Autonomous High Throughput Real Time PCR Platform for Large Scale Environmental Monitoring

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ABSTRACT

We present the design and development of a continuous flow, semi-autonomous, quantitative Polymerase Chain Reaction (qPCR) system. This battery-powered system operates under programmable control and is able to detect many different primer probe combinations as is required for environmental analyses. Our design focuses on a system with re-usable components for low levels of consumables, automated fluid handling and low power consumption. It also maintains user control over essential parameters including sample volume, temperature, and cycle time. We use a two-phase emulsion system connected to a continuous flow, two-temperature zone cylindrical thermocycler. The fluorescence from each channel is excited using an LED coupled through a fiber optic bundle and emission detected using optical fiber coupling to a 64 channel PMT. Data collection and quantitative PCR analysis is performed using a laptop computer. This platform can be transported for in-situ field applications.

Keywords: real-time PCR, high throughput, emulsion, continuous flow, PMT

1 INTRODUCTION

Real time monitoring and characterization of microbial populations over large temporal and spatial scales are essential in the design and development of early warning systems and containment strategies. Consequently; accurate, exhaustive, long-term and continuous acquisition of the target data is critical [1]. Historically, in-situ information collected from marine environments was limited to conductivity, temperature and depth (CTD). Now there is great interest in studying a broader spectrum of chemical and biological variables in the environment without transporting samples to the laboratory for processing. Environmental monitoring requires collection of liter scale sample volumes from multiple locations. These samples are then transported to laboratory testing facilities after specially treating them to "freeze" the activity of the microorganisms. The preserved samples are then analyzed days or even months afterwards to ascertain the biological composition of the collected sample. This methodology has the potential of introducing artifacts in the analyzed results, and requires significant labor, time and cost associated with the collection and preservation of those samples [1], [2]. Zehr et. al. [3] reports on the use of molecular biological methods for the study of marine organisms and provides a comprehensive review of the various approaches that can be implemented on a sensing platform for rapid in-situ environmental analysis.

Autonomous field operations are thus often desired in bio-analytical instrumentation for in-situ monitoring. The Monterey Bay Aquarium Research Institute (MBARI) has developed an environmental sample processor (ESP) that is capable of performing in-situ analysis of marine water samples but is limited by the number of reagents it can process [4]. Size and power constraints also limit the ESP's ultimate portability. Using the MBARI ESP front-end to collect and isolate DNA from water samples, we are developing a qPCR back-end to combine the purified DNA with multiple analyte solutions and detect dozens of genes.

Here we present the preliminary design and development of a back-end continuous flow quantitative PCR (qPCR) system, suitable for low power, high-throughput, semi-autonomous field operations. Key design parameters include re-usable components for low levels of consumables, automated fluid handling, low power consumption, and high throughput sample processing that can be deployed for field analysis. This design utilizes a two-phase, continuous flow, emulsion based PCR design to reduce sample cross-talk and increase sample throughput. This technique enables the introduction of multiple PCR reagents into a continuous oil stream to quantitatively detect dozens of genes from a given water sample in 1-2 hours.

2 MATERIALS AND METHODS

This device can be divided into four functional components; Fluid handling, PCR thermocycling, Optical coupling and Fluorescence detection and analysis.

2.1 Fluid Handling

One of the hurdles of achieving high-throughput PCR on a portable platform is overcoming sample to sample cross-contamination. One approach is to carry cleaning solutions to flush the system between each sample run but this reduces throughput and portability and also increases operational complexity, size and cost. Another approach, single use consumables, requires more complex

autonomous operation (switching between consumables) and does not avoid cross-contamination in shared downstream fluid-pathways.

The two-phase emulsion based approach implemented here uses oil as a carrier phase to isolate the sample and reagent plugs from wetting instrument surfaces to reduce the number of cleaning steps required for operation. To minimize the likelihood of PCR reagents interacting with solid surfaces, we have chosen a hydrophobic and fluorophilic Fluorinated Ethylene Propylene (FEP) tubing along with Fluorinert® FC-40 oil (Sigma Aldrich, USA) as the carrier phase. Not only does the oil preferentially wet the fluorinated surfaces, it has a low viscosity of 4.8 cPs, which helps reduce the overall backpressure in the system to <10 psi.

Instrument quantification was performed using custom Taqman® hydrolysis probes (5' FAM and 3' MGB/NFQ) and primers (Applied Biosystems, USA) for cbbL (a photosynthetic gene) obtained along with environmental samples derived from seawater provided by MBARI. All reactions were run at final concentration of 1x Taqman Universal PCR Mastermix with AmpliTaq® gold, a hot start polymerase (Applied Biosystems, USA), 0.175 µM/µL of recombinant Taq DNA polymerase (Fermentas, Canada), and 900 nM each forward and reverse primer and 250 nM hydrolysis probe. Addition of the recombinant Taq DNA polymerase allowed cycling without an initial hot start lag time.

