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Direct detection of genomic DNA with fluidic force discrimination assays

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ABSTRACT

Herein, we describe the direct detection of genomic DNA using fluidic force discrimination (FFD) assays. Starting with extracted bacterial DNA, samples are fragmented by restriction enzymes or sonication, then thermocycled in the presence of blocking and labeling sequences, and finally detected with microbead-based FFD assays. Both strain and species identification of extracted *Bacillus* DNA have been demonstrated in <30 min, without amplification (e.g., PCR). Femtomolar assays can be achieved with this rapid and simple procedure.

The unambiguous identification of biological agents that cause disease, illness, or even death has become critical, in particular for biodefense, food safety, and medical diagnostics. Molecular beacons [1,2], nanoparticle-based labeling [3–5], and biosensors based on SPR [6,7], QCM [8,9], or piezoelectronics [10,11] are but a few of the detection strategies being utilized for identification of nucleic acid sequences. The crucial difference between nucleic acid detection strategies and immunoassays is target availability. In immunoassays, immunoreagents can directly capture the target (e.g., bacteria, viruses) from solution. In contrast, detection of nucleic acids requires first extracting and then collecting that material. Although recent years have witnessed great strides in automating these processes [12–18], this additional sample handling adds both time and complexity to the analysis.

After extracting nucleic acids, most detection strategies require amplification (e.g., PCR, nonenzymatic, or even isothermal amplification strategies) to increase signal to a detectable level. As amplification strategies have improved and become more commonplace, real-time PCR (rtPCR)[2] has filled the need for rapid, unambiguous identification. Starting with extracted DNA, rtPCR is capable of both producing results in as little as 1 min and detecting low copy numbers of target DNA [19–21]. Unfortunately, the impressive results possible with rtPCR come with the risk of contamination and amplification of nontarget sequences [7]. Moreover, real-world sample matrices often interfere with the amplification process or even degrade the target via DNase hydrolysis. For these reasons, strategies for detection of nucleic acids which avoid amplification are highly desirable.

We have developed the fluid force discrimination (FFD) assay, a solid-phase assay for multiplexed detection that is simple, uses few reagents, is rapid (<25 min), and directly detects the biological target without amplification. We have previously demonstrated that FFD assays can be used for multiplexed, femtomolar nucleic acid hybridization [22] and/or attomolar immunoassays [23]. In FFD assays, biomolecular targets are captured onto a microarray and then labeled with microbeads. Next, nonspecifically bound bead labels are preferentially removed by application of controlled, laminar fluidic forces. The density of beads remaining on each spot after FFD indicates the target identity and concentration. The use of microbead labels enhances the analytical sensitivity by enabling label detection at extremely low label densities ($<<10^4$ beads/cm^2), down to single labels in a typical microarray spot (~100-μm diameter). Unlike approaches that rely on amplification, microbead assays are ideal for the analysis of samples in complex matrices because they do not suffer from the many forms of matrix interference that plague other labels (e.g., autofluoresence, electrochemical background). Finally, if the microbead labels are paramagnetic, FFD assays can be performed in a field-portable detection system incorporating magnetoresistive sensor array chips [22–26].

In the work presented here, we describe the application of FFD assays for the direct detection of genomic DNA from three closely related bacteria: *Bacillus thuringiensis (BT)* and two strains of

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[2] *Abbreviations used*: *BA*, *Bacillus anthracis*; *BT*, *Bacillus thuringiensis*; FFD, fluidic force discrimination; rtPCR, real-time polymerase chain reaction.

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Bacillus anthracis (BA). To successfully target a 54 base pair (bp) segment of each *Bacillus* sample ($\sim 5 \times 10^6$ bp genome), we must fragment the genome into smaller pieces. Successful detection has been achieved following fragmentation with either restriction enzymes or sonication; sonication is our preferred method owing to a significant savings in multiplexing strategy and assay time. Next, the fragmented DNA is mixed with blocking and labeling oligo sequences and thermocycled. The added oligo sequences isolate the target region in a ssDNA conformation that is then available for hybridization. Finally, the ssDNA target is captured at the microarray surface, labeled with a microbead, and FFD applied. We will discuss the impact of each of these sample preparation steps on detection and demonstrate how when correctly performed, these steps enable femtomolar detection of extracted *Bacillus* targets, including both species and strain recognition, in only minutes.

Materials and methods

Extraction of genomic material

Genomic material was extracted from vegetative *BA-Sterne* and *BT* samples as described below. Genomic DNA extracted from *BA-Ames* was obtained from the Department of Defense Critical Reagents Program (Frederick, MD, USA).

BT spores were cultivated from Thuricide (AG Organics, Prosper, TX, USA), a caterpillar insecticide containing $\sim 6 \times 10^6$ viable spores per milligram of insecticide concentrate. In this procedure, 5 mL of concentrate was added to 45 mL of nutrient broth and the suspension incubated overnight at 37 °C. The nutrient broth consisted of 5 g of peptone (ATCC, Manassas, VA, USA), 3 g beef extract (ATCC), 1 L distilled water, and finally adjusted to pH 6.8. After incubation, *BT* vegetative cells were harvested and washed with TE buffer (Sigma-Aldrich). Finally, genomic DNA was extracted using the Epicentre (Madison, WI, USA) gram-positive DNA purification kit according to manufacturer instructions. The amount of extracted DNA was determined by UV/Vis measurements using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The NanoDrop reports the measured optical density at 260 nm in nanograms per microliter. Using this protocol, a typical extraction from a 10^8 cfu/mL BT vegetative cell sample resulted in the recovery of $\sim$15 ng/μL of genomic DNA. For all experiments reported herein, we have converted the optical density readings into molarity using the known genome lengths and average molecular weight for a base pair. Note that all reported concentrations therefore represent the whole genome and not just the 54 bp target area. We have opted to use molarity as the universal measure in this paper because colony counts in plate cultures (cfu/mL) do not reproducibly reflect DNA concentration. The presence of lysed and nonviable cells contributes DNA that *is detected* with FFD assays, but those cells do not lead to colony formation [27].

