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A Novel Screening Method for Competitive FRET-Aptamers Applied to *E. coli* Assay Development

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Abstract A novel high-throughput screening method is described in which a family of DNA aptamers selected against *E. coli* outer membrane proteins (OMPs) is subjected to PCR in the presence of fluorophore-dUTP conjugates using Deep Vent® exo- polymerase. The fluorophore-doped aptamers and their complementary strands are then heated to render them single-stranded and screened in filter well microtiter plates for fluorescence resonance energy transfer (FRET) assay potential. Using this system, a superior competitive FRET-aptamer designated EcO 4R was identified and the location of its putative binding pocket was determined by individually testing FRET potential in each of the secondary loop structures. By labeling the binding pocket with Alexa Fluor (AF) 647 and binding the aptamer to heavily Black Hole Quencher-3 (BHQ-3)-labeled *E. coli* bacteria, detection of as few as 30 live unlabeled *E. coli* per ml was achieved in a competitive displacement FRET assay format. The far red fluorescence emission enables detection in largely blue-green autofluorescent matrices. In addition, the competitive transfer of AF 647-EcO-4R aptamer to unlabeled *E. coli* cells after a 15 min equilibration period was verified by fluorescence microscopy. The present study also demonstrated that high aptamer affinity is not well correlated with competitive FRET potential.

Keywords Aptamer · Coliform · High-throughput screening · SELEX · Waterborne

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Introduction

Over the past decade, a number of prominent research groups have recognized the potential of DNA or RNA aptamers to be engineered as FRET biosensor elements [1–12]. Aptamer FRET strategies have centered on transfer of energy between fluorophores and mildly quenching nucleotides [5, 6, 9] in "signaling aptamers" or between fluorophores and quenchers on the 3′ and 5′ ends of a stem-loop structure in the manner of molecular beacons or aptly named "aptamer beacons" [4, 7, 8, 10, 11]. While these FRET approaches have worked fairly well, their signal-to-noise ratios and consequently their sensitivity have appeared limited in some cases.

In an attempt to overcome potential sensitivity limitations and achieve a unique intellectual property position, our group adopted a different approach. We reasoned that the best mode for FRET-aptamer responsivity might be to place a fluorophore and its optimum spectrally overlapping quencher directly in the putative aptamer binding pocket within the Förster distance of 60–85 Å. Such an approach should allow the target analyte to easily separate the fluorophore and quencher beyond the Förster distance, thereby triggering a sizeable increase in fluorescence. While we have also been developing an "intrachain" approach to placement of a fluorophore and its spectrally-matched quencher within the same DNA chain, our simpler competitive FRET approach (Fig. 1) [1–3] was used in the present work for high-throughput screening and assay development. We patterned our competitive FRET-aptamer assay strategy after that of competitive immuno-FRET assays [13, 14] in which we generally fluorophore or quencher-label the target analyte and then label the suspected aptamer binding pocket at various locations with the opposite moiety (quencher or fluorophore) until we achieve optimal FRET sensitivity upon

competitive displacement by the lowest possible levels of unlabeled analyte. This approach has proven quite productive, but we have discovered that we cannot simply search for the highest affinity aptamer by an ELISA-like microplate assay and expect that aptamer sequence to also exhibit optimal sensitivity and specificity in a competitive displacement FRET assay format [1–3].

Therefore, as reported here, we have developed a relatively rapid high-throughput FRET screening method to identify the best possible subset of competitive FRET aptamers from pools of tens to hundreds of sequences down selected from the final SELEX (Systematic Evolution of Ligands by Exponential enrichment) pool [15, 16]. The physical principle of our test involves labeling aptamers by PCR with more "permissive" DNA polymerases which lack editing functions (e.g., Deep Vent® exo-) and readily accept modified nucleotide substrates [17] such as fluorophore-dNTP conjugates. The double-stranded fluorophore-doped aptamer PCR product is then melted into a single-stranded (ss) aptamer, allowed to bind quencher-labeled analytes, and washed in a filter-well microplate (Fig. 1). Upon introduction of unlabeled target analytes, the fluorophore-labeled aptamers are liberated and fluoresce in proportion to the amount of unlabeled target present in a sample until equilibrium is reached (Fig. 1). The physical principle of competitive displacement, therefore, forms the basis for detection which can be easily expanded to high-throughput screening when filter well microtiter plates are used to enable rapid formation and purification (washing) of the fluorophore-labeled aptamer plus quencher-labeled bacteria FRET complexes for each different aptamer sequence to be assessed.

Experimental

DNA aptamer development, cloning and sequencing

A lawn of *E. coli* strain 8739 (Crooks strain) was grown on a blood agar plate overnight at 37 °C. Bacteria were washed from the plate into 10 ml of cold 1.5 M $MgCl_2$ in sterile nuclease-free water. The thick suspension of bacteria was left overnight at 4 °C to allow the chaotropic action of $MgCl_2$ to extract OMPs. One ml of bacterial suspension was centrifuged at 14,000 rpm for 5 mins on a microfuge and the supernate was added to 1 ml of stock 2.8 micron Dynal tosyl-coated magnetic beads (2×10^9 MBs, Invitrogen Corp. Carlsbad, CA) for 2 hrs at 37 °C. OMP-conjugated MBs were collected for 2 mins on a Dynal MPC-S® magnetic collection device and the supernate was

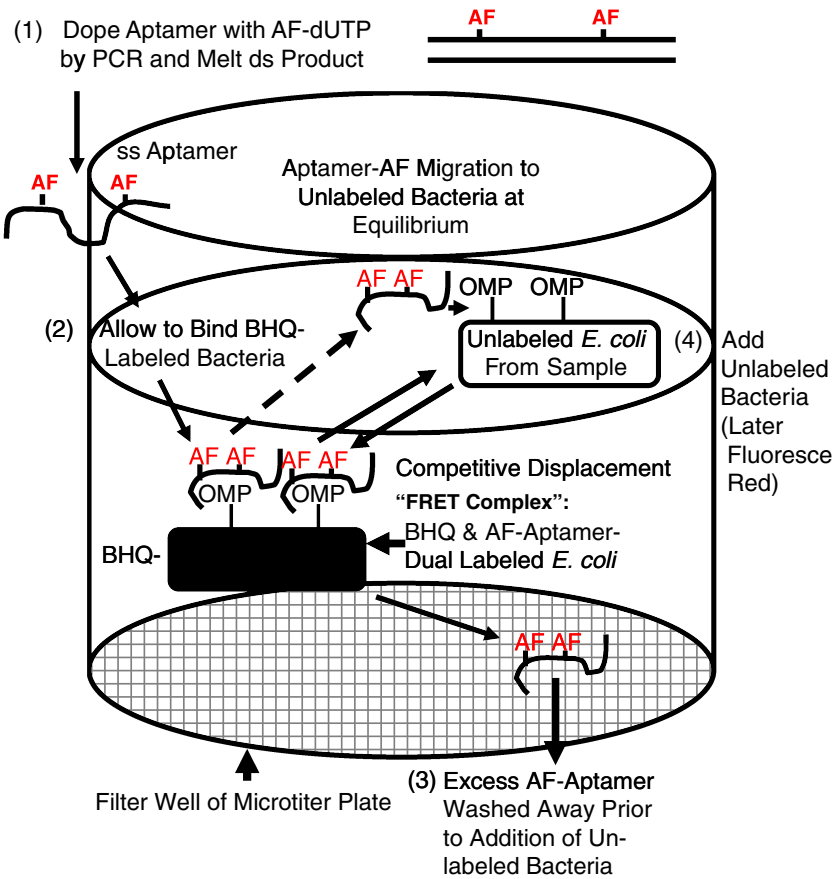


Fig. 1 Diagram illustrating the competitive displacement assay principle and competitive high-throughput FRET-aptamer screening method for bacteria in a single filter well. Major steps in the process are numbered to indicate order of performance. Abbreviations: *AF* Alexa Fluor, *BHQ* Black Hole Quencher, and *OMP* outer membrane protein

carefully withdrawn with a pipette tip. OMP-MBs were then resuspended by vortexing briefly in 1X Binding Buffer (1XBB; 0.5 M NaCl, 10 mM Tris-HCl, and 1 mM $MgCl_2$, pH 7.5–7.6,) and washed by agitation for 5 mins. MBs were collected and washed three times in this manner and then resuspended in 1 ml of 1XBB.