To automate the fluid processing of the instrument, we have designed a fluid routing scheme illustrated in Figure 1. In this scheme purified DNA samples mix with PCR reagents from a primer bank and injected into the thermocycler. Mechanical pinch valves combined with flexible tubing maintain continuity in the fluid path. The T-junction and flexible tubing are also made or coated with fluorinated compounds. The sample and the reagent are introduced into the sample-mixing chamber and then pushed out using oil. Because of the hydrophobic and fluorinated tubing used, even during aspiration, the oil forms a layer around the reagents as it preferentially wets the surface, thereby minimizing carryover and cross-contamination. This process is repeated for multiple analyte solutions until all the reaction volumes are aspirated into the main line, and then flowed through the thermocycler simultaneously.

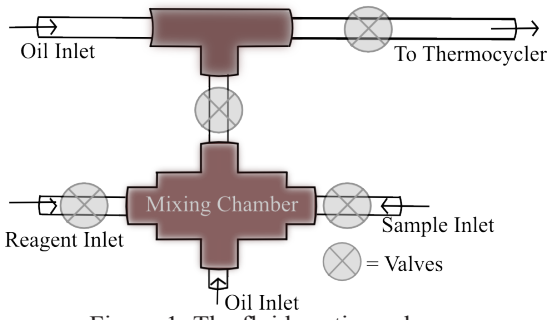

Figure 1: The fluid routing scheme

2.2 PCR Thermocycling

Previous works have presented continuous flow PCR using tubing wrapped around a three temperature thermal mass [5]. The heated zones are held at their respective temperatures and only the small thermal mass of the fluid flowing through the tubing is cycled. This eliminates the need for active cooling mechanisms and enhances the efficiency of the thermocycler making it well suited for low power designs. We designed a thermocycler optimized for TaqMan® PCR assays which calls for two temperature zones, typically a denaturation temperature of (95ºC) and an annealing temperature of (60ºC)

Our thermocycler design consists of a 3" tall cylindrical tube of aluminum (3" OD and 1/16" thickness) machined into two separate pieces for two different temperature zones. The ~9.4" perimeter was divided according to a 2:1 length ratio of the annealing/extension zone (60ºC) to the denaturation zone (95ºC). The zones are held in place with plastic rings at the top and bottom. The aluminum pieces were fitted with thin film resistive Kapton heaters (Omega, USA) on the inside for heating and thermocouples on the outer surface for temperature feedback. 40 turns of FEP tubing (1mm OD and 0.5mm ID) were wrapped around the cylindrical thermocycler and fed through a 40-channel optical fiber coupling block as shown in Figure 2. The inside and outside of the thermocycler was then covered with ½" thick low-density foam for insulation.

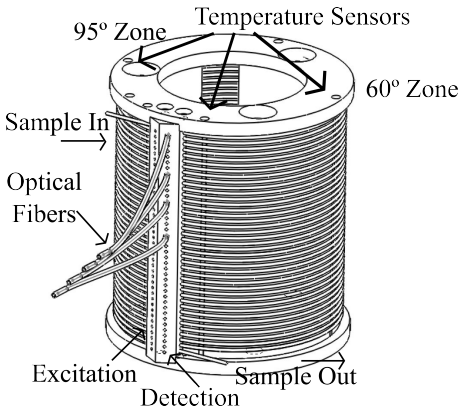

Figure 2: Schematic of the continuous flow PCR device depicting the two zone thermocycler, tubing, temperature sensors, optical fiber coupling and inlet and outlet ports.

Maintaining a proper thermal profile is critical to attaining accurate PCR results. A thermocouple feedback based control circuit was designed to maintain the set temperatures. K-type thermocouples are used at four separate points (two in each of the heat zones). The thermocouple readouts were then amplified using Analog Devices AD8497 thermocouple amplifiers and read into an Arduino microcontroller board. A PID control program was written for the Arduino microcontroller that compared the temperatures independently in the four channels to the set temperature and generate a pulse width modulated (PWM)

signal to control the heaters. With this setup we were able to have a temperature profile within 0.5ºC of the set-point temperature.

