction. Competition for resources, the interactions between primer
2 sets, and primer set hybridization efficiencies all have an effect on sensitivity (11). To
3 ensure proper amplification of all diagnostic regions, primer and probe melting
4 temperatures and GC content must be close enough for adequate hybridization.
5 Homologies between primers and probes should be checked in order to minimize
6 primer/dimer formation. Since an inadequate amount of Taq enzyme has been shown to
7 negatively affect sensitivity (11); we added twice the amount we normally added for our
8 singleplexed reactions. We empirically tested primer and probe set combinations,
9 gradually building up the number of reactions in the multiplex. Sensitivity can be
10 compromised not only in the PCR assay, but during the bead array portion of the assay
11 too. Linker design is an important factor; in our assay, we achieved the best coupling
12 efficiency with a 5′ amine-labeled 18C spacer.

13 Another platform for high throughput microbial detection is microarray technology. A
14 photolithography microarray was successfully developed to identify 18 pathogens
15 simultaneously (26), while a spotted microarray composed of conserved viral sequences
16 was successfully developed to identify unknown viruses including the SARS virus (24).
17 While detection is very sensitive and specific, microarray platforms require significant
18 design experience for construction, and bioinformatics expertise for data analysis. The
19 costs of photolithography arrays are particularly prohibitive for routine microbial
20 diagnostic use (~$400/array). The liquid array platform is attractive in its simplicity of
21 design and analysis, and a 10-plex reaction can be run for $1.20 in material costs.

22 Immunoassays are another means to identify microorganisms, and can also be used in a
23 liquid array platform (19). The advantage of an immunoassay is that microbial cells do
24 not need to be disrupted for identification, since antigens found on cell surfaces are used

1 for positive identification. A screening process similar to that used for identifying unique
2 nucleic signatures must be performed for candidate antibodies to detect diagnostic cell
3 surface antigens. Finding candidate antibodies and antibody production is much more
4 time consuming, however. Sensitivity of an immunoassay is usually around 10,000
5 colony forming units (CFU)/ml (20) while a nucleic acid assay is about 200 CFU/ml (2,
6 26).
7 Our array assay relies on the ability of a flow cytometer to resolve multiple
8 microsphere-based assays. A previous version of this technology has been used to detect
9 viral load in human plasma samples using nucleic acid targets (7). Recently, similar
10 liquid array systems (Luminex) were used for nucleic acid and antigen detection of
11 bacterial targets. Four pathogens were identified using a single PCR assay to amplify
12 variable regions in the 23S ribosomal RNA gene. The liquid array system differentiated
13 the products specific to the 4 pathogens (9). Autonomous detection of bacterial antigen
14 targets in air was achieved using an autonomous pathogen and detection system (APDS)
15 (19, 20).
16 In an event of a BW release in the environment, there would be an exponential increase
17 in the number of samples to be monitored (16). To that effect we have developed a liquid
18 array based assay for the 4 biothreat agents *B. anthracis, Y. pestis, F. tularensis and B.*
19 *melitensis*. We have demonstrated the efficacy of these multiplex assays in a high through
20 put format at a demonstration exercise, which is currently being used as a model for the
21 design of other similar high throughput laboratories. A total of 384 samples were
22 analyzed in an 8-hour workday by two technicians using one robotic sample preparation
23 station, one 96-well thermal cycler and one 96-well flow cytometer. Thus three shifts per
24 day with two technicians each shift would translate to a high-throughput of more than

2000 samples/day. While developed for BW surveillance, this format could easily be adapted for environmental monitoring of foodborne, agricultural, water, and bloodbank pathogens. Additionally, multiplex PCR-liquid bead detection could be especially useful in autonomous detection platforms, which currently use immunological assays in a liquid bead detection format (20).

In summary, we found the multiplex PCR coupled liquid bead array to offer some important benefits for microbial detection. All-in-one reactions save on labor, reagents and consumable costs. The high-throughput platform shortens analysis time while giving definitive hybridization based detection results. The capacity of the liquid bead array assay to accommodate up to 100 different diagnostic regions is especially attractive. The number of primer pairs amplified in a multiplex reaction will most likely be limited to below 100; in that case different multiplex PCR reactions sets could be performed and then merged for simultaneous detection using the liquid bead array assay. This work is part of a larger effort to advance detection technologies for civilian counterterrorism response (13). We are currently expanding the multiplex PCR portion of the assay to detect more pathogens and also working toward increased automation to handle 10,000 samples/day.

