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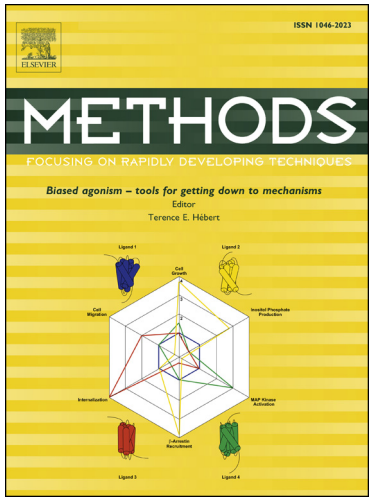

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In Vitro Selection of RNA-cleaving DNAzymes for Bacterial Detection

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Abstract. DNAzymes refer to single-stranded DNA molecules with catalytic activity and can be isolated from synthetic random-sequence DNA pools using the technique of in vitro selection. DNAzymes that cleave RNA, known as "RNA-cleaving DNAzymes", represent one of the best-studied classes of DNAzymes and have been widely used for the development of biosensors and bioassays for various analytes. We have been interested in developing RNA-cleaving DNAzymes as bacterial sensors and these DNAzymes are engineered to perform three linked functions: recognition of a bacterial biomarker, RNA cleavage, and fluorescence generation. These fluorogenic DNAzymes emit fluorescence automatically in the presence of a bacterium of interest and can be used to set up a simple "mix-and-read" assay to detect this bacterium. In this article, we will discuss this DNAzyme system and present a proven strategy for isolating highly specific bacteria-responding DNAzyme probes from random-sequence DNA pools. We will also provide an in vitro selection protocol successfully used to derive RNA-cleaving fluorogenic DNAzyme probes that are capable of recognizing a targeted strain of *Clostridium difficile*.

Keywords: DNAzyme; in vitro selection; RNA cleavage; bacterial detection; biosensor; *Clostridium difficile*

1. Introduction

Pathogenic bacteria constitute serious threats to public health and safety because they can cause frequent, deadly and costly outbreaks. Quick and accurate detection of specific pathogens often serves as the first step towards preventing an outbreak and is implemented as a crucial strategy for minimizing the impact of an on-going outbreak. Many excellent methods for bacterial detection have been developed and they have provided medical professionals with powerful means to manage and contain bacterial pathogens. Widely used methods for bacterial detection include cell culturing, antibody-based assays and nucleic acid amplification techniques such as polymerase chain reaction (PCR). However, each of these techniques comes with inherent drawbacks. Cell-culture methods are often long and take days or even weeks to complete. Antibody-based assays need multiple steps to retrieve antigens from cells and often are not sensitive enough to detect bacterial cells at low concentrations. PCR also needs a step of extracting DNA from cells and can produce false-positive signals as it can pick up DNA from dead bacteria. Therefore, there is great need for developing new, particularly simpler, methods that can identify specific bacterial pathogens. This paper will focus on a method for bacterial detection using catalytic DNA molecules.

It is now widely known that single-stranded DNA and RNA molecules are capable of adopting well-defined structures to carry out molecular recognition and catalysis [1-3]. DNA and RNA sequences capable of binding specific targets are called aptamers; those with ability to speed up a chemical transformation are termed DNAzymes and ribozymes; all these molecules are more generally referred to as functional nucleic acids. A good number of ribozymes and RNA aptamers have ben employed by natural biological systems to carry out some important cellular functions [4,5]. However, the development of in vitro selection technique [6-8] has led to the discovery of a great array of man-made ribozymes and RNA aptamers [2,9]. In vitro selection has also given the birth to DNAzymes [10,11] and DNA aptamers [12,13], both of which have not been found in natural systems. A large body of literature amassed over the past 40 years has presented compelling evidence to demonstrate

that both DNA and RNA in single-stranded form are functionally powerful enough to achieve precise molecular recognition and efficient enzymatic catalysis, setting up the stage for exploiting functional nucleic acids as highly versatile molecular tools. DNA aptamers and DNAzymes are especially attractive for practical applications because DNA is highly stable and can be produced easily and cost-effectively via automated solid-phase synthesis.

Two unique advantages associated with the development of functional nucleic acid-based molecular probes for biosensing applications are linked to the technique for making them: high probability of success and the ability to tune probe properties. In vitro selection begins with the use of a synthetic DNA pool that offers enormous sequence diversity. A DNA pool that contains 10^{13}-10^{16} different sequences can be routinely obtained through standard DNA synthesis (10^{13} is equivalent to ~1350× the world population at the moment). The ability to simultaneously screen these many molecules in a single mixture greatly increases probability of success. In addition, the entire process of in vitro selection is carried out in test tube, which allows an experimenter to incorporate specific procedures and/or conditions to search for functional nucleic acids with tailored properties. This second advantage will be re-visited below when we will discuss the engineering of DNA probes to recognize specific bacteria.

Both DNA aptamers and DNAzymes are suitable molecular probes for biosensing applications, which have been discussed in many reviews [e.g. ref. 14-16]. The choice of DNA aptamer vs. DNAzyme for a specific application often reflects personal preference. However, each class of functional DNA may offer some benefits over the other class. For example, a DNAzyme that can be activated by a specific target (often denoted “aptazyme”) has to carry out both molecular recognition and enzymatic catalysis, while a DNA aptamer that recognizes the same target only needs to perform a single binding function. Therefore, theoretically it might be more challenging to develop an aptazyme than an aptamer. However, for specific targets such as metal ions, DNAzymes may be easier to obtain as DNAzymes often require the assistance of metal ions as cofactors. One technical advantage

associated with the DNAzyme selection is that the catalytic activity can act as a powerful mechanism for selection simply because catalytically active sequences have a huge advantage in competing for survival. In comparison, the techniques used for the aptamer selection often come with substantial background binding, which may delay the emergence of aptamers.

2. In vitro selection of RNA-cleaving DNAzymes for bacterial detection

There are many DNAzyme systems that can be potentially exploited for biosensing applications [17-25]. Our group has been interested in studying RNA-cleaving DNAzymes and examining their potential as biosensors. There is a rich history of studying RNA-cleaving nucleic acid enzymes. Several classes of RNA-cleaving ribozymes have been discovered in nature to date, which include the hammerhead ribozyme [26], the hairpin ribozyme [27], the hepatitis delta virus (HDV) ribozyme [28], the Varkud satellite (VS) ribozyme [29], and the glucosamine-6-phosphate (Glms) activated ribozyme [30]. The first-ever DNAzyme also targeted RNA for cleavage [10]. Since then, many in vitro selection studies have been conducted to isolate RNA-cleaving DNAzymes, which have led to the establishment of a large assembly of DNA sequences that cleave RNA [10, 31-59]. Generally speaking, RNA-cleaving DNAzymes are catalytically proficient [15, 60, 61] and thus biosensors engineered with these DNAzymes can offer a quick response time [14-16]. Moreover, RNA cleavage results in the separation of two shorter nucleic acid segments (Figure 1), which provides a convenient way for coupling to various signal transduction platforms. There has been tremendous progress made with exploiting RNA-cleaving DNAzymes for biosensing, highlighted by many biosensors for detection of metal ions, small-molecule metabolites, and more recently bacterial and mammalian targets [62-82]. This paper focuses on our own efforts in developing RNA-cleaving DNAzymes for the detection of specific bacteria.

We are particularly interested in developing RNA-cleaving DNAzymes that can generate fluorescence as a result of RNA cleavage. This is achieved through the use of a chimeric DNA/RNA substrate containing a single RNA unit flanked by two nucleotides modified with a pair of fluorophore

and quencher (Figure 1). The RNA unit is intended as the cleavage site and the cleavage of this unit by a DNAzyme will lead to spontaneous increase in fluorescence due to the separation of the fluorophore from the quencher [38]. Thus, such a DNAzyme is encoded with fluorescence-signaling capability and can be used to develop a 'mix-and-read' type assay for the detection of a target of interest. We term this type of DNAzyme "RNA-cleaving fluorogenic DNAzyme", or simply "RFD". Over the past decade, we have isolated many RFDs and examined their catalytic and signaling properties [38, 39, 67-69, 81-90]. More recently, we began to develop RFDs that that can be activated in the presence of a specific bacterium, such as *Escherichia coli* and *Clostridium difficile* [72, 80, 82, 91-96].

