Automatic & Rapid Molecular Detection of *E. coli* and Enterococci in Raw Recreational Water Samples Using the Fully automated Rheonix CARD™ Technology Platform

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INTRODUCTION

We have developed a fully automated molecular diagnostic platform that is capable of automatically performing all aspects of a molecular and/or immunologic assay. Once a "raw" sample is applied, all sample preparation, analysis and readout functions are automatically performed without any additional "hands on" efforts. Since the Rheonix CARD™ (Chemistry And Reagent Device) platform eliminates virtually all hands on efforts, sophisticated tests can be easily performed by individuals of varying skill level. Due to the ease of performance, the Rheonix CARD™ platform is well suited for recreational water testing settings.

DEFINITION OF PROBLEM

Current testing of recreational waters for the presence of fecal indicators relies upon time consuming and labor intensive culture methods. The resulting 1-2 delay in achieving results not only jeopardizes the health and safety of bathers, but can also cause unnecessary concern on the part of individuals who may have used a particular beach in the days immediately leading up to a beach-closing announcement. Because of the inherent safety and health hazards associated with these delays, the U.S. EPA is currently under a court order to reduce the time to results to "same day."

DISCUSSION

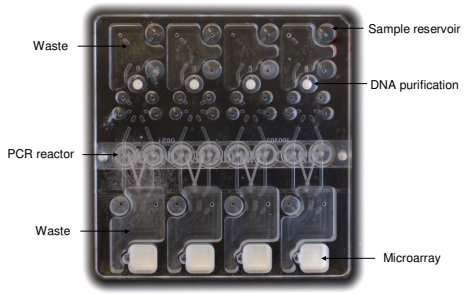
The goal of the present study was to develop a fully automated molecular diagnostic system whereby an analyst can introduce a raw water sample and achieve easy-to-interpret results within 3-4 hours. Using the fully automated Rheonix CARD™ Technology platform, once a raw water sample is introduced, cell concentration and lysis, followed by DNA isolation, multiplex PCR amplification and readout of results can be accomplished with virtually no "hands on" efforts.

The Rheonix CARD™ Technology platform utilizes a proprietary disposable device capable of automatically performing sophisticated molecular assays under the complete control of software contained in either a portable, battery operated system for onsite use, or a higher throughput workstation intended for laboratory use. The disposable Rheonix CARD™ device contains all pumps, valves, microchannels, reaction vessels, reagents and read out capabilities required to automatically perform all of the necessary assay steps. PCR primer pairs and probes were designed to amplify and detect the single copy genes for the enterobacterial common antigen and the elongation factor Tu (*tuf*) for detection of *E. coli* and enterococci, respectively. Similarly, primer pairs and probes were also designed to detect multicopy rRNA genes in these two groups of target microbes. Pretreatment of cells with propidium monoazide (PMA), which differentially permeates nonviable cells, allows the determination of the viability status of the target cells.

RESULTS

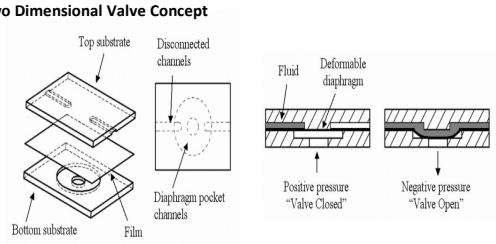
In order to develop the fully automated recreational water test, we first defined and tested PCR primer pairs and probes that could be used to specifically detect the microbes of interest. The organisms targeted included E. coli and four enterococci (*E. faecium, E. faecalis, E. gallinarum, and E. avium*). For E. coli we selected the ECA (enterobacterial common antigen) and the multicopy 16S rRNA genes. For the enterococci, we selected the *tuf* gene encoding the enterococci elongation factor EF-Tu. Once optimized on the bench top, the assay was migrated to the Rheonix CARD™ device, capable of running four simultaneous assays (Figure 1)

Figure 1: Rheonix CARD™ Device



The self-contained Rheonix CARD™ device contains all pumps, valves, mixing chambers, microchannels, PCR chambers and microarrays for detection. Using a proprietary process, the plastic device is formed by laminating three layers of plastic (Figure 2). The upper layer contains discontinuous channels while the lower layer contains diaphragm pockets. Sandwiched between them is a thin deformable layer of plastic that can be moved upward or downward, depending upon the application of positive or negative pressure, respectively. The actuation of this layer can create an closed or open valve. Additionally, a series of these diaphragms can be assembled to form a bi-directional pumping mechanism