ACKNOWLEDGEMENTS

We thank Chip Beckwith and Mary McBride for carefully reviewing this manuscript. This work was supported by the U.S. Department of Energy, NN-20, Chemical and Biological Non-Proliferation Program. This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract W-7405-ENG-48.

REFERENCES

1. **Anonymous.** 2001. Evaluation of *Bacillus anthracis* contamination inside the Brentwod Mail Processing and Distribution Center, District of Columbia, October, 2001. Morb. Mortal. Wkly. Rep. **50:**1129-1133.

2. **Belgrader, P., C. J. Elkin, S. B. Brown, S. N. Nasarabadi, R. G. Langlois, F. P. Milanovich, and B. W. Colston, Jr.** 2003. A reusable flow-through polymerase chain reaction instrument for the continuous monitoring of infectious biological agents. Anal. Chem. **75:**3446- 3450.

3. **Bertolini, E., A. Olmos, M. M. Lopez, and M. Cambra.** 2003. Multiplex nested reverse transcription-polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in olive trees. Phytopathology **93:**286 -92.

4. **Collins, D. M., D. M. Gabric, and G. W. DeLisle.** 1989. Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. FEMS Microbiol. Lett. **60:**175 -8 (1989).

5. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski.** 2001. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J. Clin. Microbiol. **39:**1553-1558.

6. **Cousins, D. V., R. Whittington, I. Marsh, A. Masters, R. J. Evans, and P. Kluver.** 1999. Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS900-like sequences detectable by IS900 polymerase chain reaction: implications for diagnosis. Mol. Cell.

Probes **14:**431- 442.

7. **Defoort, J. P., M. Martin, B. Casano, S. Prato, C. Camilla, and V. Fert.** 2000. Simultaneous detection of multiplex-amplified human immunodeficiency virus type 1 RNA, Hepatitis C virus RNA, and Hepatitis B virus RNA using a flow cytometer microsphere-based hybridization assay. J. Clin. Microbiol. **38:**1066-1071.
8. **D'Esopo, M.** 2003. Go Figure. Biosecurity and Bioterrorism: Biodefense Strategy, Practice and Science **1:**55.
9. **Dunbar, S. A., A. Coe, V. Zee, K. G. Oliver,K. L. Karem, and J. W. Jacobson.** 2003. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP^{TM} system. J. Microbiol. Methods **53:** 245-252.
10. **Estes, M. C., and J. S. Sevall.** 2003. Multiplex PCR using real time DNA amplification for the rapid detection and quantitation of HTLV I or II. Mol. Cell.Probes **17:**59- 68.
11. **Exeter, M. M., and M. A. Lewinski.** 2002. Sensitivity of multiplex real-time PCR reactions, using the lightcycler and the ABI PRISM 7700 Sequence Detection System, is dependent on the concentration of the DNA polymerase. Mol. Cell. Probes **16:**351- 357.
12. **Fitch J. P., S. N. Gardner, T. A. Kuczmarski, S. Kurtz, R. Myers, L. L. Ott, T. R. Slezak, E. A. Vitalis, A. T. Zemla, and P. M. McCready.** 2002. Rapid development of nucleic acid diagnostics. Proc. IEEE. **90:**1708-1721.
13. **Fitch, J. P., E. Raber, and D. R. Imbro.** 2003. Technology challenges in responding to biological or chemical attacks in the civilian sector. Science **302:**1350-1354.

14. **Fulton, R. J., R. L. McDade, P. L. Smith, L. J. Kienker, and R. J. Kettman, Jr.** 1997. Advanced multiplexed analysis with the FlowMetrix system. Clin. Chem. 43:1749-1756.

15. **Green, E. P., M. L. Tizard, M. T. Moss, J. Thompson, D. J. Winterbourne, J. J. McFadden, and J. Hermonk-Taylor.** 1989. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. **17:** 9063-73.

16. **Higgins, J. A., M. Cooper, L. Schroeder-Tucker, S. Black, D. Miller, J. S. Karns, E. Manthey, R. Breeze, and M. L. Perdue.** 2003. A field investigation of *Bacillus anthracis* contamination of U.S. Department of Agriculture and other Washington, D.C., Buildings during the anthrax attack of October, 2001. Appl. Environ. Microbiol. **695**93 -599.