Traditionally, the development of a diagnostic test begins with the identification of a specific biomarker or a group of biomarkers for the disease of interest. However, the conventional biomarker discovery represents a colossal effort for any disease – the process is tedious, lengthy, expensive and often unproductive. Our group has experimented a new concept of creating a diagnostic test without first identifying a specific biomarker [92]. More specifically we have developed an approach that permits the development of cell-specific RFD probes using unpurified cellular mixture as the complex target. Our approach has two key cornerstones. The first one is the "many-against-many" approach – the random DNA library (which contains as many as 10^{16} different sequences) is screened against the complex mixture from a specific cell (which contains many candidate biomarkers). In other words, the random DNA library offers many candidate RFDs whereas the complex cellular mixture offers many potential biomarkers (Figure 2). The second cornerstone is the positive-selection and counter-selection combination, as illustrated in Figure 3. In each selection cycle, the cellular mixture of an intended cell is used as the positive-selection target and the cellular mixture from an unintended cell type is used as the counter-selection target. The use of the combined positive-selection/counter-selection strategy is to ensure the derived RFDs have high specificity for the intended cell but exhibit no or minimal cross-activities for the unintended cell through the elimination of RFDs that respond to the molecules present in both cells.

We have successfully validated the above concept in three separate studies. In the first study, we used model bacterial cell *E. coli* [72]. Specifically, the crude extracellular mixture (CEM) left behind in the *E. coli* culture (denoted CEM-EC) was used as the positive-selection target. To tune the specificity, the CEM from *Bacillus subtilis* (CEM-BS) was used as the "counter-selection" target. By this approach, we were able to generate an RFD, named RFD-EC1, that responded well to CEM-EC but not to CEM-BS as well as CEMs from a host of other bacteria (Figure 4). RFD-EC1 exhibits high sensitivity and a large dynamic range – the probe can detect as few as 100 *E. coli* cells and as many as 10^7 cells [95].

In the second study, we developed a selective RFD probe for MDA-MB-231, a triple-negative breast cancer cell [81]. The probe, named AAI2-5, was derived using the crude intracellular mixture (CIM; i.e. cell lysate) from MDA-MB-231 as the positive selection target, and the CIM of the normal breast cancer cell line MCF-10A as the counter-selection target. AAI2-5 is capable of detecting MDA-MB-231 at a crude protein concentration of as low as 0.5 μg/mL, which is equivalent to ~5,000 cell/mL. AAI2-5 is able to distinguish MDA-MB-231 from many other cell lines, including normal breast cells, other subtypes of breast cancer cells, and other types of cancer cells [81].

Our most recent study was conducted to isolate RFDs that can recognize a specific strain of *C. difficile* [82]. The in vitro selection experiment was carried out using the CEM derived from a BI/027 strain of *C. difficile* as the positive selection target, and the CEM prepared from *E. coli*, *B. subtilis* and CD630 (a non-BI/027 strain of *C. difficile*) as the counter-selection target. This approach led to the identification of RFD-CD1, an RFD that is not only able to distinguish *C. difficile* from many other types of bacteria (Figure 5A), but is also highly strain-specific (Figure 5B): when it was tested with 12 strains of *C. difficile*, it is only active with BI/027. Molecular probes with such a high specificity have been rarely reported. From the three studies described above, we believe that the featured method can be generalized to develop specific RFDs for a given cell line of interest by carefully selecting cell lines as counter-selection targets.

The DNAzyme probes derived above are intrinsically fluorogenic and are able to produce bright fluorescence upon target binding. Although this property will make RFDs immediately useful for fluorescence-based assays, there is increasing interest in developing simpler, colorimetric assays that are more suitable for point-of-care applications. We have recently developed an approach to take advantage of the classic litmus test for colorimetric detection of bacteria using RFDs [80]. Our expanded litmus test platform is illustrated in Figure 6. The method involves the creation of an RFD-urease (Ur) conjugate and immobilizing it onto magnetic beads (MB). When the target is present, the RFD is cleaved, which releases the urease from MB into solution. Upon magnetic separation, the urease-containing solution is used to hydrolyze urea into ammonia, which causes a pH increase. Therefore, this approach translates the presence of a target of interest into pH change, which can be conveniently detected using pH-sensitive dyes or pH papers. Since this test takes advantage of magnetic separation that is easy to implement and pH-sensitive dyes or pH papers that are cheap and widely available, it is well suited for point of care applications. We have validated this approach using *E. coli*-detecting probe RFD-EC1 [80]. The key to this expanded litmus test is the use of cleavage-based RFD probes, and therefore, it can be applied for the detection of any target for which an RFD can be derived.

3. Description of Methods

3.1 Bacterial sample preparation

When living microbes are grown under nutritious conditions, they constantly exchange materials with their environments and leave behind a mixture of small and macromolecular molecules that can be highly characteristic for each bacterium. The idea of using this left-behind mixture, which is termed “crude extracellular mixture (CEM)”, is to simplify the tedious biomarker identification process that is required to develop biosensor for each bacterium. We have demonstrated that through the power of in vitro selection, RFDs can be isolated to respond to the CEM from a specific bacterium.

The preparation of CEM from the bacterium of interest is important to the entire selection process and for downstream biosensor applications. Protein and small molecule expression profiles vary between different bacterial growth phases. Therefore, it is ideal to collect CEM from a growth phase that is compatible with downstream detection applications. For example, we collected CEM from *C. difficile* at ~ OD_{600} 1, which is the stationary phase for *C. difficile*. At this phase, *C. difficile* produces the most toxin-related proteins that contribute to its pathogenicity [97]. This gives us a higher chance of isolating RFDs that function under clinically relevant conditions.

In addition, we have found that there are varying amounts of nucleases in CEM from different bacteria. It is important to conduct a nuclease assay using a small amount of the initial DNA library to determine the level of nuclease-dependent cleavage before the start of selection. If there is a high level of nuclease degradation, heat-treatment of CEM at an elevated temperature should be considered for deactivating nucleases. Of course, the caveat of this approach is that some potential protein targets are also deactivated during the process.

3.2 Population diversity

In the in vitro selection approach, population diversity is generated through a random-sequence DNA library where the number of possible sequences for DNA molecules containing *n* random-sequence nucleotides is 4^n. Therefore, the size of the random-sequence domain would dictate the diversity of the starting library. A longer random domain provides opportunities for creating more complex structures which can be beneficial when selecting for efficient catalysts [98]. However, long random domains (n>80) can potentially form inhibitory motifs that subvert the enrichment of active species. Long motifs are also present in smaller copy number compared to short motifs in a DNA pool. This presents a significant disadvantage for long motifs, as they are often out-competed by shorter motifs. This phenomenon, known as 'the tyranny of short motifs', is observed in the most widely studied 8-17 RNA-cleaving DNAzyme, which has a catalytic core of only 14 nucleotides and has been

found in multiple independent selection experiments [61]. Another important factor for consideration is sequence sampling. A DNA library made of 25 random-sequence nucleotides corresponds to a sequence space of 10^{15} molecules, translating into ~1 nanomoles of DNA. Due to the physical limit of DNA synthesis, the initial library contains generally 10^{14} to 10^{16} molecules. This means that any library with the random-sequence domain longer than 25 nucleotides would be under-sampled. We have used libraries with 40 and 70 random-sequence nucleotides and had success with both [72,82].

3.3 Choice of experimental parameters

A key advantage of the in vitro selection technique is its flexibility to accommodate different experimental parameters to achieve specific purposes. For bacterial detection, high recognition specificity is critically important. As discussed earlier, this can be achieved using a strategy of conducting both counter selection and positive selection. Counter selection is carried out to eliminate sequences with cross-activities for unintended bacterial species or strains. This step is typically done by first incubating the DNA pool with the CEM from one or a group of bacteria and then isolating DNA sequences that do not promote cleavage. These DNA molecules are then subjected to the positive selection step where the CEM of the intended bacterium is used. The cleaving sequences are isolated, amplified, and used for the next cycle of selective enrichment.

Catalytic proficiency is another important parameter to consider during selection because fast-cleaving RFDs allow for the design of quick-responding biosensors. To isolate fast-cleaving DNA molecules, we typically adopt a strategy of progressively reducing the time of incubation between the CEM and the DNA pool. In earlier rounds, a longer time is used because active sequences are scarce and preservation of population diversity is important. In later rounds, usually after the appearance of a detectable cleavage signal, the reaction time is gradually shortened to favor the isolation of most active DNAzymes.