MB-SELEX was performed essentially as previously described [1, 3, 15, 18, 19]. All oligonucleotides were obtained from Integrated DNA Technologies, Inc. (IDT; Coralville, IA). The degenerate SELEX template library sequence was: 5′-ATCCGTCACACCTGCTCT-N_{36}-TGGTGTTGGCTC CCGTAT-3′, where N_{36} represents the randomized 36-base region of the DNA library. Primer sequences were: 5′-ATACGGGAGCCAACACCA-3′ (designated forward) and 5′-ATCCGTCACACCTGCTCT-3′ (designated reverse) to prime the template and nascent strands, respectively. The random library was reconstituted in 500 μl of sterile nuclease-free water and heated to 95 °C for 5 min to ensure that the DNA library was completely ss and linear. The hot template solution was added to 100 μl of OMP-MBs (2×10^8 beads) with 600 μl of sterile 2X Binding Buffer (2XBB). The DNA library and OMP-MB suspension (1.2 ml) was mixed at room temperature (RT) for 1 hr. OMP-MBs with any bound DNA (round 1 aptamers) were magnetically collected with the Dynal MPC-S® unit. The DNA-OMP-MB complexes were washed three times in 400 μl of sterile 1XBB. Following the third wash, the DNA-OMP-MB pellet (about 75 μl) was used in a PCR reaction to amplify the bound DNA as follows. The MB pellet was split into 15 μl aliquots and added to five Easy Start™ Micro 50 tubes (Molecular BioProducts, Inc., San Diego, CA), which contained most of the nonperishable components of a PCR reaction beneath a wax seal. A total of 3 μl of 1:10 primer mix (10% forward primer plus 10% reverse primer) in nuclease-free deionized water or ~ 20 nanomoles of each primer per ml plus 1 μl (5 U) of Taq DNA polymerase (Fisher Scientific, Inc., Pittsburgh, PA) and 5 μl of 2 mM $MgCl_2$ were added to each of the five tubes. PCR reactions were supplemented with 0.5 μl of Perfect Match™ *E. coli* single-strand binding protein (SSBP, Stratagene Inc., La Jolla, CA) to inhibit high molecular weight concatamer formation. PCR was carried out as follows: an initial 95 °C phase for 5 min, followed by 30 cycles of 1 min at 95 °C, 1 min at 53 °C, and 1 min at 72 °C followed by a 72 °C completion stage for 7 min, and refrigeration at 4 °C. This constituted the first of five rounds of MB-SELEX. To begin the second round and all subsequent rounds, four complete tubes of the five original PCR tubes were heated to 95 °C for 5 min to release bound DNA from the OMP-MBs. The fifth tube was always retained and refrigerated as a back-up for that round of the SELEX process. All available DNA (25 μl per tube) was siphoned out of the hot tubes without removing the OMP-MBs before the tubes cooled significantly and the DNA was pooled. The 100 μl of hot DNA was added to 100 μl of fresh OMP-MBs in 200 μl of 2XBB and allowed to mix for 1 hr at RT. Thereafter, the selection and amplification process was repeated for four more rounds of MB-SELEX with checking for 72 bp aptamer PCR product by ethidium bromide stained 2% agarose electrophoresis after each round. Following round 5, aptamers were cloned into chemically competent *E. coli* using a Lucigen GC cloning kit (Middleton, WI) and clones were sent to Sequetech, Inc. (Mountain View, CA) for proprietary GC-rich DNA sequencing [18, 19].

Enzyme-linked aptamer plate assay

To evaluate relative affinity rankings for each of the 44 candidate OMP aptamers (Table 1), an enzyme-linked plate assay [3, 18, 20] was conducted by first immobilizing 100 μl of 1:10 diluted *E. coli* 8739 OMPs in 0.1 M $NaHCO_3$ (pH 8.5) overnight at 4 °C in a covered polystyrene 96-well plate. The plate was decanted and washed three times in 250 μl of 1XBB. The 44 different 5′-biotinylated *E. coli* OMP aptamers (4.50 nmoles each) from Integrated DNA Technologies (Coralville, IA) were rehydrated in 100 μl of 1XBB and applied to their corresponding plate assay wells for 1 hr at room temperature (RT; ~25 °C) with gentle mixing on an orbital shaker. The plate was decanted and washed three times in 250 μl of 1XBB for at least 5 min per wash with gentle mixing. One hundred μl of a 1:2,000 dilution of streptavidin-peroxidase from a 5 mg/ml stock solution (Southern Biotech, Inc., Birmingham, AL) in 1XBB was added per well for 30 min at RT with gentle mixing. The plate was decanted and washed three times with 250 μl of 1XBB per well as before. One hundred μl of One-Component® ABTS substrate (Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD) was added per well for 10 min at RT. Absorbance was quantified using a microplate reader at 405 nm.

High-throughput FRET screening method

To prepare for the high-throughput FRET screening method depicted in Fig. 1, two ml of *E. coli* 8739 bacterial suspension from the lawn of bacteria described previously were labeled by addition of 100 μl of 5 mg/ml Black Hole Quencher (BHQ)-2-succinimide (Biosearch Technologies, Inc., Novato, CA) in DMSO in a 37 °C incubator for 2 hr. BHQ-2 labeled bacteria were pelleted in two separate microfuge tubes (1 ml per tube) at 14,500 rpm for 5 min followed by resuspension in 1XBB and 3 washes in 1 ml of 1XBB. BHQ-2-labeled and unlabeled bacteria were counted under a phase-contrast microscope (400X magnification) using an Improved Neubauer hemocytometer to estimate cell concentration.