17. **Hoffmaster, A. R., R. F. Meyer, M. P. Bowen, C. K. Marston, R. S. Weyant, G. A. Barnett, J. J. Seyvar, J. A. Jernigan, B. A. Perkins, and T. Popovic.** 2002. Evaluation and validation of a real-time polymerase chain reaction assay for rapid identification of *Bacillus anthracis.* Emerging Infect. Dis. **8:**1178-1182.

18. **Honore, B., P. Madsen, and H. Leffers.** 1993. The tetramethylammonium chloride method for screening of cDNA libraries using highly degenerate oligonucleotides obtained by backtranslation of amino acid sequences. J. Biochem. Bioph. Methods **27:**39.

19. **McBride, M. T., S. Gammon, M. Pitesky, T. W. O'Brian, T. Smith, J. Aldrich, R. G. Langlois, B. Colston, and K. S. Venkateswaran.** 2003. Multiplexed liquid arrays for simultaneous detection of simulants of biological warfare agents.

1 Anal. Chem. **75:**1924 -1930.

2 20. **McBride, M. T., D. Masquelier, B. J. Hindson, A. J. Makarewicz, S. Brown,**
3 **K. Burris, T. Metz, R. G. Langlois, K. Wing Tang, R. Bryan, D. A. Anderson,**
4 **K. S. Venkateswaran, F. P. Milanovich, and B. W. Colston, Jr.** Autonomous
5 detection of aerosolized *Bacillus anthracis* and *Yersinia pestis.* Anal. Chem.
6 **75:**5293-5299.

7 21. **Mumford, R. A., K. Walsh, I. Barker, and N. Boonham.** 2002. Detection of
8 Potato mop top virus and Tobacco rattle virus using a multiplex real-time
9 fluorescent reverse-transcription polymerase chain reaction assay. Phytopathology
10 **90:**448-453.

11 22. **Ochman, H., J. G. Lawrence, and E. A. Croisman.** 2000. Lateral gene transfer
12 and the nature of bacterial innovation. Nature **405:**299-304.

13 23. **Saade, M., F. Aparicio, J. A. Sanchez-Navarro, M. C. Herranz, A. Myrta,**
14 **B. DiTerlizzi, and V. Pallus.** 2000. Simultaneous detection of the three ilarviruses
15 affecting stone fruit trees by nonisotopic molecular hybridization and multiplex
16 reverse-transcription polymerase chain reaction. Phytopathology **90:**1330-1336.

17 24. **Wang, D., L. Coscoy, M. Zylberberg,, P. C. Avila, H. A. Boushey, D. Gamen,**
18 **and J. L. DeRisi** 2002. Microarray-based detection and genotyping of viral
19 pathogens. Proc. Nat. Acad. Sci. USA **99:**15687-15692.

20 25. **Weller, S.A., J. G. Elphinstone, N. C. Smith, N. Boonham, and D.E. Stead.**
21 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex,
22 real-time, fluorogenic PCR (TaqMan) assay. Appl. Environ. Microbiol. **66:**2853 -
23 2858.

24 26. **Wilson, W.J., C. L. Strout, T. Z. DeSantis, J. L. Stilwell, A. V. Carrano, and**

G. L. Andersen. 2002. Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. Mol. Cell. Probes **16:**119-127.

1 **Table 1.** Organism, corresponding primers and probe lengths, GC, and Tm, product size

2 and detection limits for the multiplex PCR coupled liquid bead array assay.

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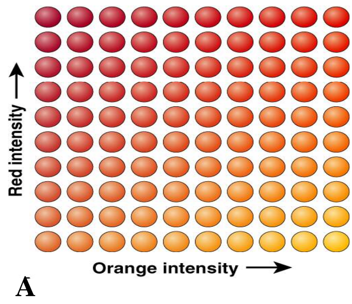
Organism/ strain	Primer Set Name	Forward Primer: Length (nt), GC, Tm	Reverse Primer: Length (nt), GC, Tm	Probe: Length (nt), GC, TM	Amplified Product Size (nt)	Multiplex Detection Limit (fg)	Singleplex Detection Limit (fg)
Bacillus anthracis Ames	BA-1	21,52,62	23,43,61	32,40,70	78	100	10
	BA-2	26,50,69	27,44,67	38,28,62	106	100	10
Brucella melitensis	BM-1	18,55,62	20,50,61	22,70,69	62	100	100
	BM-2	21,42,62	21,52,63	28,50,66	70	100	100
Yersinia pestis Kim	YP-1	24,50,68	23,57,68	35,52,71	147	100	10
	YP-2	23,48,63	23,48,63	29,45,66	191	100	250
	YP-3	22,55,65	25,48,65	28,54,69	183	100	250
Franciscella tularensis	FT-1	28,32,61	28,39,61	26,50,66	85	10,000	10
	FT-2	30,33,62	25,40,63	30,40,65	88	10,000	10
Myoglobin PCR control	MG-1	21,55,62	21,50,69	30,50,69	157	500,000	Not determined