3.4 Polymerase chain reaction (PCR)

PCR is the most commonly used method for DNA amplification to generate progeny populations that can be subjected to further selection. However, with any amplification method, unintended selection pressures can be introduced. First, amplification efficiency may not be the same for different DNA sequences in the population, which creates an inadvertent selective pressure in favour of species that are more easily amplified by the polymerase. Mishybridization between primers can also create undesirable artifacts that undermine the selection progress. Therefore, the sequence of primers must be carefully designed to avoid primer hairpin, self-dimer and hetero-dimer formation to maximize PCR efficiency. In addition, the primer binding sites that flank the random sequence domain may influence the outcome of a selection experiment. We generally avoid the use of primer-binding sites that form strong interactions with the substrate sequence, as the random-sequence domain might need to interact with the substrate to create a catalytically active structural fold.

During the early rounds of selection (generally rounds 1 to 3), active DNA molecules only represent a very small fraction of the pool. In order to preserve the DNA sequence diversity, we typically use all or most of the cleavage product as the input template for PCR. We then progressively reduce the amount of the cleavage product so that more copies are produced for each DNA template.

4. Materials

From this point forward, we will use the in vitro selection experiment that has led to the isolation of strain-specific *C. difficile*-detecting RFDs as the case study to discuss the details of the method.

4.1 DNA oligonucleotides

The sequences of all oligonucleotides are listed below.

Name	Sequence
Library (L1)	5′-CACGGATCCTGACAAG-N_{40}-CAGCTCCGTCCG

Forward Primer (FP)	5′-CACGGATCCTGACAAG
Reverse Primer 1 (RP1)	5′-CGGACGGAGCTG
Reverse Primer 2 (RP2)	5′-A_{20}-S_9-CGGACGGAGCTG (S9 is a triethylene glycol linker)
Ligation Template (LT)	5′-CTAGGAAGAGTCGGACGGAGCTG
Fluorogenic Substrate (FS1)	5′-ACTCTTCCTAGC-F-rA-Q-GGTTCGATCAAG A (F = fluorescein-labeled dT, Q = DABCYL-labeled dT, rA = adenine ribonucleotide)
Deep Sequencing Forward Primer (DF)	5′-AATGATACGGCGACCACCGAGATCTACACTCTTT CCCTACACGACGCTCTTCCGATCTCACGGATCCTGACAAG
Deep Sequencing Reverse Primer (DR)	5′-CAAGCAGAAGACGGCATACGAGATGCCTAAGTGAC TGGAGTTCAGACGTGTGCTCTTCCGATCTCGGACGGAGCTG

4.2 Chemicals and enzymes

Radioactive nucleotides are purchased from Perkin Elmer. Enzymes are purchased from Thermo Scientific with the exception of DNA polymerase, which is purchased from Biotools. Non-radioactive nucleotides are purchased from Thermo Scientific. All other chemicals are purchased from Sigma-Aldrich if not otherwise specified. All multi-component solutions are made in-house using autoclaved, deionized water, filtered through the Milli-Q® Intergral Water Purification System (EMD Millipore, Massachusetts, USA).

- ATP: 100 mM in water
- [γ-^{32}P]ATP: 1.66 µM (6000 Ci/mmol)
- [α-^{32}P]dGTP: 3.33 µM (3000 Ci/mmol)
- 10× dNTP mix: 2 mM dATP, 2 mM dGTP, 2 mM dCTP and 2 mM dTTP
- Hot 10× dNTP mix: 2 mM dATP, 2 mM dTTP, 2 mM dCTP and 0.2 mM dGTP
- EDTA: 0.5 M, pH 8.0
- NaOH: 0.25 M
- Trimethylamine tris hydrofluoride (TEA•3HF)
- Butanol (Thermo Scientific)

- Ethanol: both 70% and 100%, stored at -20°C
- Sodium acetate: 3 M, pH 5.2
- 1× Elution buffer: 10 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA, pH 7.5
- 2× Selection Buffer (2× SB): 100 mM HEPES, 300 mM NaCl, 30 mM $MgCl_2$, and 0.02% Tween 20, pH 7.5
- 2× Stop solution: 200 mM EDTA and 14 M urea
- 2× PAGE gel loading buffer: 14 M urea, 100 mM Tris-borate, 0.6 M sucrose, 0.1% (w/v) SDS, 1 mM EDTA, 0.025% (w/v) xylene cyanol, and 0.025% (w/v) bromophenol blue, pH 8.3
- 10% denaturing PAGE: 7 M urea, 1× TBE, 10% 29:1 bis/acrylamide
- Ammonium persulfate: 10% (w/v) in water
- TEMED (BioShop Canada)
- TBE buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.5
- SYBR safe DNA Gel Stain (Thermo Scientific)
- T4 polynucleotide kinase (PNK): 10 units/µL
- 1× PNK reaction buffer A: 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, and 0.1 mM spermidine, pH 7.6
- T4 DNA ligase: 5 units/µL
- T4 DNA ligase 1× reaction buffer: 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, and 0.5 mM ATP, pH 7.8
- *Tth* DNA polymerase: 5 units/µL
- 10× PCR buffer: 750 mM of Tris-HCl, 20 mM $MgCl_2$, 500 mM KCl, and 200 mM $(NH_4)_2SO_4$, pH 9.0

4.3 Cell lines and cell culture reagents

C. difficile BI/027-H strain is cultured from a patient at St. Joseph's Healthcare Hamilton (Ontario, Canada). Other *C. difficile* strains are obtained from the American Type Culture Collection (ATCC). *E. coli* K12 BW25113Δrna and *Bacillus subtilis* are routinely cultured and maintained in our laboratory.

- Chopped meat glucose medium (Sigma-Aldrich)
- Luria Bertani (LB) Medium (BioShop Canada)
- Complete protease inhibitor cocktail (Roche)

5. Experimental Protocols

5.1 Oligonucleotide synthesis

All oligonucleotides are purchased from IDT DNA Technologies with the exception of FS1, which is purchased from Keck Oligo Synthesis Facility at Yale University, USA.

5.2 Oligonucleotide purification

Standard DNA purification: All DNA oligonucleotides are purified by 10% denaturing polyacrylamide gel electrophoresis (PAGE). Our lab typically uses an adjustable slab electrophoresis unit Model ASG-400 (CBS Scientific, CA, USA), with glass plates at a size of 16 cm x 27 cm. The gel running time is dependent on the length of the sequence. Upon completing gel running, wrap the gel on both sides with Saran Wrap film and visualize on a fluorescent TLC (thin-layer chromatography) plate (20 cm × 20 cm silica gel 60 F_{254}; EM Science) using a handheld UV lamp. Cut the DNA bands and elute with 1× elution buffer. Recover the DNA through ethanol precipitation as described in section 5.3. We recommend that the DNA library, primers and ligation templates be purified on separate PAGE gels to avoid cross-contamination.

PAGE gel elution: For standard DNA oligonucleotides, cut the DNA bands within PAGE gels

into small pieces and submerge in 1× elution buffer and shake at 37°C overnight. Retain only the supernatant and recover the DNA through ethanol precipitation. For oligonucleotides containing RNA (such as FS1), perform crush-elution (which reduces the elution time in order to prevent RNA hydrolysis). Crush PAGE gel into a fine paste using a pipette tip and submerge in 1× elution buffer. Shake the mixture vigorously with a vortex mixer at room temperature for 20 min and spin the gel particles down using a tabletop centrifuge. Retain the supernatants and recover the DNA through ethanol precipitation.