Table 1 DNA aptamer sequences selected against *E. coli* 8739 OMPs

EcO – 1 For	**ATACGGGAGCCAACACCA** TGGTACAAGCAAACCAATATTAGGGCCCAGACATCG **AGAGCAGGTGTGACGGAT**
EcO – 1 Rev	**ATCCGTCACACCTGCTCT** CGATGTCTGGGCCCTAATATTGGTTTGCTTGTACCA **TGGTGTTGGCTCCCGTAT**
EcO – 2 For	**ATACGGGAGCCAACACCA** TGATACCCTAAGGTAGGGGAGGCCTAAGCGCCACGT **AGAGCAGGTGTGACGGAT**
EcO – 2 Rev	**ATCCGTCACACCTGCTCT** ACGTGGCGCTTAGGCCTCCCCTACCTTAGGGTATCA **TGGTGTTGGCTCCCGTAT**
EcO – 3 For	**ATACGGGAGCCAACACCA** CGCATCCCCCGCCGGGCCCGCGCCCCGCTCGCAGAC **AGAGCAGGTGTGACGGAT**
EcO – 3 Rev	**ATCCGTCACACCTGCTCT** GTCTGCGAGCGGGGCGCGGGCCCGGCGGGGGATGCG **TGGTGTTGGCTCCCGTAT**
EcO – 4 For	**ATACGGGAGCCAACACCA** TAATATGCCGTAAGGAGAGGCCTGTTGGGAGCGCCGT **AGAGCAGGTGTGACGGAT**
EcO – 4 Rev	**ATCCGTCACACCTGCTCT** ACGGCGCTCCCAACAGGC***CTCTCCTTACG***GCATATTA **TGGTGTTGGCTCCCGTAT**
EcO – 5 For	**ATACGGGAGCCAACACCA** GGAAAAAAAGAGCCTGTGAAGATTGTAATATCAGTT **AGAGCAGGTGTGACGGAT**
EcO – 5 Rev	**ATCCGTCACACCTGCTCT** AACTGATATTACAATCTTCACAGGCTCTTTTTTTCC **TGGTGTTGGCTCCCGTAT**
EcO – 7 For A	**ATACGGGAGCCAACACCA** CAAAAGCCTTACCTAACTGCCAACAATGAATAGCA **AGAGCAGGTGTGACGGAT**
EcO – 7 For B	**ATACGGGAGCCAACACCA** TACCTGACCCCCCGCCGCAATCCTAGTCTACCTCCG **AGAGCAGGTGTGACGGAT**
EcO – 7 Rev A	**ATCCGTCACACCTGCTCT** CGGAGGTAGACTAGGATTGCGGCGGGGGGTCAGGTA **TGGTGTTGGCTCCCGTAT**
EcO – 7 Rev B	**ATCCGTCACACCTGCTCT** TGCTATTCATTGTTGGCAGTTAGGTAAGGCTTTTGT **TGGTGTTGGCTCCCGTAT**
EcO – 8 For	**ATACGGGAGCCAACACCA** CGACTAACACGACCGTTGGGGGGGGCTCGCGCGGGC **AGAGCAGGTGTGACGGAT**
EcO – 8 Rev	**ATCCGTCACACCTGCTCT** GCCCGCGCGAGCCCCCCCCAACGGTCGTGTTAGTCG **TGGTGTTGGCTCCCGTAT**
EcO – 9 For	**ATACGGGAGCCAACACCA** GTCCCCGCCCAGCCGTGAGCCGTACCCCCGCACACC **AGAGCAGGTGTGACGGAT**
EcO – 9 Rev	**ATCCGTCACACCTGCTCT** GGTGTGCGGGGGTACGGCTCACGGCTGGGCGGGGAC **TGGTGTTGGCTCCCGTAT**
EcO – 10 For	**ATACGGGAGCCAACACCA** TCCCGCCCCGTTTTTGCTCTTGCAGGCCCAACCTTG **AGAGCAGGTGTGACGGAT**
EcO – 10 Rev	**ATCCGTCACACCTGCTCT** CAAGGTTGGGCCTGCAAGAGCAAAAACGGGGCGGGA **TGGTGTTGGCTCCCGTAT**
EcO – 11 For	**ATACGGGAGCCAACACCA** TCCCGCCCCGTTTTTGCTCTTGCAGGCCCAACCTTG **AGAGCAGGTGTGACGGAT**
EcO – 11 Rev	**ATCCGTCACACCTGCTCT** ACTTGGCTTGCGACTATTATTCACAGGGCCAAAGAC **TGGTGTTGGCTCCCGTAT**
EcO – 12 For	**ATACGGGAGCCAACACCA** TAGTGTTGGACCAATACGGTAACGTGTCCTTGG **AGAGCAGGTGTGACGGAT**
EcO – 12 Rev	**ATCCGTCACACCTGCTCT**CCAAGGACACGTTACCGTATTGGTCCAACACTA **TGGTGTTGGCTCCCGTAT**
EcO – 17 For	**ATACGGGAGCCAACACCA** CACGACCCAAGGAATTGGAAAAACACCGACATTCCA **AGAGCAGGTGTGACGGAT**
EcO – 17 Rev	**ATCCGTCACACCTGCTCT** TGGAATGTCGGTGTTTTTCCAATTCCTTGGGTCGTG **TGGTGTTGGCTCCCGTAT**
EcO – 18 For	**ATACGGGAGCCAACACCA** CGTCCTCCACCCCCGCCCGGACCGCGTCGCCGTCGC **AGAGCAGGTGTGACGGAT**
EcO – 18 Rev	**ATCCGTCACACCTGCTCT** GCGACGGCGACGCGGTCCGGGCGGGGGTGGAGGACG **TGGTGTTGGCTCCCGTAT**
EcO – 19 For A	**ATACGGGAGCCAACACCA** GAGGGTTCTAGGGTCACTTCCATGAGAATGGCTCAC **AGAGCAGGTGTGACGGAT**
EcO – 19 For B	**ATACGGGAGCCAACACCA** CCACGACTCCCCCCGCCTCCCTCGCGTCCCCAGGCC **AGAGCAGGTGTGACGGAT**
EcO – 19 Rev A	**ATCCGTCACACCTGCTCT** GGCCTGGGGACGCGAGGGAGGCGGGGGGAGTCGTGG **TGGTGTTGGCTCCCGTAT**
EcO – 19 Rev B	**ATCCGTCACACCTGCTCT** GTGAGCCATTCTCATGGAAGTGACCCTAGAACCCTC **TGGTGTTGGCTCCCGTAT**
EcO – 20 For	**ATACGGGAGCCAACACCA** GCAGCGCTGGAGAACTGTATAGTAAGAGGCCCTGTG **AGAGCAGGTGTGACGGAT**
EcO – 20 Rev	**ATCCGTCACACCTGCTCT** CACAGGGCCTCTTACTATACAGTTCTCCAGCGCTGC **TGGTGTTGGCTCCCGTAT**
EcO – 21 For	**ATACGGGAGCCAACACCA** TCTCTCTTAGGATACAAAGCCAAACTGAGCCCGTGC **AGAGCAGGTGTGACGGAT**
EcO – 21 Rev	**ATCCGTCACACCTGCTCT** GCACGGGCTCAGTTTGGCTTTGTATCCTAAGAGAGA **TGGTGTTGGCTCCCGTAT**
EcO – 22 For	**ATACGGGAGCCAACACCA** GGGGGTGGCGAACATGGTATAACTTGATAAGTGTGA **AGAGCAGGTGTGACGGAT**
EcO – 22 Rev	**ATCCGTCACACCTGCTCT** TCACACTTATCAAGTTATACCATGTTCGCCACCCCC **TGGTGTTGGCTCCCGTAT**
EcO – 23 For	**ATACGGGAGCCAACACCA** CTCCGACACCGGCCGCCGGCACCACCCACTCCCCCT **AGAGCAGGTGTGACGGAT**
EcO – 23 Rev	**ATCCGTCACACCTGCTCT** AGGGGGAGTGGGTGGTGCCGGCGGCCGGTGTCGGAG **TGGTGTTGGCTCCCGTAT**
EcO – 24 For	**ATACGGGAGCCAACACCA** TCCGGCGCGCCCTCCTCCCCCACTGCTCCCCGCCCG **AGAGCAGGTGTGACGGAT**
EcO – 24 Rev	**ATCCGTCACACCTGCTCT** CGGGCGGGGAGCAGTGGGGGAGGAGGGCGCGCCGGA **TGGTGTTGGCTCCCGTAT**
EcO – 25 For	**ATACGGGAGCCAACACCA** TACGCAGAGGTCCCCTACCCAGGCCAGCCGGATGCC **AGAGCAGGTGTGACGGAT**
EcO – 25 Rev	**ATCCGTCACACCTGCTCT** GGCATCCGGCTGGCCTGGGTAGGGGACCTCTGCGTA **TGGTGTTGGCTCCCGTAT**

All sequences are presented 5′ to 3′ from left to right. Fixed primer regions are *bolded*. C and G are shaded variously to show the high GC content of most aptamer candidates and general lack of consensus sequences but commonality of some short sequence runs. The putative loop 5 binding region (CTCTCCTTACG) of the EcO 4R aptamer for competitive FRET is highlighted *underlined* and *italicized*. This sequence occurs only once in the sequenced library

The 44 different aptamer sequences which resulted from OMP-MB-SELEX listed in Table 1 were synthesized and lyophilized in separate wells of a 96-well microplate by Integrated DNA Technologies, Inc. at exactly 4.50 nanomoles per well. Wells were rehydrated in 100 µl of nuclease-free water with gentle mixing for 30 mins and 30 µl (1.35 nanomoles) were withdrawn and added to the tops of 44 EasyStart Micro 100™ pre-made PCR mix tubes (Molecular BioProducts, Inc., San Diego, CA). Also added to each tube was 6 µl of 100 µg/ml forward and reverse primers (5′-ATACGGGAGCCAACACCA-3′ and 5′-ATCCGTCACAC CTGCTCT-3′ respectively), 2 µl of 0.1 mM Alexa Fluor 546-14-dUTP (Invitrogen), 10 µl of 10X ThermoPol buffer and 2 µl of Deep Vent® exo- DNA polymerase (New England Biolabs, Ipswich, MA). PCR was conducted by first heating tubes to 95 °C for 5 min followed by 20 cycles of 95 °C for 45 s, 53 °C for 45 s, and 72 °C for 45 s. PCR was completed by heating at 72 °C for 7 min and cooling to 4 °C.