Figure 1. A. The liquid array employs polystyrene beads embedded with different ratios of red and infrared fluorescent dyes to yield 100 unique spectral addresses. B. Each PCR product sequence to be detected is assigned one set of uniquely dyed beads and the beads are conjugated with their assigned probe, a reverse complement internal PCR product sequence. Standard biotin-labeled PCR products from a multiplexed PCR assay are allowed to hybridize to the bead set and are then labeled with a second reporter dye, streptavidin phycoerythrin (SAPE). C. An automated flow cytometer (Luminex, Austin, TX) uses two lasers to detect the type and quantity of PCR product: a 635 nm laser detects the spectral address of the unique bead set assigned to the PCR product, and a 532 nm laser measures the SAPE reporter dye to determine the quantity of PCR products hybridized to each bead. A computer software program records fluorescence readings.



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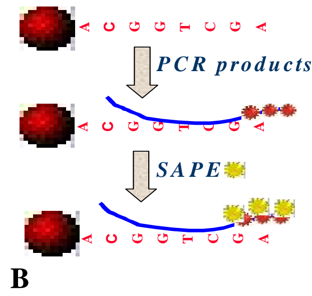

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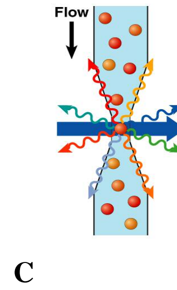

C

Figure 2 Results of the 10-plex PCR coupled liquid bead array assay. **(a)** Mean fluorescence detection of PCR products for 7 template concentrations using the liquid bead array, in triplicate. BA-1, BA-2: *B. anthracis* specific primer sets; BM-1, BM-2: *B. melitensis* specific primer sets; YP-1, YP-2, YP-3: *Y. pestis* specific primer sets; FT-1, FT-2: *F. tularensis* specific primer sets. **(b)** Dot plot of the12-plex liquid bead hybridization (including two liquid array controls), as reported by Luminex software. The white circles represent each bead type's spectral address; a flow cytometer classifies the bead type and amount of PCR product attached to each bead's surface, expressed as MFI. **(c)** Corresponding gel electrophoresis (4% agarose gel) of the same10-plex amplification products.