The same extraction procedure was followed for the cultivation of *BA* vegetative cells beginning with *BA-Sterne* spores obtained from the Critical Reagents Program. In this case, 100 μL of *BA-Sterne* spore mixture (>10^8 cfu/mL) was added to 10 mL nutrient broth and incubated for 24–48 h at 37 °C. The cells were harvested and genomic DNA was extracted as described above. Again, extraction concentration was determined by UV/Vis measurements.

DNA fragmentation

Extracted DNA was fragmented by two different methods. The first method used restriction enzymes (New England Biolabs, Ipswich, MA, USA) to cut out the target sequence. Bacterial genomes were evaluated for restriction enzymes that cut on either side of the target sequence, but leave the target sequence intact. The selected enzymes were Eco0109 and Pci1 for *BT* samples, TspR1 and BsrG1 for *BA-Sterne* samples, and EcoR1 and Acu1 for *BA-Ames* samples. Many cuts were made in each genome and the resultant section containing the 54 bp target sequence was 286 bp long for *BT*, 407 bp long for *BA-Sterne*, and 344 bp long for *BA-Ames*. All enzymes were used in accordance with the manufacturer's instructions with respect to reaction temperature and buffer. For each bacterium, the first enzyme (which reacts at a lower temperature) was added to the genomic sample for 1 h; next the second enzyme was added for an additional 1 h; and finally all enzymes were inactivated by thermal treatment at 80 °C for 20 min.

The second fragmentation method used was sonication. DNA samples were sonicated on a Branson Sonifier 150 (Danbury, CT, USA) for 1 min, unless otherwise indicated in the text. The instrument was fitted with a 1/8-inch-diameter horn and used at a power output of 13 W at the tip.

Thermocycling, blocking, and labeling

All DNA sequences, including the targets, are listed in Table 1. Selection of the target regions was based on known PCR primer locations and with the largest possible sequence variation. Biotinylated capture and poly(dA)-terminated label probes were then designed with respect to T_m and G–C content. Following fragmentation of extracted genomic DNA, samples were prepared for detection by isolating the target sequence in a ssDNA format. To each fragmented sample, blocking sequences (Integrated DNA Technologies) and the label probe (Integrated DNA Technologies) were added such that each was at a final concentration of 1 μM. The mixture was then thermocycled on a Stratagene (La Jolla, CA, USA) Robocycler Gradient 96. The thermocycle procedure heated the mixture to 95 °C for 4 min, followed by 55 °C for 1 min, and then the mixture was immediately used for FFD assays.

Table 1
Probe sequences for the detection of *Bacillus* species.

Assay probe	Sequence (5′–3′)
BT capture	CAG AAC CAT AGA CCC ATA ATT CGT—biotin
BT target	ACG AAT TAT GGG TCT ATG GTT CTG CTA ATT GGA ATG ATA AGA TTA AGA CTG TAA
BT label	AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TTA CAG TCT TAA TCT TAT CAT TCC
BT block 1	TCT TGA ATT GTA AAT GCT TCC CCT TT
BT block 2	AAG GGG AAG CAT TTA CAA TTC AAG A
BT block 3	CTG CTA ATT GGA
BT block 4	TAG TGG TTG TAG ATA CAA ACG
BA-Ames capture	TTG ATA GTC CGT CAA TTA ATC TTG—biotin
BA-Ames target	CAA GAT TAA TTG ACG GAC TAT CAA TTA ACC CTC CTG TAT CTT GCA ACC TTT GAT
BA-Ames label	AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA ATC AAA GGT TGC AAG ATA CAG GAG
BA-Ames block 1	CAA TTA ACC CTC
BA-Ames block 2	TTA ATA TCA TAT GGT TGA ATA TCA AG
BA-Ames block 3	TAT ACT GCT TTC TTA CAT C
BA-Sterne capture	CGA TGC TGT GGC TCG ATA TAA—biotin
BA-Sterne target	TTA TAT CGA GCC ACA GCA TCG TGA TGT TTT ACA AAC GAA CAA GAA ATA AAT CTA
BA-Sterne label	AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA TAG ATT TAT TTC TTG TTC GTT TGT AAA
BA-Sterne block 1	TGC GAA AGC T
BA-Sterne block 2	AGC ATC GTG ATG TTT TAC AGC
BA-Sterne block 3	CTT TTC TGT AGA ATT CTT GGA ACA AA
Positive control	AAA AAA AAA AAA AAA AAA AAA AAA A—biotin
PCR primer	Sequence (5′-3′)
BT forward primer	TGT TTG GGA AGA ACG TGA GG
BT reverse primer	GAA TTG TAA ATG CTT CCC CTT T
BA forward primer	CAG CTT AAG GAA CAT CCC ACA
BA reverse primer	TCT TGA CAG CAT CCG TTG AT