The wax layer was removed from each tube and a 5 µl sample of PCR products was drawn from nine tubes at random to check for successful amplification and AF 546-14-dUTP incorporation by running a 20% TBE-polyacrylamide electrophoresis gel and photographing the gel before and after staining in ethidium bromide on a UV transilluminator (Fig. 3). PCR tubes were heated to 95 °C for 2–5 min to convert all DNA to its single-stranded form. All 100 µl of AF 546-doped PCR products were rapidly transferred to their respective wells in a black filter well plate (Part No. 5044, 0.45 µm pore size GHP filter well plate, PALL Life Sciences, Ann Arbor, MI) that was pre-loaded with 100 µl of BHQ-2-labeled *E. coli* 8739 (approximately 10^5 cells/well).

The AF 546-labeled aptamer candidates and BHQ-2-labeled bacteria were gently swirled for 20 min at RT and then unbound AF 546-labeled aptamers were washed out by suction using a PALL Life Sciences vacuum manifold. Each well was then washed twice in 200 µl of 1XBB and finally resuspended in 100 µl of 1XBB. One hundred µl of unlabeled *E. coli* 8739 at a final concentration of approximately100 cells/well were added to each well and the wells were allowed to equilibrate with gentle swirling for 15 min at RT. Fluorescence intensity readings were acquired using a Cary Varian Eclipse® spectrofluorometer with microplate reader attachment set to excite at 555 nm with 5 nm slits, 900 V photomultiplier tube (PMT) setting, and scanning from 560–700 nm. Following the 100 cells/well microplate scan, an additional 90 µl of more concentrated unlabeled *E. coli* 8739 was added to each well to bring the approximate unlabeled cell concentration to 1,000 cells/well, the plate was swirled gently for an additional 15 min and fluorescence readings were repeated. Samples of the before and after FRET interaction groups were subjected to fluorescence microscopy using an Olympus BH-2 microscope equipped with a rhodamine filter cube and Motic color CCD camera system and image acquisition software.

Competitive FRET-aptamer assays

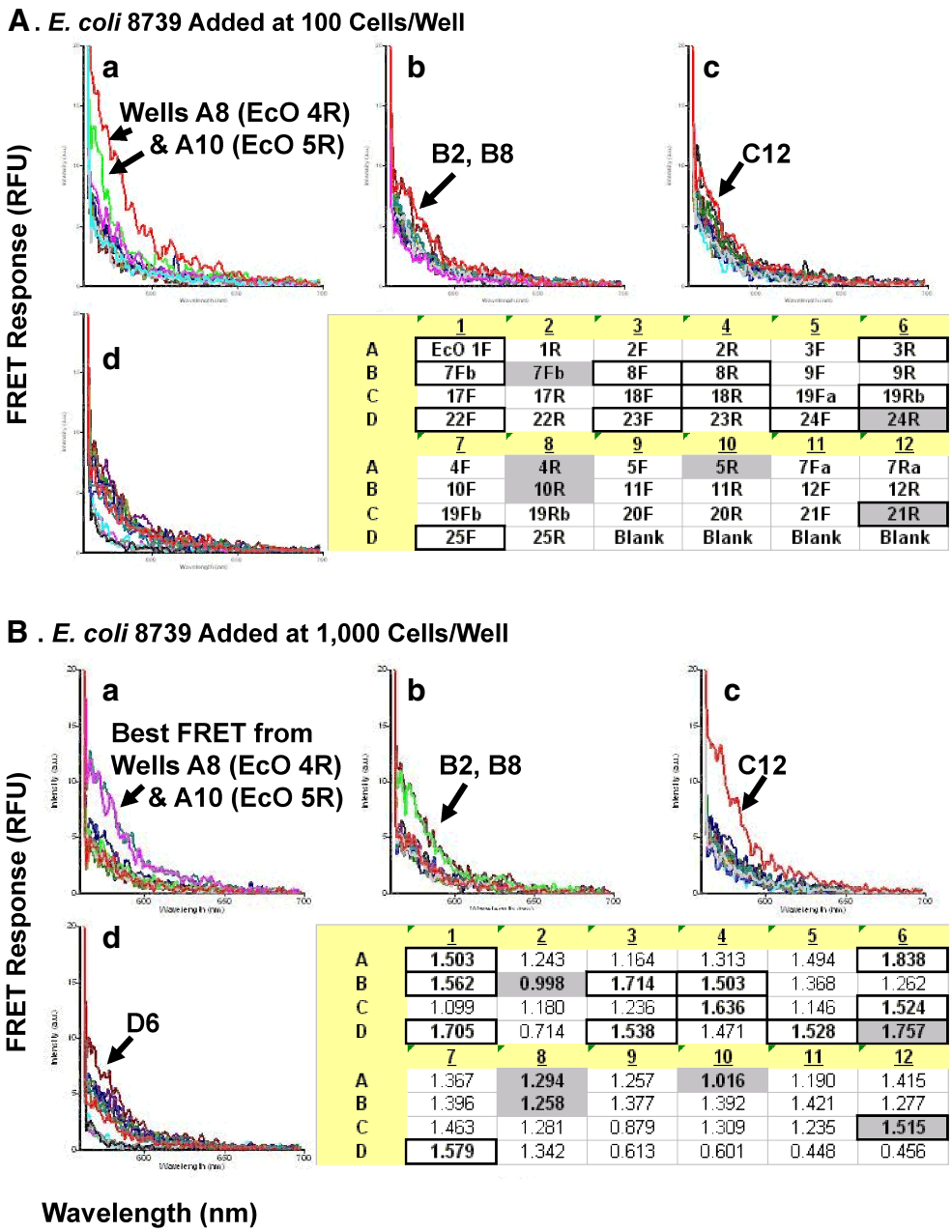
Once the field of most sensitive FRET-aptamers had been narrowed to two sequences (EcO 4R and EcO 5R) or their complementary forward-primed sequences based on the FRET results in Fig. 2 (wells A8 and A10), the secondary structures of these top two aptamer sequences were deduced at RT using internet-based Vienna RNA software [21, 22] with DNA parameters [23] as shown in Fig. 4. Approximately 1.1 mg of each of the aptamer sequences was then synthesized by Integrated DNA Technologies with a Uni-Link™ primary amine moiety located at the approximate center of each loop structure for a total of ten different Uni-Link™ amine-labeled oligonucleotides. Each of these internally amine-labeled aptamers was then labeled with 100 µl of AF 647-succinimide from an oligonucleotide labeling kit (Invitrogen) for 2 hrs in a 37 °C incubator, followed by purification through a 1XBB-equilibrated PD-10 (Sephadex G-25; GE Healthcare) column. The one ml fractions of each of the purified AF 647-DNA aptamers were reacted with approximately 10^7 BHQ-3-succinimide-labeled *E. coli* 8739 cells for 30 min at RT with mixing in 1XBB and then washed three times in 1 ml of 1XBB after pelleting at 14,500 rpm for 5 min per wash to produce a "FRET complex" consisting of bound AF 647-aptamer on BHQ-3-labeled bacteria.

Generally, 100 to 300 µl of each of the FRET bacterial complexes (10^6 to 3×10^6 FRET-labeled cells) were added to each of nine polystyrene cuvettes (Perfector Scientific, Inc., Atascadero, CA) in which one ml suspensions of *E. coli* 8739 had been serial ten-fold diluted. A tenth cuvette received no unlabeled bacteria and acted as the baseline (zero) control in each case and all cuvettes were brought to a total volume of 2 ml with 1XBB. The cuvettes were gently swirled in racks on an orbital shaker for 20 min and fluorescence was assessed with a Cary Varian Eclipse spectrofluorometer using an excitation of 650 nm with 5 nm slits, a 900 V PMT setting and scanning of emission intensity from 655 to 700 nm.

Similar competitive FRET assay technique was used for the *E. coli* titration and cross-reactivity studies. To verify cell concentrations and limits of detection, 100 µl of the lowest detectable FRET reactions were plated on five separate blood agar plates by the spread plate method and incubated overnight at 37 °C for colony counts.