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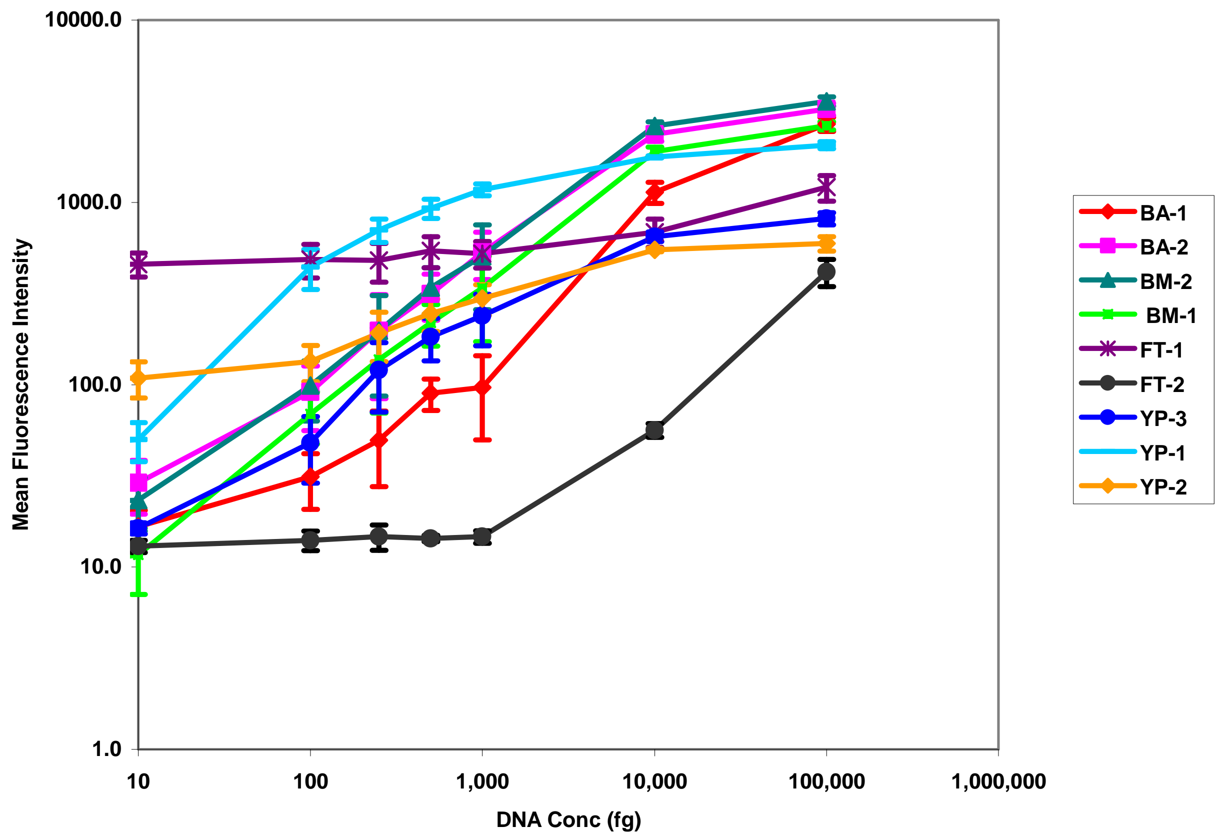

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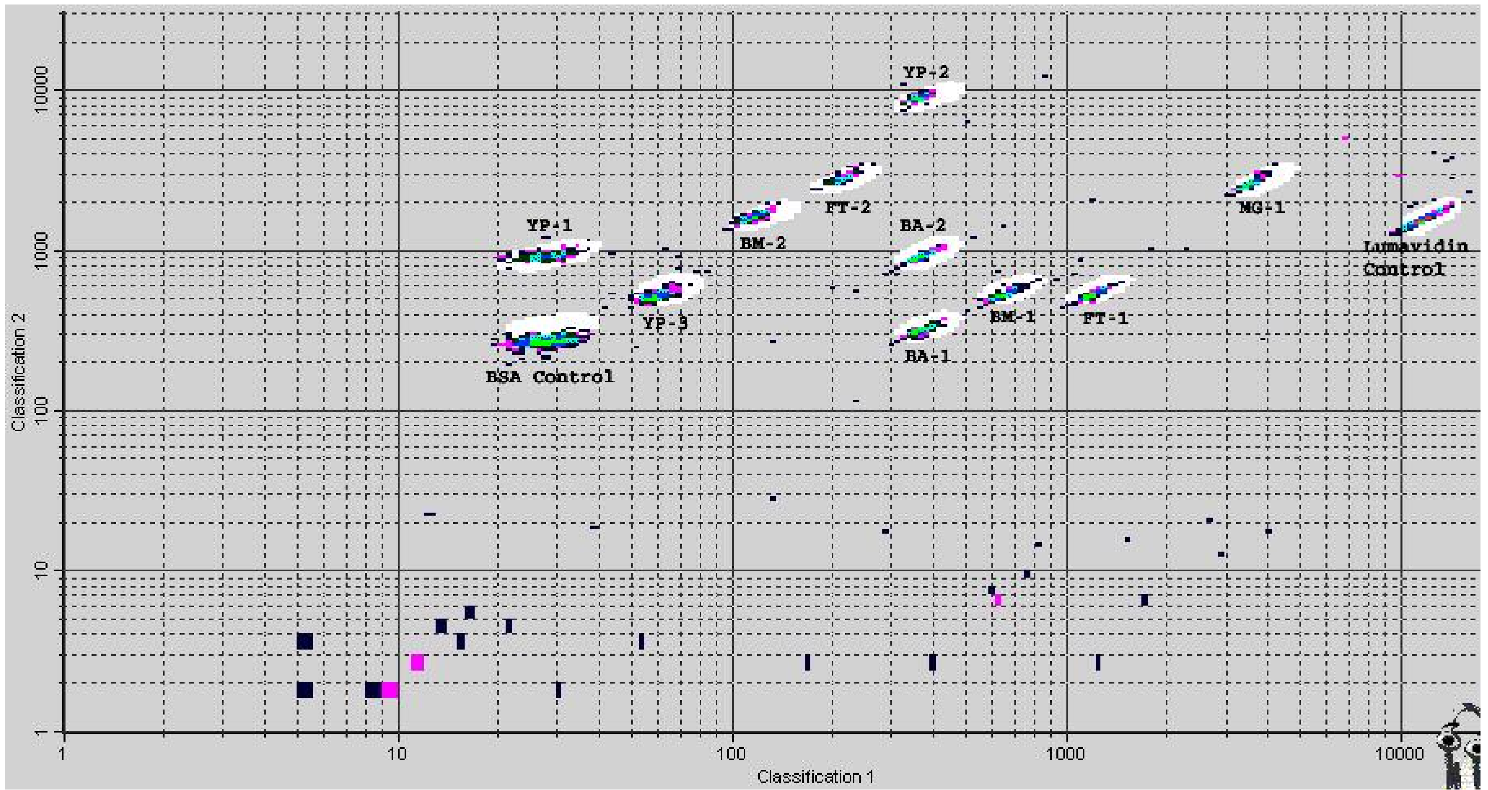