FS1 de-protection: The 2′-hydroxyl group on the adenine ribonucleotide in FS1 is protected by a *tert*-butyldimethylsilyl group. It is important to remove this protective group to create a cleavable substrate. Ethanol-precipitate 50 nmol of FS1 (dissolved in 50 µL of H_2O) as described in section 5.3. It is necessary to make sure that FS1 is completely anhydrous, therefore dry the pellet in a speedvac concentrator (Thermo Scientific) for at least 30 min with mild heat. Re-suspend the dried pellet in 200 µL of anhydrous dimethyl sulfoxide and 250 µL of triethylamine trihydrofluoride. Heat the sample at 65°C while vortexing periodically to ensure the pellet is fully dissolved, and then incubate at 65°C overnight. Next day, split the solution evenly into 2 centrifuge tubes and recover the DNA through butanol precipitation as follow: add 25 µL of 3 M sodium acetate (pH 5.2) and 1 mL of butanol into each tube and vortex vigorously to ensure thorough mixing. Place the samples at -20°C for 30 min and centrifuge at 22,000 g for 20 min. Decant the supernatant and wash the pellet 4 times with 70% ethanol. Dry the pellet using a speedvac at room temperature, dissolve the pellet in H_2O and add equal volume of 2× PAGE gel loading buffer. Load the sample onto a denaturing PAGE gel to purify FS1 according to the protocols above.

5.3 DNA recovery by ethanol precipitation

Add sodium acetate (3 M, pH 5.2) to a DNA solution in a 1:10 ratio (for example, add 10 μL of sodium acetate to 100 μL of DNA in H_2O), and then add 2.5× volume of pre-chilled 100% ethanol (250 μL of 100% ethanol is added in the above example). Vortex the mixture thoroughly and chill at -20°C for 20 min. Centrifuge the mixture at 22,000 g for 20 min. Remove the supernatant and wash the pellet with 500 μL of 70% ethanol and centrifuge again at 22,000 g for 10 min. Dry resulting pellet is in a speedvac. Note that all DNA oligonucleotides can be dried with heat to speed up the process while oligonucleotides containing RNA is dried at room temperature to prevent RNA cleavage.

5.4 Determination of oligonucleotide concentrations

Oligonucleotide concentration is measured at 260 nm using any UV-vis spectrophotometer, such as NanoVue (GE Healthcare). The concentration is calculated depending on the oligonucleotide length and composition according to the Beer-Lambert equation. A simple online calculator is available at http://biotools.nubic.northwestern.edu/OligoCalc.html.

5.5 Radioactive labeling of oligonucleotides

In a reaction volume of 50 μL, mix 200 pmol of an oligonucleotide with 10 μCi of [γ-^{32}P]ATP and 10 units of PNK in 1× PNK buffer A. Incubate the mixture at 37°C for 30 min to allow the phosphorylation of DNA. Recover the resulting radioactive DNA by ethanol precipitation and purify it on a 10% denaturing PAGE gel.

5.6 Preparation of crude extracellular mixture (CEM) from bacteria

Clostridium difficile: Inoculate 5 mL of liquid chopped meat broth with *C. difficile* from a frozen stock and grow to an OD_{600} of ~1 under anaerobic condition with gas mixture of 80% N_2, 10% CO_2, 10% H_2 in a Whitley Anaerobic Workstation (Don Whitley Scientific). Transfer the bacterial

culture into new microcentrifuge tubes and heat at 90°C for 5 min. The purpose of the heating step is to inactivate the nucleases and ribonucleases present in the mixture. Centrifuge the culture at 22,000 g at 4°C for 5 min. Recover the supernatant, termed CEM henceforth, and add a protease inhibitor cocktail (Roche Life Science) according to the manufacturer's suggested concentration. Pass the CEM through a 0.22 µm filter using a syringe and aliquot into microcentrifuge tubes and stored at -20°C until further use.

Other bacteria: Grow all other bacteria used in this study according to their designated growth conditions. Use LB broth for aerobic bacteria and chopped meat broth for anaerobic bacteria. Grow all bacteria cultures to OD_{600} ~1 and prepare the CEM as described above.

5.7 In vitro selection of *C. difficile*-responsive RFDs

Selection marker preparation: Often times, the cleavage product from counter selection or positive selection is not visible on the PAGE gel. Therefore, a selection marker is made artificially by inducing RNA cleavage using NaOH and the marker sample is loaded next to the selection sample to indicate the position of the cleavage product. For every round of selection, mix ~10% of the ligated library with 5 µL of 0.25 M NaOH and heat to 90°C for 10 min to induce cleavage. Perform ethanol precipitation to recover DNA and dissolve it in 10 µL of 1× PAGE gel loading buffer and use as the marker.

For description below, refer to Figure 7 for graphic explanation.

Ligation (Step 1) and purification (Step 2) of DNA pool: Incubate 500 pmol of the fluorogenic substrate FS1 with 10 µCi of [γ-^{32}P]ATP and 20 units of PNK in 1× PNK buffer A in a reaction volume of 100 µL at 37°C for 15 min to allow radioactive phosphorylation of the 5′ end. Then, add nonradioactive ATP (from a 100 mM stock) to a final concentration of 1 mM and incubate for an additional 20 min at 37°C. Quench the reaction by heating at 90°C for 5 min. Add equimolar DNA

library L1 and ligation template LT to the sample, heat the mixture at 90°C for 1 min and cool to room temperature for 10 min. Initiate the ligation reaction by adding 15 μL of 10× T4 DNA ligase buffer and 2 μL (10 units) of T4 DNA ligase in a total volume of 150 μL, and incubate the reaction mixture at room temperature for 2 h. Recover the DNA by ethanol precipitation and separate the ligated DNA on a 10% denaturing PAGE. Expose the radioactive PAGE gel to a phosphorimaging plate for 5 min and obtain an image on a Typhoon 9200 Imager (GE Healthcare). Cut out and crush-elute the gel piece containing the ligated DNA library. Precipitate the DNA with ethanol and store at -20°C until further use.

Counter selection (Steps 3 and 4): Dissolve the ligated DNA library in 50 μL of 2× SB. At the same time, mix the CEMs from *E. coli, B. subtilis, C. difficile* CD630 in equal amounts. Incubate 50 μL of the mixed CEM with the ligated library (50 μL) at room temperature for 5 h. Quench the reaction by adding 100 μL of 2× stop solution and recover the DNA by ethanol precipitation. Purify the uncleaved pool by 10% denaturing PAGE, crush-elution and ethanol precipitation.

Positive selection (Steps 5 and 6): Dissolve the uncleaved pool from the counter selection step in 50 μL of 2× SB and incubate with 50 μL of the CEM from *C. difficile BI/027-H* for 30 min at room temperature. Quench the reaction by adding 100 μL of 2× stop solution and recover the DNA by ethanol precipitation. Purify the cleaved product by 10% denaturing PAGE, DNA elution and ethanol precipitation. Dissolve the DNA pellet in 10 μL of H_2O and use for the PCR step next.

PCR1 (Step 7): Set up two PCR reaction mixtures (50 μL each). Mix 1 μL of FP (25 μM), 1 μL of RP1 (25 μM), 33.5 μL of H_2O, 5 μL of 10× PCR buffer, 5 μL of the dNTP mix, and 0.5 μL of DNA polymerase (2.5 units) with 5 μL of the cleaved DNA product (half of the total recovered sample). PCR is carried out under the following condition: an initial denaturation step at 94°C for 1 min; 12 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, extension at 72°C for 45 s; a final extension step at 72°C for 1 min. Load 5 μL of the PCR1 products on a 2% agarose gel stained with 0.005% (v/v)

SYBR safe DNA Gel Stain. Image the agarose gel using the Typhoon 9200 to confirm successful amplification.

PCR2 (Step 8): Dilute 1 μL of PCR1 product into 20 μL with H_2O and take 1 μL of this diluted PCR1 product as the template for PCR2. Similar to PCR1, prepare a 50 μL PCR reaction mixture using primers FP and RP2. The PCR conditions are the same as those indicated for PCR1. In order to obtain at least 100 pmol of PCR2 product for the next round of in vitro selection, perform 8 individual 50 μL PCR reactions simultaneously. Analyze the PCR product on a 2% agarose gel as described for PCR1 to ensure proper amplification. If needed, adjust the number of PCR cycles to obtain a higher quantity of PCR products.

Regeneration of DNA pool (Steps 9 and 10): Combine all 8 PCR2 mixtures and recover DNA by ethanol precipitation. Purify the coding strand by 10% denaturing PAGE. Note that RP2 contains a 20-nucleotide overhang at the 5′ end separated by a triethylene glycol linker, which cannot be amplified by DNA polymerase. This makes the non-DNAzyme-coding strand 20-nucleotide longer than the DNAzyme strand. Excise the bottom band on the PAGE gel and perform DNA elution and ethanol precipitation. Dissolve the DNA in 50 μL of water and use it for the next round of selection.