Surface plasmon resonance

Three top *E. coli* aptamer candidates (EcO 3R, EcO 4R, and EcO 8F) were selected based on their enzyme-linked



	1	2	3	4	5	6
A	EcO 1F	1R	2F	2R	3F	3R
B	7Fb	7Fb	8F	8R	9F	9R
C	17F	17R	18F	18R	19Fa	19Rb
D	22F	22R	23F	23R	24F	24R
	7	8	9	10	11	12
A	4F	4R	5F	5R	7Fa	7Ra
B	10F	10R	11F	11R	12F	12R
C	19Fb	19Rb	20F	20R	21F	21R
D	25F	25R	Blank	Blank	Blank	Blank

	1	2	3	4	5	6
A	1.503	1.243	1.164	1.313	1.494	1.838
B	1.562	0.998	1.714	1.503	1.368	1.262
C	1.099	1.180	1.236	1.636	1.146	1.524
D	1.705	0.714	1.538	1.471	1.528	1.757
	7	8	9	10	11	12
A	1.367	1.294	1.257	1.016	1.190	1.415
B	1.396	1.258	1.377	1.392	1.421	1.277
C	1.463	1.281	0.879	1.309	1.235	1.515
D	1.579	1.342	0.613	0.601	0.448	0.456

Fig. 2 High-throughput competitive FRET-aptamer screening results with **a** low added levels of unlabeled live *E. coli* (100 bacterial cells/well) and **b** higher added levels (1,000 cells/well) to compete off fluorophore-labeled aptamers from quencher-labeled bacterial complexes. Excitation was at 550 nm (for AF 546-doped aptamer PCR products) and the PMT was set to 900 V. Also shown in **(a)** is the corresponding assay plate matrix of aptamer designations for reference and in **(b)** the absorbance values at 405 nm for each well of the enzyme-linked plate assay which directly correspond to the aptamers named in the matrix shown in **(a)** above. Shaded values in **(b)** represent the absorbance for the best FRET aptamer wells (A8, A10, B2, B8, C12, and D6) while boxed values represent the highest affinity aptamer wells with $A_{405} > 1.50$

plate assay or their high-throughput FRET screen results and were purchased with 5′ biotin tags from Integrated DNA Technologies. A 50 µM solution of each aptamer was covalently attached to a fresh CM5 chip (GE Healthcare, Piscataway, NJ) previously saturated with streptavidin. Each aptamer was then tested on a Biacore X100 (GE Healthcare), with various concentrations ranging from 6.9×10^5 to 1.1×10^7 cells/ml or more of three different *E. coli* strains O157:H7, 8739, and HB101 as well as *Salmonella enterica* strain 13311, *Enterococcus faecalis* and *Campylobacter jejuni* strain 29428. Varied concentrations of each bacterial strain were passed over each aptamer at a flow rate of 30 µl/min with an association time of 30 s. The dissociation time was set for 600 s and a regeneration step consisting of 50 mM NaOH between each sample injection for 30 s was included.

Results

We attempted the high-throughput FRET-aptamer screening scheme outlined in Fig. 1 for low (100 *E. coli* 8739 cells per well) and high (1,000 *E. coli* 8739 cells per well) cell concentrations to narrow the determination of which candidate *E. coli* OMP aptamer sequences shown in Table 1 were most promising for FRET-aptamer assay development. Figure 2 illustrates the general results of these studies in which wells A8 and A10 (EcO 4R and 5R aptamer

templates respectively or their forward-primed complementary strands) yielded consistently higher FRET even at low bacterial concentrations. It is of interest to note that aptamer sequences in some wells such as A10, B2, B8, C12 and D6 exhibited an increased FRET response to increased levels of unlabeled bacteria while the aptamers in well A8 (EcO 4R and 4F) continued to yield one of the most intense fluorescence responses at both the low and high levels of unlabeled bacteria. For this reason, the aptamer sequences from wells A8 (EcO 4R and 4F) and well A10 (EcO 5R and 5F) were chosen for further FRET analysis and dissection of their potential binding pockets to better understand and exploit their FRET potential. Clearly, the forward-primed cDNA strands (EcO 4F and 5F aptamers) could also be responsible for some or all of the FRET from wells A8 and A10, but oddly the wells in which these aptamers acted as templates (A7 and A9) did not exhibit a noteworthy FRET response. Therefore, we focused our attention on further study of the secondary loop structures of EcO 4R and 5R (wells A8 and A10 respectively).

Figure 2 also illustrates the fact that, in general, aptamer affinity as evaluated by the aptamer-based analogue to ELISA [3, 15, 18, 20, 24] did not correlate well with the ability of a particular aptamer sequence to exhibit competitive FRET. The matrix in the upper panel of Fig. 2 indicates the location of each aptamer sequence with corresponding absorbance values from the enzyme-linked plate assay taken at 405 nm in the lower panel. The shaded values indicate the relative affinity of the best FRET-aptamers which ranged from <1.0 to slightly >1.5, while the highest affinity aptamers (boxed in the lower panel of Fig. 2) all had absorbance values >1.5 and ranged beyond 1.8. It is of interest to note that there is little correlation between aptamer affinity as reflected in the enzyme-linked plate assay results and performance in the FRET screening assay (i.e., wells A8 and A10 show only moderate affinity for *E. coli* with A_{405}=1.294 and 1.016 respectively and yet dominated the FRET responses even at 100 added bacteria per ml).

Some of the difference in aptamer affinity versus FRET capacity may be accounted for by differences in the ability of some aptamer templates to incorporate AF 546-14-dUTP even with the permissive Deep Vent® exo- DNA polymerase during PCR [17] as shown in Fig. 3. Figure 3a demonstrates lack of incorporation of AF 546-14-dUTP (boxed in Fig. 3a) in two of the nine PCR samples chosen at random, but successful or partially successful PCR in all nine samples (Fig. 3b). Clearly, if fluorophores are completely lacking in a potential FRET-aptamer structure, no competitive FRET can be expected. Two more repeated attempts to improve the PCR-based incorporation with up to 10 more rounds of PCR (30 rounds total) did not yield any improvement in AF 546-14-dUTP incorporation or significant changes in the high-throughput FRET screening results (data not shown). These results may be a reflection of an inability to effectively incorporate fluorophore-modified dUTP into some GC-rich nascent strands. The unsuccessful AF 564-14-dUTP PCR incorporation reactions generally used templates with 50% or more GC content. Still, the high-throughput method did appear to reveal some potentially strong FRET responders such as those in wells A8 (Eco 4R), A10 (EcO 5R), etc. (Fig. 2).

We used web-based Vienna RNA software [21, 22] with DNA parameters [23] and a default temperature of 25 °C to determine the secondary structures and color-coded probabilities of the stem-loop structures for the promising EcO

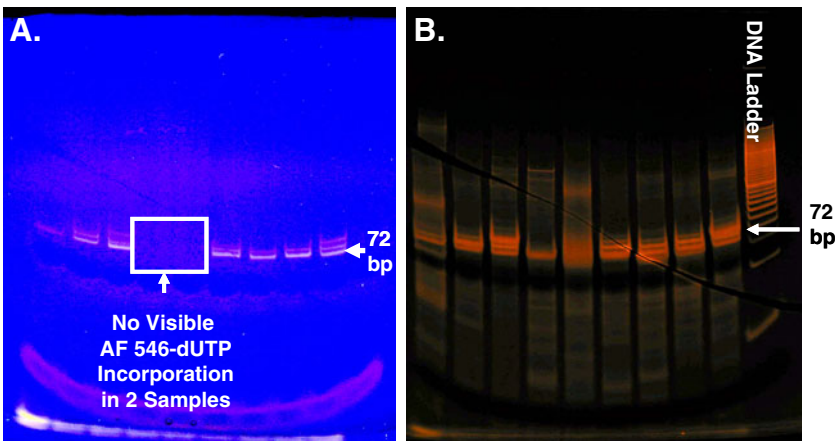


Fig. 3 **a** Unstained 20% polyacrylamide electrophoresis gel on a UV transilluminator and **b** same gel after ethidium bromide staining and a buffer wash showing that most samples from the FRET screening experiments produced successful PCR products **(b)**, but two of the nine PCR products (boxed) were unable to incorporate AF 546-14-dUTP (pink bands at left in **(a)**). The lack of pink bands in the boxed region in **(a)** suggests that some GC-rich templates inhibit or prohibit fluorophore-modified dUTP incorporation by PCR even with Deep Vent exo- polymerase. These observations may account for the failure of some aptamers to exhibit FRET in the screening data shown in Fig. 2. The far right lane in **(b)** consists of a DNA ladder for size reference (aptamer PCR products are 72 bp in length)

4R and EcO 5R aptamers as shown in Fig. 4. From this information, we defined five potential binding pockets as marked in Fig. 4 for each of the candidate aptamers and we purchased the ten different DNA aptamers labeled with amino linkers placed in the approximate middle of each loop structure for labeling with AF 647-succinimide (Invitrogen) and purification through Sephadex G-25. The various loops were labeled with Uni-Link™ amine-succinimide AF 647 (Integrated DNA Technologies) as follows with numbering of bases from the 5′ end. For Eco 4R, labels were placed between bases 12/13, 25/26, 34/35, 42/43, and 53/54 and for EcO 5R, labels were placed between bases 13/14, 17/18, 30/31, 38/39, and 48/49 to locate AF 647 at or near the center of each respective secondary loop structure as illustrated by red hash marks between the bases in Fig. 4.