2.3 Optical Coupling

In our design, real-time fluorescence measurements from the droplets/plugs were captured for each cycle using fiber optic coupling between the 60ºC to 95ºC aluminum pieces. This novel technique makes use of a custom built close packed bundle of 37 optical fibers (Polymicro, USA) to launch the excitation from an LED light source into individual wraps of tubing passing through the coupling block as shown in Figure 3a. The emission from the fluorescent reporter was then collected using another set of optical fibers coupled through an emission filter to the face of a 64-channel multi-anode PMT in an 8x8 configuration (H8500, Hamammatsu, USA) as shown in figure 4. The output from the PMT was recorded using a Vertilon SIB064A interface board interfaced to a laptop computer through a PhotonIQ IQSP482 DAQ system (Vertilon, USA).

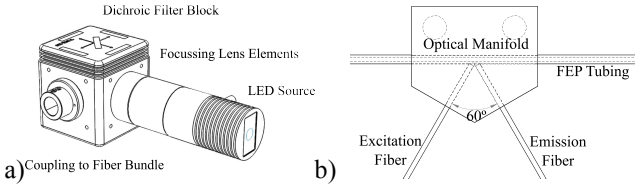

Figure 3: a) Schematic of the multichannel LED excitation block. b) Cross-section of the fiber coupling

A 470nm LED (M470L2, ThorLabs, USA) was used as the excitation source. In order to attain a narrow excitation bank around 470nm, a dichroic filter was used as illustrated in Figure 3a. To couple the excitation and emission fibers to the tubing a fiber optic manifold was developed so that the fibers are at an angle of 30º to the normal and 60º to each other as illustrated in Figure 3b. The optical manifold was integrated in the thermocycler right after the 60ºC zone, which is the optimum location for taking fluorescence intensity measurements. An optical coupling gel was used to ensure better coupling between the optical fibers and the index matched FEP tubing.

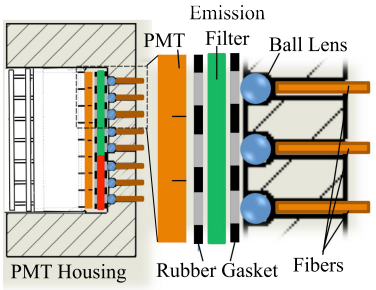

Figure 4: Schematic of detection block showing how the optical fibers interface with the 64-channel PMT block

On the detector side, each fiber optic carrying the emission signal for the corresponding PCR cycle was coupled to a custom fabricated optical assembly as illustrated in Figure 4. The emission output is first collimated using 3mm ball lenses, then passed through a green (FF01 540/50 Semrock, USA) interference filter followed by a 3mm glass absorption filter (FSQOG515 Newport, USA) before being received at the PMT surface for the FAM fluorophore detection. However, by simply changing out the interference filter, other fluorophore wavelengths can be detected as well.

2.4 Fluorescence Detection and Analysis

Although care was taken to ensure uniform excitation and emission out of all fiber coupled 37 channels, non-uniformities in the coupling efficiency at both the excitation source and the detector as well as inside the optical manifold gave rise to varying efficiencies of emission signals. To rectify this we normalized the intensity measurements against a standard solution of 200 nM fluorescein (Sigma Aldrich, USA) in 0.1M pH 8.0 Tris HCl buffer solution. The 200nM solution was chosen because it was quantitatively determined that the fluorescence intensity of a fully amplified PCR reaction would match the 200nM fluorescein concentration. As the droplets or plugs passed through our optical manifold the peak intensity of each droplet/plug was measured and recorded using the interface that came along with the Vertilon system.

The recorded intensity measurements were then analyzed using custom MATLAB® scripts. Because of the continuous nature of this instrument, we have developed a robust MATLAB® script that can analyze simultaneous data from all the 37 channels as well as track the droplet order from one cycle to the next. The sizes of the droplets/plugs were chosen so that they were larger than the dimensions of the tubing, thereby ensuring the preservation of the droplet sequence across all cycles. To account for the possibility of coalescence and/or breakup of the fluid plugs propagating through the tubing, our detection algorithm was designed to collate the data and distinguish between different reaction numbers and volumes. After extracting the recorded average peak intensities they were normalized and the relative fluorescence intensity plotted as a function of PCR cycle as illustrated in Figure 5.

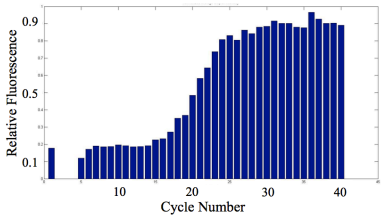

Figure 5: PCR amplification curve for $10e^8$ copies/10 µL concentration of cbbL gene.

The threshold for the real-time PCR reaction (C_t) was determined as the cycle number where the level of signal

increased beyond the baseline signal. Since the background signal level is a function of the instrument and the reaction, the Ct threshold value was determined to be 0.29 on the Relative Fluorescent scale based on the average background signal levels from multiple runs.