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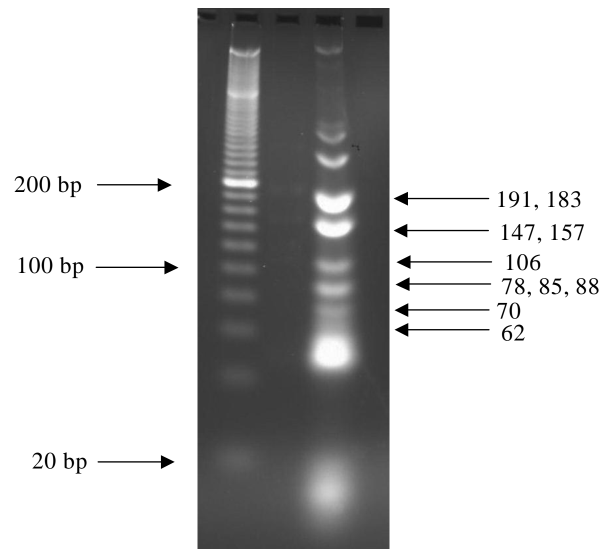

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1 **Fig. 3.** Comparison of BA-1 and BA-2 detection sensitivity in single assay and multiplex

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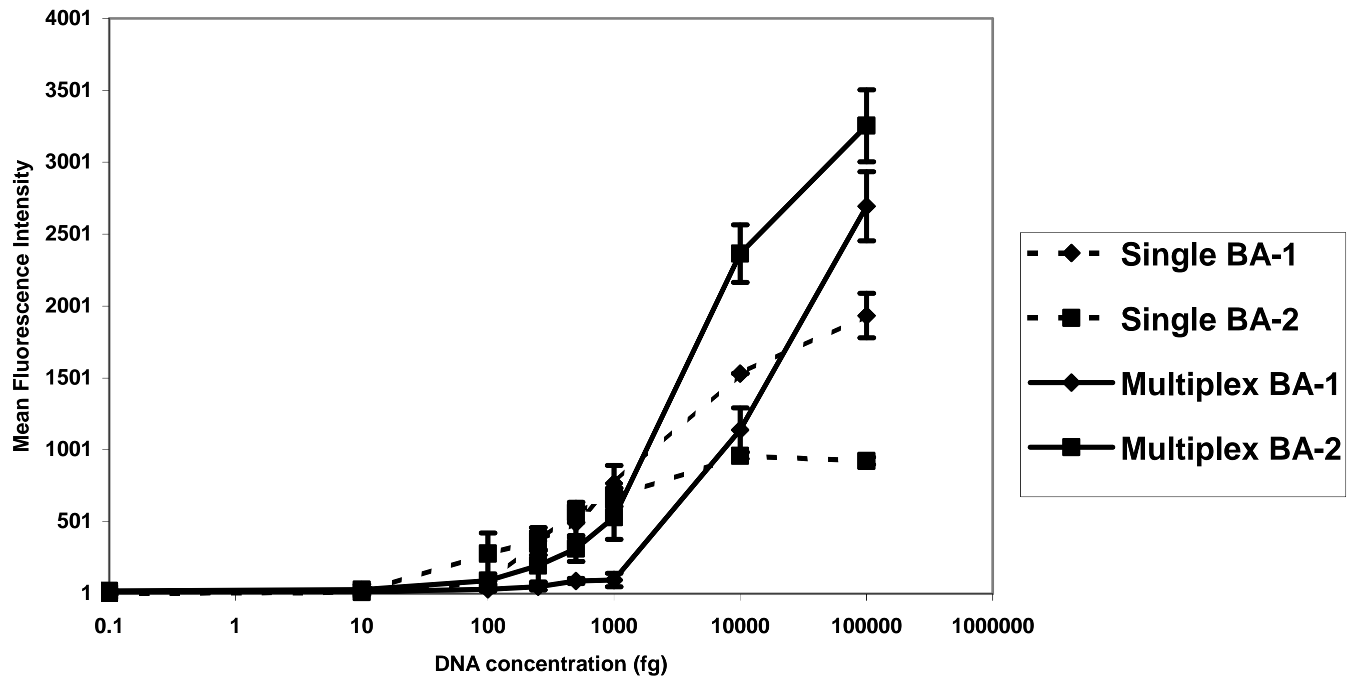