Repeating selection cycle: In total, 19 rounds of in vitro selection are conducted according to the protocols described above. After round 1, ligate ~100 pmol of purified PCR2 product with equimolar amount of FS1 and LT with the identical procedure described for round 1.

5.8 Sequencing

Preparation of DNA sample for sequencing: Amplify the DNA pool from the final round of selection by PCR to obtain sufficient DNA for sequencing. There are two PCR steps. PCR1 is conducted using FP and RP1 following the same protocol as described above. Dilute 1 μL of the PCR1 product with H_2O to 100 μL, use 2 μL as the template for PCR2 using deep sequencing primers DF and DR following the same protocol above for PCR1. Note that the numbers of amplification cycles are

adjusted, typically between 12 and 15 cycles. Perform four PCR reactions and the PCR products are purified by 2% agarose gel electrophoresis. Perform DNA extraction from agarose gel using any one of the commercially available kits from Qiagen, Promega or Invitrigen.

Sequencing: Purified PCR products are sequenced via paired-end next-generation sequencing (NGS) using an Illumina Miseq system at the Farncombe Metagenomics Facility, McMaster University.

Sequence analysis: After obtaining the raw sequencing data, sort forward and reverse reads by tag and then export the reads as FASTQ files using the Illumina Basespace platform. Remove primer domains and merge the paired-end reads using PANDAseq 2.6, and sequences possessing perfect complementarity between paired-end reads are output in FASTA format for further analysis [99]. Extract high quality sequences using PRINSEQ v0.20.4 [100], and eliminate sequences with any bases of Phred scores <20 (base-call probability <99%). Then group sequences into clusters (also known as classes) using the clustering algorithm CD-HIT-EST [101]. The following input parameters are used: identity threshold (-c), 0.9; word length (-n), 7; (-d), 0; (-g), 1. Organize and rank grouped classes by size, which are defined by the number of sequences in that class.

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Figure legends

Figure 1. The chemical transformation catalyzed by an RFD (RNA-cleaving fluorogenic DNAzyme). The DNAzyme cleaves a phosphodiester bond using a nearby 2′-hydroxyl as the nucleophile. The special nucleic acid substrate to be cleaved has two features: (1) it contains a single ribonucleotide cleavage site embedded in a DNA sequence, and (2) the cleavage site is immediately sandwiched between two DNA nucleotides modified with a fluorophore (F) and a quencher (Q). Therefore, the RFD can generate a real-time fluorescent signal as it performs its catalytic function, simply because the cleavage event results in physical separation of the fluorophore-quencher pair.

Figure 2. Schematic illustration of the "Many against Many" approach for deriving RFD probes that respond to molecules from a cell. The DNA library provides many different DNA sequences (as many as 10^{16} sequences; only one is shown) whereas a cell produces many potential targets (many thousands of small molecules and proteins, represented by different shapes in the graph).

Figure 3. Engineering RFD Specificity using a combined counter-selection and positive-selection strategy. Assume the DNA library contains three types of RFD molecules: catalytically inactive sequences (blue), the ones that cross-react with unintended cell lines (red), and those that are reactive with the intended cell line (green). The counter-selection step serves to eliminate the cross-reacting sequences and the positive-selection step facilitates the enrichment of the desired cell-specific RFDs.

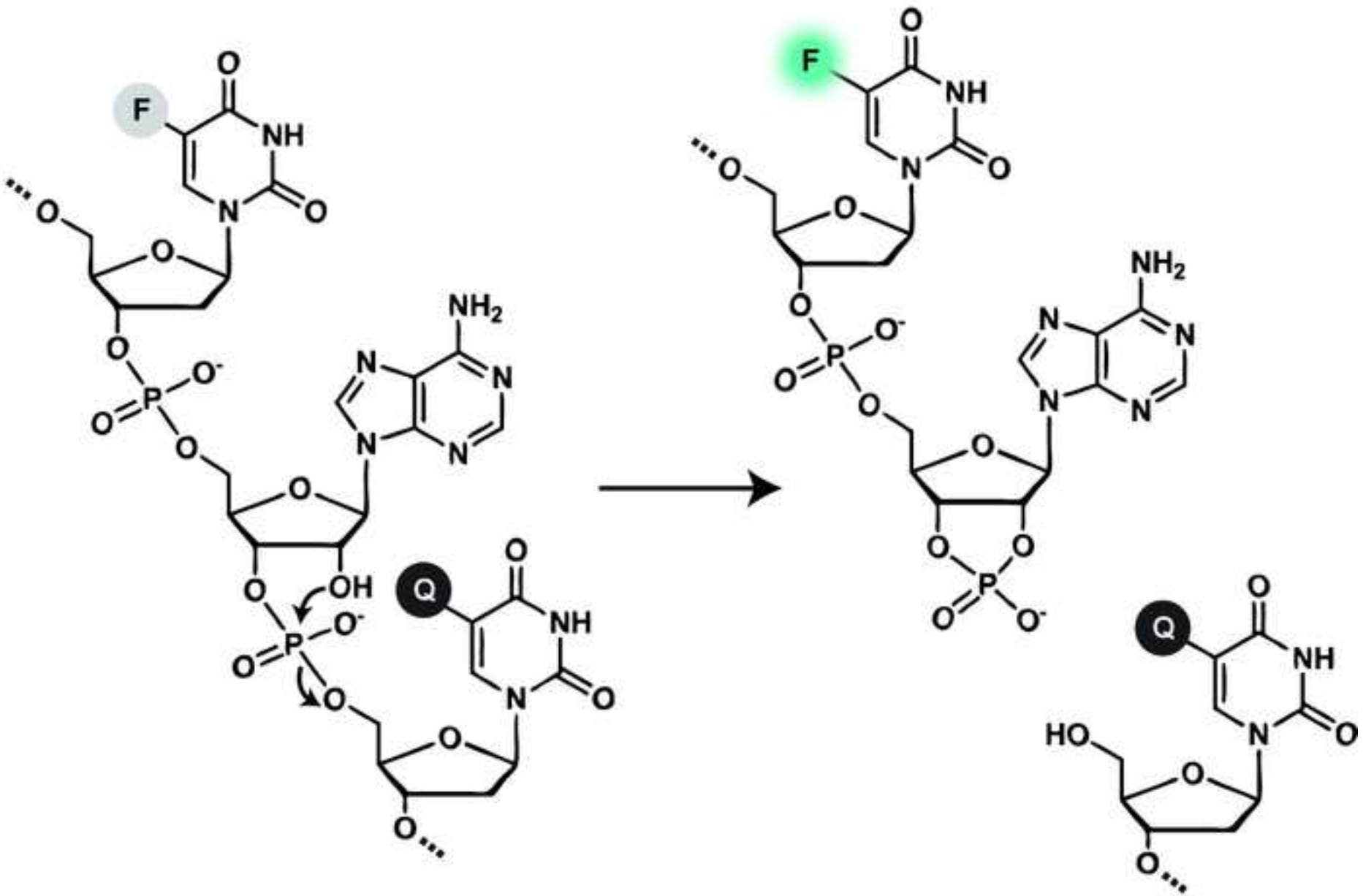
Figure 4. RFD for *E. coli*. (A) The sequence of RFD-EC1. The sequence is written from 5′ to 3′ direction. (B) Recognition specificity of RFD-EC1. RFD-EC1 is highly specific as it can only be activated by *E. coli*. The RFD was added to each bacterial sample at 5 minutes and the mixture was indicated for 25 more minutes while the fluorescence intensity of each mixture was monitored. 4 different bacterial cells were used for this test, which included *Escherichia coli, Bacillus subtilis, Leuconostoc mesenteroides, and Lactobacillus planturum* .