Upon fluorescence screening of the ten potential EcO 4R and 5R aptamer binding loops, the number 5 loop of EcO 4R between positions 42 and 43 (counting from the 5′ end) gave the strongest FRET response to a series of dilutions of *E. coli* 8739 as shown at the bottom of Fig. 4. None of the other loop structures labeled with AF 647 gave any noteworthy FRET responses (data not shown for brevity).

We performed several steps to begin optimizing the competitive FRET-aptamer assay using the EcO 4R aptamer labeled with AF 647 at position 42/43 including varying the FRET-aptamer to BHQ-3 ratio and the amount of AF 647-EcO 4R bound to BHQ-3-labeled *E. coli* ("FRET reagent"). By tripling the amount of FRET reagent to the equivalent of 3×10^6 FRET-reagent-labeled bacterial cells per assay, we were able to achieve an improved signal-to-noise ratio, but also elevated the background fluorescence about two-fold (compare Fig. 5a and b with the bottom panel of Fig. 4). The highest detectable unlabeled *E. coli* 8739 dilutions (10^{-5} dilution in Fig. 5a and b) in 1XBB was subsequently determined to represent an average limit of detection of 30 colony forming units/ml (30 cfu/ml) by spread plate counts on five separate blood agar plates in the two separate trials. Figure 5a and b also illustrate that the unlabeled *E. coli* themselves have no appreciable fluorescence in the red region of the spectrum above 650 nm; a fact that was later confirmed by fluorescence microscopy (Fig. 6).

Our group also performed preliminary cross-reactivity evaluations for the EcO 4R-AF 647 FRET assay and found that EcO 4R exhibits relatively low cross-reactivity with

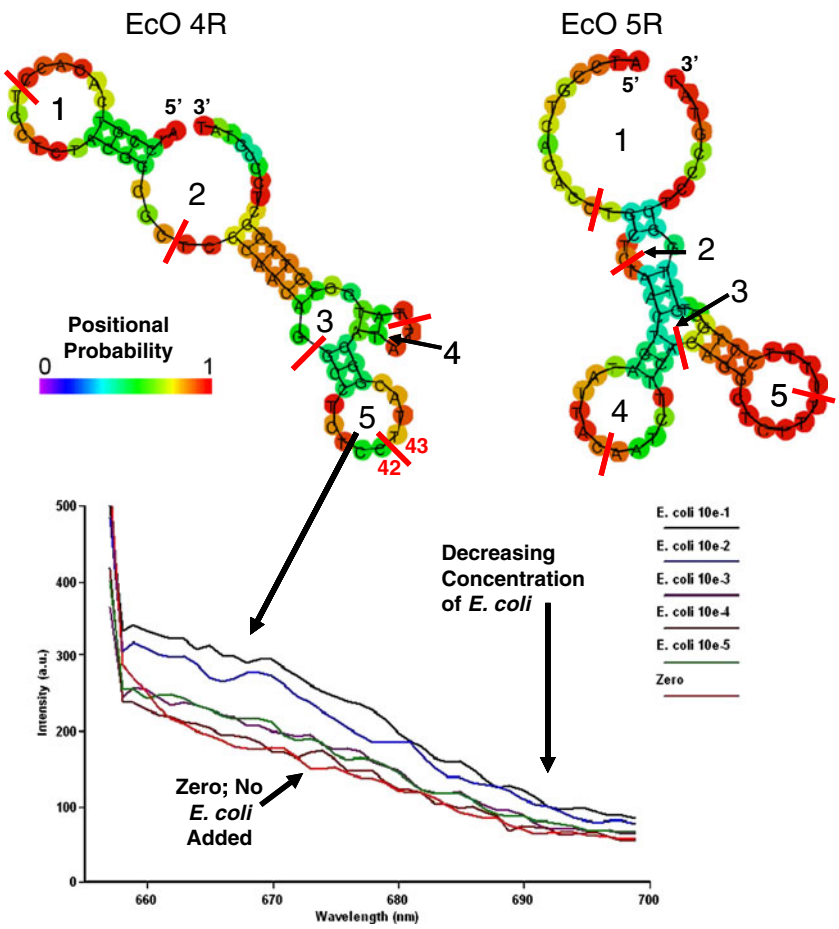


Fig. 4 Secondary structures of the EcO 4R and 5R aptamers by use of web-based Vienna RNA software using DNA parameters at 25 °C. Color-coded bases indicate the probability of stem-loop structures with red indicating a certain probability value of 1.0. Each of the loop structures was individually labeled with AF 647-succinimide at primary amines placed between bases at the indicated hash marks in each loop and defined in the text. Only loop 5 of EcO 4R labeled with AF 647 between bases 42 and 43 (from the 5′ end) produced any noteworthy FRET when reacted with ten-fold dilutions of unlabeled live *E. coli* 8739 as shown at the bottom of the figure