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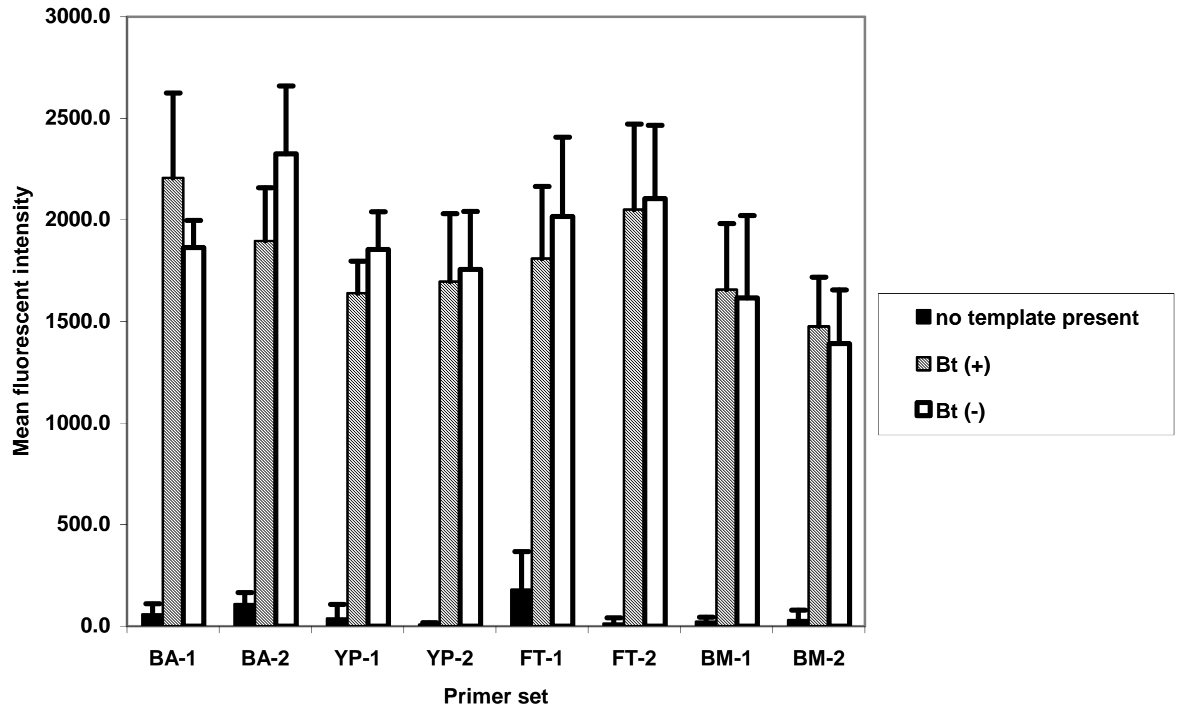
Figure 4. MFI detection of pathogen DNA when spiked with different *B. thuringensis* spore backgrounds in comparison to controls containing no *B. thuringensis* spores. *B. anthracis* and *F. tularensis* were tested in 1×10^7 bead-beaten spores/ml background, *B. melitensis* in 1×10^7 unbeaten spores/ml, and *Y. pestis* in 1×10^5 bead-beaten spores/ml.



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