Figure 2: Two Dimensional Valve Concept

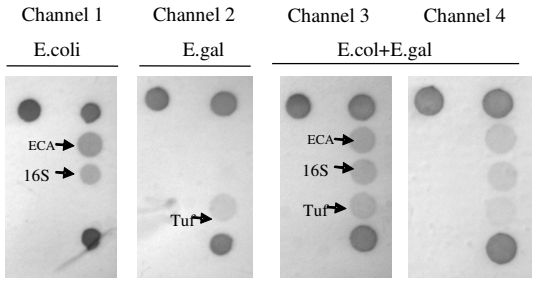


The fully automated Rheonix CARD™ Assay procedure consists of the following steps:

1. Introduction of "raw" sample to sample reservoir on Rheonix CARD™ Device (the only human intervention).
2. Lysis of cells and isolation of DNA.
3. Multiplex PCR amplification of gene targets of interest. One of each PCR primer pair set is biotinylated at its 5' end.
4. Denaturation of PCR Amplicons and delivery to a low density DNA microarray.
5. Introduction of streptavidinylated HRP and TMB substrate.
6. Capture of resulting reverse dot blot (RDB) image and determination of presence of specific microbes of interest.

Although the only "manual" operation is the concentration of the 100 ml sample with an ElutraSep (Canton, GA) hollow fiber concentration system, our current efforts are also integrating this step as well. Using the above procedure, mixtures of *E. coli* and *E. gallinarum* (100 CFU/100 ml sample) were analyzed on the Rheonix CARD™ device capable of running four simultaneous assays. As noted in Figure 3, analysis of samples containing either *E. coli* or *E. gallinarum* alone or in combination yielded strong RDB signals of the expected gene targets.

Figure 3: Reverse Dot Blots of *E. coli* and Enterococci samples



In order to establish the viability status of the microbes, we exploited the capability of propidium monoazide (PMA) to selectively infiltrate nonviable cells. Photoactivation of the PMA-treated cells causes the PMA to bind to and precipitate DNA, thereby rendering it unavailable for PCR. We found that, as compared with viable cells, PMA treatment of nonviable cells dramatically reduced the total amount of DNA that could be isolated from cells and concurrently reduced the total amount of PCR amplicons detected (Table I). We are currently designing the Rheonix CARD™ device to automatically perform the PMA treatment, which will permit samples to be simultaneously analyzed with and without treatment of PMA.

Table I: Differential effect of propidium monoazide on viable and nonviable cells

	Viable Cells		Nonviable Cells	
	- PMA	+ PMA	- PMA	+ PMA
DNA Yield	+++	+++	+++	+
PCR Amplicons	+++	+++	+++	+

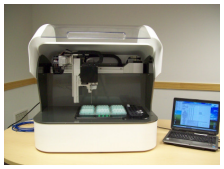
The Rheonix CARD™ platform can be used in either a "point-of-use" or central lab settings. The portable Rheonix CARD™ Controller (Figure 3) can run up to four separate simultaneous assays and operates on either AC or DC power. The EncompassMDx™ workstation (Figure 4) can run up to 24 simultaneous assays and is intended for central lab settings. Both are under the control of software which controls and monitors all aspects of the assays.

Figure 3

Rheonix CARD™ Portable Controller

Figure 4

EncompassMDx™ Workstation



CONCLUSIONS

The Rheonix CARD™ system is capable of performing fully automated molecular analysis of recreational water samples. Requiring only three hours to complete the entire assay (including all preparative, analytical and detection steps), the system can easily and rapidly assess recreational water for the presence of fecal indicators. In addition, since there are no "hands on" efforts required (other than the initial introduction of the sample), individuals of varying skill level can easily perform very sophisticated molecular diagnostic tests. Coupled with the use of inexpensive injection molded plastic, the Rheonix CARD™ devices provides an economical means to obtain sensitive molecular detection of fecal indicators in recreational water and assist the U.S. EPA in satisfying its current court order to achieve "same day" results.

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