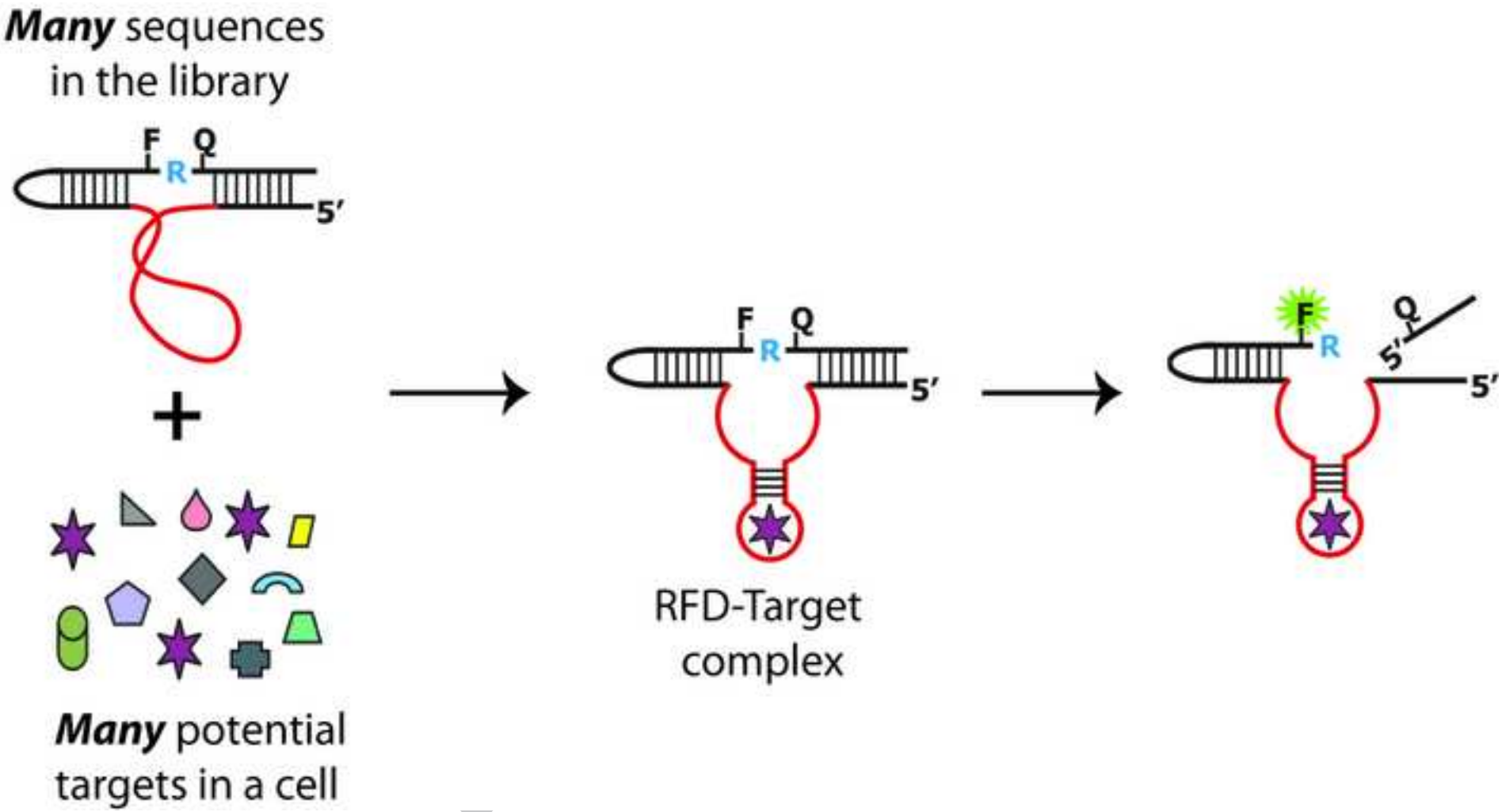
Figure 5. RFD for *C. difficile*. (A) The sequence of RFD-CD1. The sequence is written from 5′ to 3′ direction. (B) Responses of the RFD to various gram-negative and gram-positive bacteria. (1) Negative control (no bacteria), (2) *Clostridium difficile*, (3) *Bacillus subtilis*, (4) *Leuconostoc mesenteroides*, (5) *Pediococcus acidilactici*, (6) *Pseudomonas peli*, (7) *Brevundimonas diminuta*, (8) *Hafnia alvei*, (9) *Yersinia ruckeri*, (10) *Ochrobactrum grignonese*, (11) *Achromobacter xylosoxidans*, (12) *Moraxella osloensis*, (13) *Acinetobacter lwoffi*, (14) *Serratia fonticola* and (15) *Escherichia coli*. (C) Responses

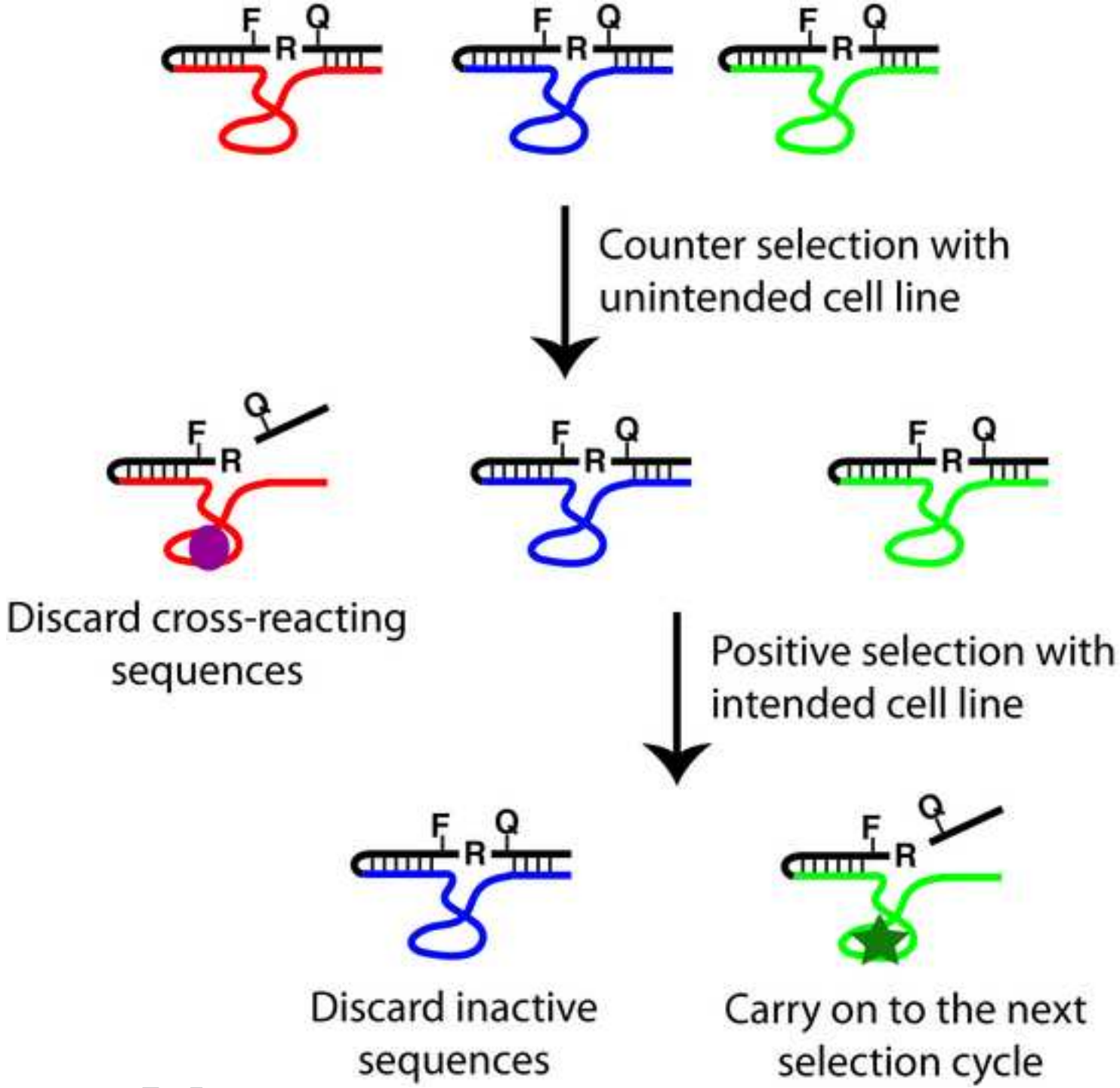
of the RFD to various *Clostridium difficile* strains. (1) BI/027-H, (2) ATCC BAA-1801, (3) ATCC BAA-1804, (4) ATCC BAA-1875, (5) ATCC 43594, (6) ATCC 43598, (7) ATCC BAA-1832 (CD630), (8) ATCC BAA-1871, (9) ATCC BAA-1872, (10) ATCC 43255, (11) ATCC BAA-1870, and (12) ATCC BAA-1814. Unclv: Uncleaved RFD-CD1; Clv: Cleaved product.