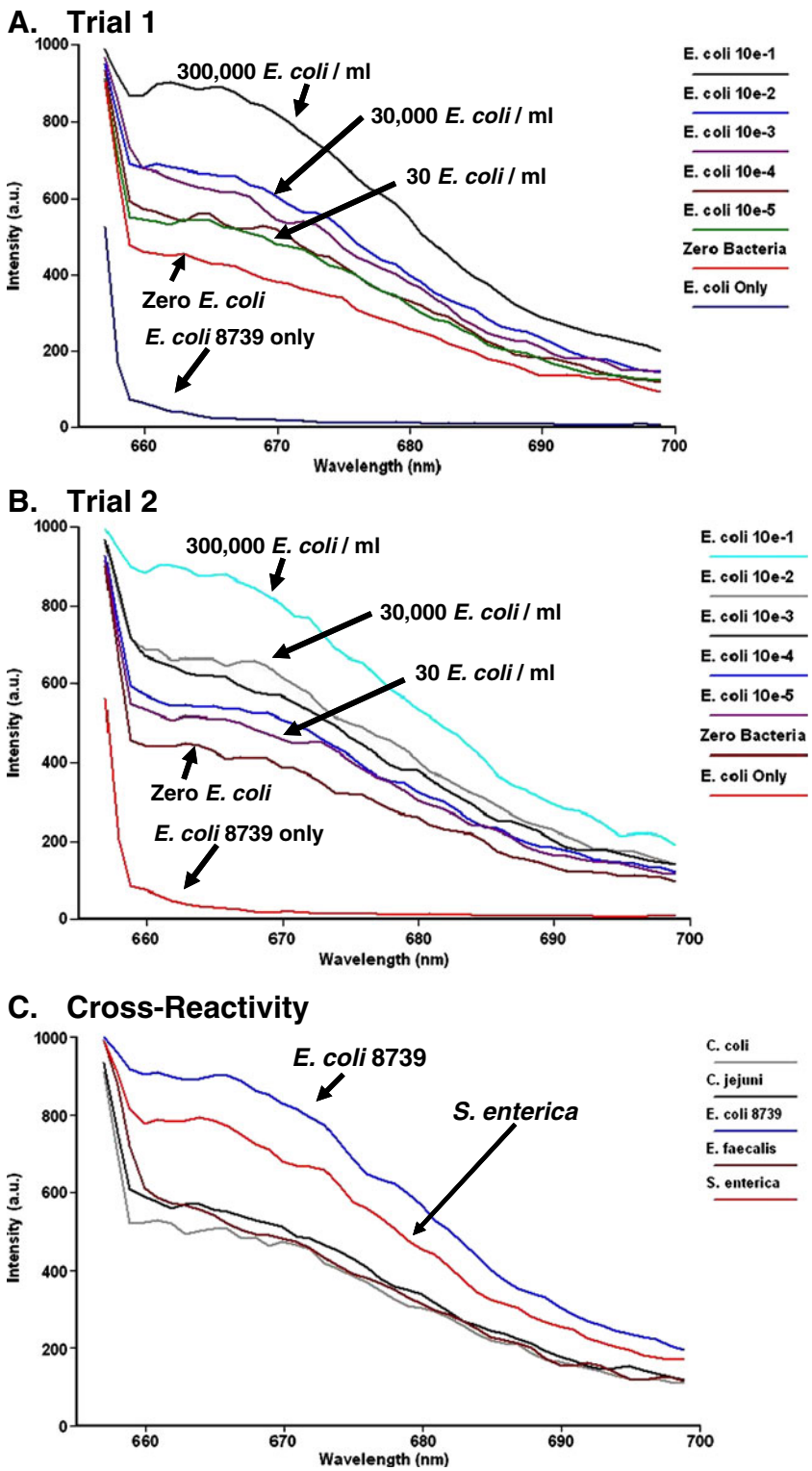


Fig. 5 **a** and **b** illustrate two separate trials of the optimized Eco 4R 42/43 (loop 5) AF-647 competitive FRET-aptamer assay versus ten-fold dilutions of live *E. coli* 8739 from 300,000 to 30 cfu/ml (verified by plate counts). *E. coli* only and FRET-aptamer without *E. coli* control spectra are shown as well. **c** shows preliminary cross-reactivity study conducted using the Eco 4R 42/43-AF 647 competitive FRET-aptamer assay. Approximately 3×10^5 live cells of each species listed were competed against the optimized FRET-aptamer assay for 15 min. In all cases, excitation was at 650 nm±5 nm and the PMT setting was 900 V

some other Gram negative organisms such as *Campylobacter coli* and *C. jejuni* and the Gram positive bacterium *Enterococcus faecalis*, but does exhibit as much as 87% cross-reactivity with *Salmonella enterica* serovar Typhimurium ATCC No. 13311 (Fig. 5c) based on peak height comparison at 665 nm.

To further validate the *E. coli* EcO 4R-position 42/43-AF 647 plus BHQ-3-succinimide-*E. coli* 8739 competitive FRET-aptamer assay, we subjected the assay to fluorescence microscopy before and after the 15 min equilibrium period as shown in Fig. 6. Results were very similar to those reported by Bruno et al. in 2001 for an antibody-

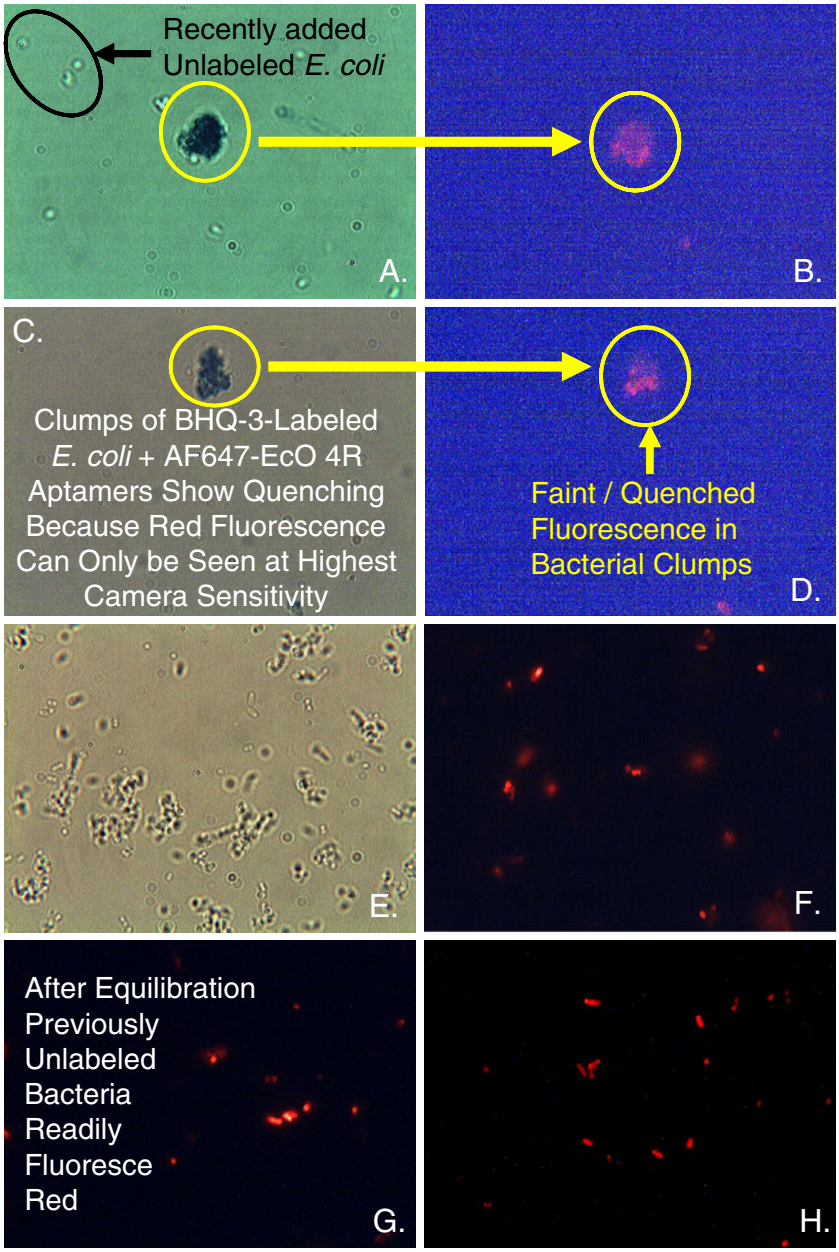


Fig. 6 **a**–**d** illustrate brightfield and fluorescence microscopy of the "FRET-reagent complexes" or aggregates of AF 647-Eco 4R aptamers bound to BHQ-3-labeled *E. coli* with recently added unlabeled *E. coli* bacteria before the 15 min equilibration period. Note the darkly stained clumps of bacteria (due to BHQ-3) that barely fluoresce even on the most sensitive camera setting and the lack of fluorescence from unlabeled bacteria initially. A green (rhodamine) filter cube was used and total magnification=1,000X. **e**–**h** show brightfield and fluorescence images taken after the 15 min equilibration process, previously unstained *E. coli* bacteria fluoresce an intense red color due to competitive displacement of some AF 647-EcO 4R aptamers off of the dual AF 647-aptamer plus BHQ-3-labeled bacteria with migration to and binding of previously unlabeled *E. coli* bacteria to yield a one step "lights on" FRET response

based immuno-FRET assay system to detect *E. coli* and *Bacillus* spores [13]. Namely, in both sets of experiments, one could see small aggregates of darkly stained and highly quenched bacteria (the "FRET reagent" before equilibration with unlabeled bacteria) that appear almost black or dark blue under brightfield microscopy and only faintly fluorescent under the fluorescence microscope even at the highest sensitivity setting on the camera. The quenching on BHQ-3-labeled bacteria is presumably due to the fact that most of the fluorophores are initially proximal to (within the very short Förster distance) BHQ-3 quenchers on the bacterial surface [13]. However, as time goes on and the fluorescently labeled aptamers (or antibodies) are allowed to equilibrate and migrate to unlabeled bacteria by competitive displacement (Fig. 1), one begins to observe fluorescently stained bacteria which were previously non-fluorescent. This change in fluorescence is apparent by comparison of the various panels of Fig. 6 in which clearly unlabeled *E. coli* 8739 (invisible or translucent in Fig. 6 panels a and e) assume red surface fluorescence after 15 min (panels F-H). The reader should recall as well that control spectra from Fig. 5, panels a and b demonstrated no appreciable red autofluorescence of unlabeled *E. coli*.