3 RESULTS AND DISCUSSIONS

Based on the dimensions of the thermocycler and the tubing, a single wrap around the heater was calculated to be 47 µL, yielding a total volume of 1.9 mL for the entire thermocycler. Taking into account additional lengths of tubing at the inlet and outlet, for a single PCR run, approximately 2.2 mL of oil was consumed per run. But because of the continuous flow nature of this instrument, multiple reactions could be run at the same time reducing the amount of carrier fluid required per reaction. For our experiments, we optimized the PCR reactions for about 20 seconds in the 95 ºC zone and 40 seconds in the 60 ºC zone. We fixed the flow rate at 47 µL/min to achieve this, although, other cycle times are easily achieved using different flow rates.

Along with the flow, the temperature was closely monitored and our PID based control was able to maintain the set point temperature within 1 ºC. The peak power draw by the heaters during start up phase was around 33 Watts, but once the heaters equilibrates, it only draws approximately 6 Watts due to our PWM based design. Most of the power was drawn by a single heater at the front of the hot 95 ºC zone when the reagents need the most temperature change, up from 60 ºC. Using the triggering mode of the Vertilon interface board, we pulsed the LED only during data capture at a rate of 30-60Hz, which reduced the overall power requirement for the LED to <1 Watt. The interface board that supplied power to the PMT draws about 10 Watts.

Using the fluid routing scheme illustrated in Figure 1, samples could be loaded continuously with no detectable cross-contamination between runs. RNase-free Diethylpyrocarbonate (DEPC) water was used in between experiments to reduce carry-over between runs. The continuous flow real-time qPCR instrument reported here was used to perform a 40 cycle PCR with both standardized genes and environmental samples provided by MBARI. 10 µL reaction volumes were aspirated in from the sample tube and then pushed through using FC40 oil.

Compared to a commercial Applied Biosystems® **StepOne™** PCR benchtop instrument, the cycle threshold (C_t) values obtained from our instrument had a constant offset of about 4 C_t higher. This is expected however, because of variations in the instrument and the difference in C_t calculation algorithms. To qualify the efficiency, sensitivity, and dynamic range of our instrument, we performed a dilution series experiment. We were able to detect copies in the range of 10^3 to 10^{10} copies per 10 µL reaction volume. Figure 6 shows the standard curve was obtained from multiple reactions and the PCR efficiency was calculated to be 96% with an R^2 value of 0.9785.

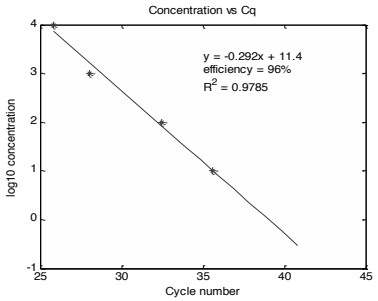

Figure 6: Standard curve obtained from the serial dilution experiment

4 CONCLUSIONS

The continuous flow PCR instrument developed here has a great potential to be developed as a platform for performing high-throughput, autonomous real-time PCR experiments in the field. It can readily be modified based on the requirements for different sets of primers or fluorescent dyes. Simply increasing the number of tubing wraps can increase the number of cycles and changing the excitation and emission filter provides the flexibility of using other types of fluorescent dyes. The set temperature points can be easily changed based on primer probe requirements.

We have focused our design towards a low power instrument that can be easily carried to a field, eliminating the need for transporting samples to full-scale laboratories. A single experiment can be performed in less than two hours with less than 60 watt-hours of power. Any standard laptop can be used for data analysis for instantaneous results. In future, we hope to further reduce the footprint and the power requirements by using an OEM data interface board from Vertilon for the data capture.

ACKNOWLEDGEMENTS

The work was performed on behalf of MBARI with financial support from the Gordon and Betty Moore Foundation. The authors would like to thank the excellent scientists at MBARI. We also thank Jeff Houkal for his help with mechanical design and manufacturing.

REFERENCES

[1] H. B. Glasgow et. al. *Journal of Experimental Marine Biology and Ecology*, vol. 300, no. 1–2, pp. 409–448, Mar. 2004.

[2] S. Kröger and R. J. Law. *Biosensors and Bioelectronics*, vol. 20, no. 10, pp. 1903–1913, Apr. 2005.

[3] J. P. Zehr, I. Hewson, and P. Moisander. *Ocean Science*, vol. 5, pp. 101–113, May 2009.

[4] C. M. Preston et. al. *PLoS ONE*, vol. 6, no. 8, p. e22522, Aug. 2011.

[5] N. Park, S. Kim, and J. H. Hahn. *Analytical Chemistry*, vol. 75, no. 21, pp. 6029–6033, Nov. 2003.