Figure 6. Bacterial litmus test. In this test, conventional pH-sensitive dyes (or simply pH papers) are used to achieve the detection of a biological target of interest (star) through the use of RFD, urease (Ur) and magnetic beads (MB). The binding of the target to the RFD that is immobilized on the magnetic bead leads to the cleavage of the RFD, accompanied by the release of urease from beads to solution. Upon magnetic separation, the released urease is taken to hydrolyze urea in the presence of a litmus dye. The hydrolysis of urea elevates the pH of the test solution, leading to the sharp color change.

Figure 7. In vitro selection scheme with the following steps: (1) Ligation of FS1 to L1; (2) purification of ligated FS1-L1; (3) negative selection with CIMs from unintended cells; (4) purification of uncleaved FS1-L1 by dPAGE; (5) positive selection with CIM from the intended cell; (6) purification of cleaved products by dPAGE; (7) PCR using FP and RP1 as primers; (8) PCR with FP and RP2 as primers (note: RP2 contains a triethylene glycol linker and A_{20} tail at the 5' end – the triethylene glycol linker prevents the poly-A tail from being amplified, making the non-DNAzyme- strand 20 nucleotides longer than the DNAzyme strand); (9) purification of DNAzyme strand by dPAGE; (10) ligation of L1 to FS1.


F
NH
O
N
NH2
OH
Q
HO

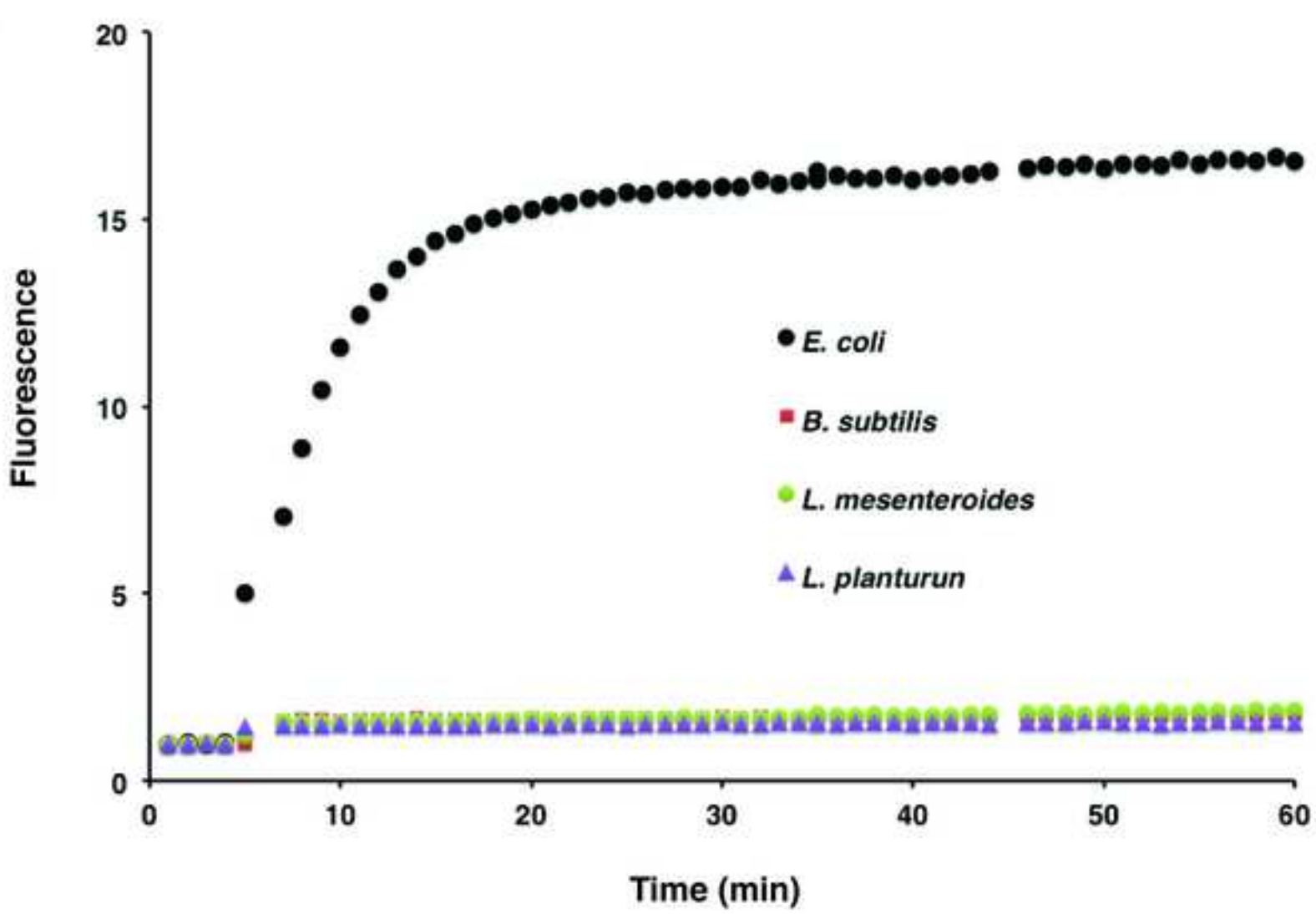




A

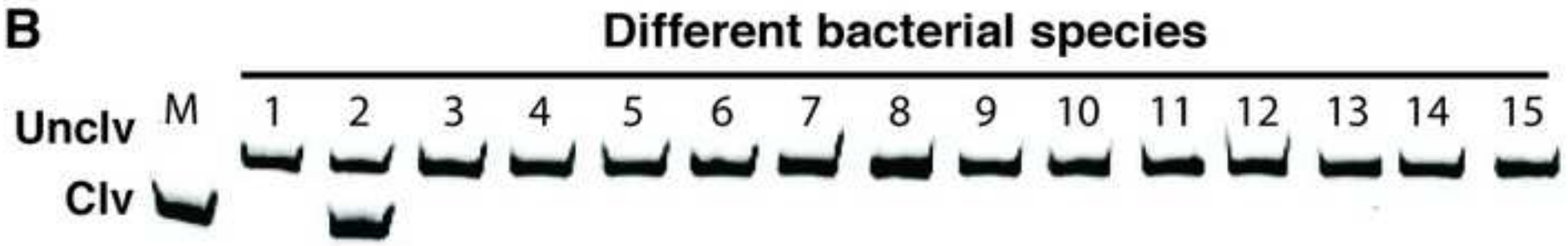
CACGGATCCT GACAAGGATG TGTGCGTTGT CGAGACCTGC GACCGGAACA
CTACACTGTG TGGGATGGAT TTCTTTACAG TTGTGTGCAG CTCCGTCCGA
CTCTTCCTAG CFRQGGTTCG ATCAAGA