Finally, to verify and compare our FRET and ELISA-like results with another purely affinity-based technique, we conducted surface plasmon resonance (SPR) studies using a Biacore X-100 instrument. Figure 7 summarizes the SPR results and shows that EcO 3R was overall the highest

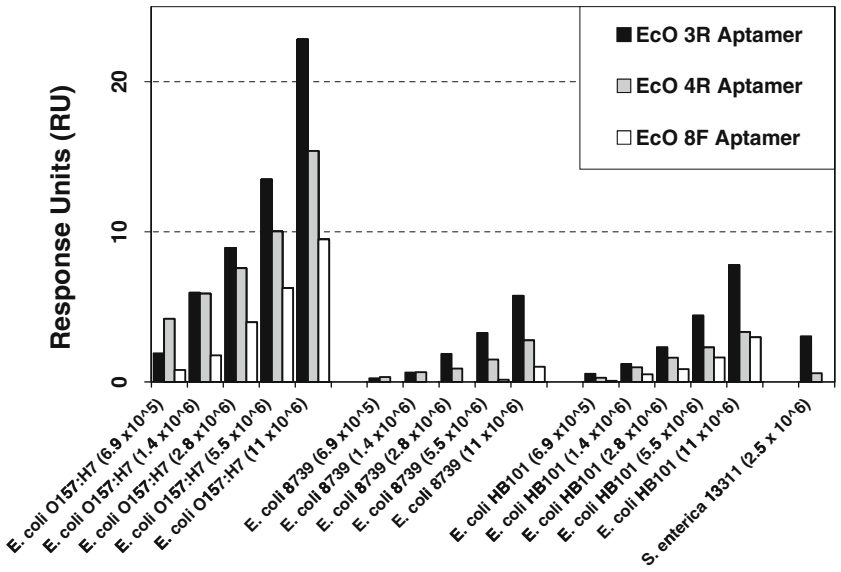


Fig. 7 Summary of SPR results for EcO 3R, 4R, and 8F (chosen to represent some of the highest affinity and best competitive FRET aptamers, see Fig. 2) to validate their relative affinity rank using a Biacore X100 as described in the text versus different concentrations of live *E. coli* 8739, O157:H7 and HB101 as well as *Salmonella enterica* strain 13311. Live *Campylobacter jejuni* cells at 2.5×10^6 cells per ml produced a negative response with each of these aptamers (not shown)

affinity aptamer sequence by SPR, which correlates very well with the ELISA-like assay results in which EcO 3R registered the highest absorbance value of 1.838 (well A6 at the bottom of Fig. 2). EcO 4R was consistently in second place with regard to affinity by SPR, and EcO 8F (a poor FRET performer from well B3 in Fig. 2) was consistently last by SPR analysis despite yielding A_{405} of 1.714 versus the 1.294 of EcO 4R. This discrepancy in affinity assessment results may reflect differences in methodology between ELISA-like plate assays and SPR. Overall, however and serendipitously, we have found that all three aptamer sequences gave their strongest SPR responses when binding the well-known pathogen *E. coli* O157:H7. Paradoxically, in opposition to the FRET cross-reactivity data in Fig. 5c, the SPR data in Fig. 7 suggest that the EcO 4R and other EcO aptamers do not bind *S. enterica* well at the level of 2.5×10^6 bacteria per ml. Similarly low or negative SPR responses were seen with 2.5×10^6 *Campylobacter jejuni* cells per ml (data not shown).

Discussion

As the world's population continues to grow, the availability and rapid identification of potable water could become increasingly important. In the U.S., the Beaches Environmental Assessment and Coastal Health Act of 2000 (Public Law 106–284) mandates daily testing of all recreational waters for fecal indicator organisms according to the very low levels set by EPA drinking water standards of 126 *E. coli* and 33 *E. faecalis* per 100 ml of water. While culturing and enzymatic methods such as the IDEXX Colilert® test are sensitive enough to detect these ultra-low levels of bacteria, they are relatively slow (requiring at least 24 hrs) and can fall prey to false positives in marine water samples [25, 26]. Consequently, there has been a great push toward rapid on-site detection of low levels of fecal bacteria to avoid prolonged closure of ocean and lake-front beaches or other recreational waters and to test drinking and treated wastewater used to irrigate crops.

While some specific high-affinity antibodies exist that can detect multiple *E. coli* strains or the toxigenic strains such as O157:H7, aptamers are beginning to compete in this arena [19, 24, 27–32]. Hence, we sought to extend our competitive FRET-aptamer assay technology [1–3] into this potentially important new area of environmental monitoring. However, along the way, we discovered that because affinity and capacity for FRET are not well correlated, we would need to develop a high-throughput FRET screening method, which led to the work reported herein. The lack of a strong correlation between affinity and FRET may be because affinity is largely based on 3-dimensional conformations and strength of binding forces, but FRET is highly dependent on placement of a fluorophore and a well-matched quencher to reside within the very short Förster distance in a binding pocket [1–3, 13, 14]. While it is true that a binding pocket could be hidden in a stem structure and result from induced fit in the presence of an analyte, if thermodynamically favorable, we presumed that naturally occurring loop structures were more likely regions to serve as binding pockets versus stems. Oddly, the successful binding pocket (no. 5) from the EcO 4R aptamer (5′-CTCTCCTTACG-3′) has not performed well as an aptamer beacon (data not shown) when we have labeled its 3′ and 5′ ends with a fluorophore and quencher, which may attest to an advantage of the competitive FRET approach.

The seemingly inverse relationship between affinity and competitive FRET was noted by our group in a previous

work as well in which a surprisingly high-affinity aptamer sequence (TTTAGT) that binds the methylphosphonic acid core of nerve agents such as sarin and soman [2, 18] exhibited competitive FRET, but only in the μg/ml range (i.e., competitive FRET sensitivity was not high for this high-affinity aptamer binding loop). We again noted in the present work that the highest affinity *E. coli* OMP aptamers identified by our ELISA-like colorimetric plate assay were not necessarily the most sensitive aptamers in a competitive displacement FRET assay format (Fig. 2). This stands to reason of course, since a high affinity FRET-aptamer is much less likely to dissociate from its target except when its equilibrium is pushed by high concentrations of unlabeled target. But, the high concentrations of unlabeled target needed to elicit a FRET response mean a concomitantly high limit of detection and low sensitivity.

In the present body of work, we have achieved the following: **1)** developed a viable high-throughput FRET screening method that can identify or reduce the number of candidate aptamer sequences for competitive FRET-based detection of bacteria, **2)** identified a robust FRET-aptamer (EcO 4R) for general *E. coli* and possibly *S. enterica* detection, and **3)** identified other DNA aptamer sequences (see Table 1, Figs. 2 and 7) that may be useful in purely affinity-based detection of *E. coli* OMPs. Compelling evidence for the validity of these achievements exists in the form of actual fluorescence spectra and fluorescence microscopic images presented herein which are at least partially corroborated by aptamer-based ELISA-like plate assay as well as SPR data.

The competitive FRET-aptamer screening system currently has some limitations including an inability to discern forward or reverse aptamers as being responsible for FRET in responsive wells. We have attempted asymmetric PCR to overcome this shortcoming (data not shown), but the more linear amplification nature of asymmetric PCR led to low fluorescence intensity and a decreased ability to identify promising FRET aptamers. We incorporated redundancy in the FRET screening system by examining forward and reverse aptamer templates which should produce approximately the same mixture of F and R sequences following 20 rounds of PCR. However, we have not observed equal FRET from matched F and R wells for a given aptamer sequence. Still, our screening system has proven somewhat useful for narrowing the field of FRET-aptamer candidates which can then be individually studied for FRET potential in suspected loop structures.

Future assay development will continue to address the limitations and will work toward detection of the very low limits imposed by the EPA, perhaps through filtration and pre-concentration of 100 ml water samples [26] and continued investigation of the ideal placement of AF 647 in EcO 4R's loop 5 in the vicinity of bases 42 and 43 so as to minimize the Förster distance to the nearest BHQ-3 molecule. In addition, we are working to identify which particular OMPs are bound by which *E. coli* aptamer sequences. This goal will be addressed by an aptamer-based analogue to Western blotting. While the high-throughput FRET screening method was developed for particulate bacterial cells, we are also working toward FRET screening for aptamers that bind small molecules including vitamins [1] and drugs. This should be entirely feasible based on previous literature and experience of our group with other aptamers and FRET-aptamers that bind and detect small molecules [1–3, 11, 18, 20].

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