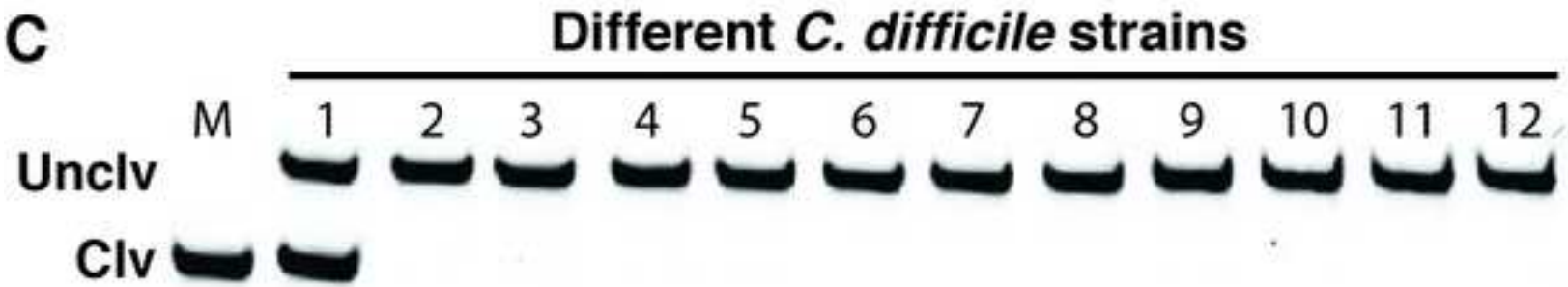
B

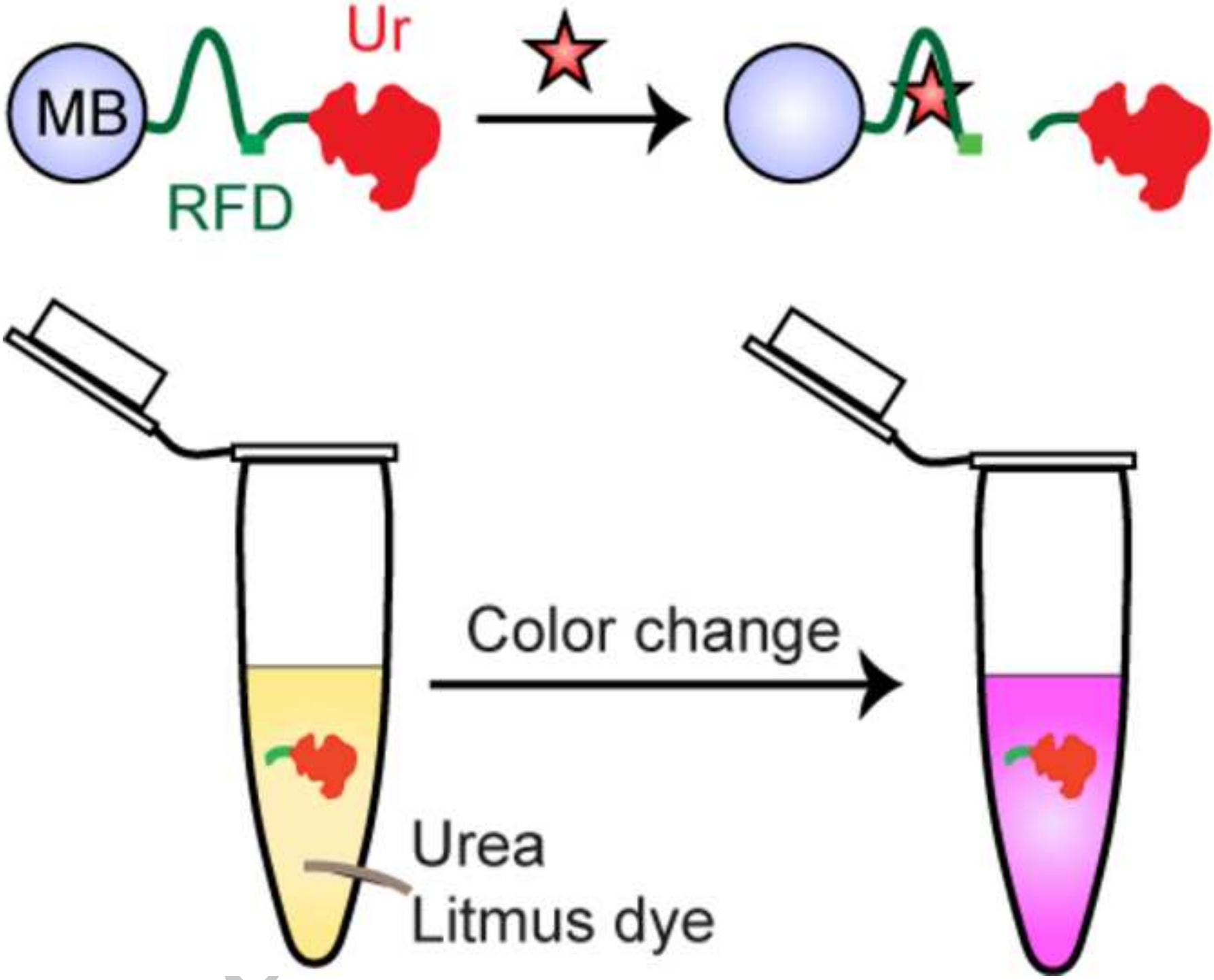


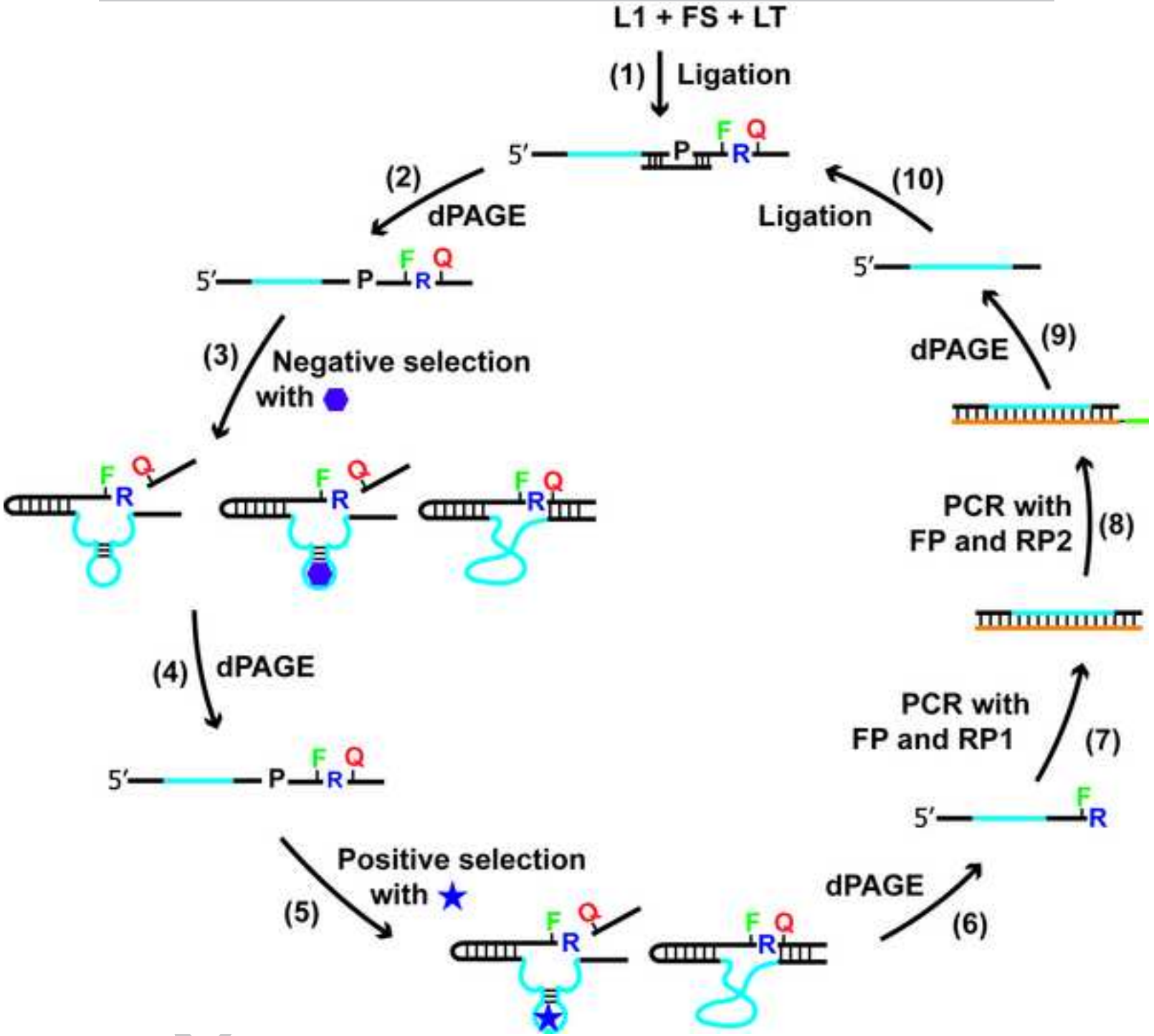
A

CACGGATCCT GACAAGGATC TGAGTGGATT GGGGCCTGCG
CGGAGTCGGG ACTATTCAGC TCCGTCCGAC TCTTCCTAGC
FRQGGTTCGA TCAAGA









Highlights

- RNA-cleaving DNAzymes are DNA-based enzymes for RNA cleavage
- Bacteria-responding RNA-cleaving DNAzymes can be isolated from a random DNA pool
- These DNAzymes can be used to set up simple assays for bacterial detection.