Sensitive, Microliter PCR with Degenerate Primers for Respiratory Virus Detection and Discovery

C. R. Phaneuf[1], K. Oh[2], N. Pak[1], D. C. Saunders[1], C. Conrardy[3], J. Landers[2], S. Tong[3], and C. R. Forest[1]
[1]Georgia Institute of Technology, Atlanta, GA, [2]University of Virginia, Charlottesville, VA, [3]Centers for Disease Control and Prevention, Atlanta, GA

Introduction: Polymerase chain reaction (PCR) is a widely used sample preparation technique for the amplification of a target DNA sequence from as little as a single copy. Despite past efforts to reduce the time and cost of this process through miniaturization, molecular biology laboratories continue to use conventional instruments that operate slowly (e.g., 0.5-2 hr/run) and require large, costly volumes (e.g., 5-50 μL). One of the many labs interested in accelerating workflow through improved PCR technology is the Pathogen Discovery Division at the Center for Disease Control (CDC), where consensus degenerate primers are used to detect existing and novel pathogens of various viral families [1]. We have worked with the CDC to design and demonstrate a microfluidic PCR system that uses inexpensively fabricated, and therefore disposable, polymer microchips that can handle as little as 1 μL reaction volumes and, with optimal surface passivation, achieve sensitivity comparable to conventional techniques. Performing the unique consensus degenerate chemistry at the microscale has not been reported previously and represents an advance towards lower cost and higher throughput pathogen screening.

Materials and Methods: Microchips were fabricated via micromilling of a poly(methyl methacrylate) (PMMA), substrate and subsequent thermal bonding to enclose 1 μL reaction chambers and filling channels [2]. Once filled, the ports of the microchip were sealed with an adhesive film (ThermalSeal, Sigma-Aldrich). Passivation techniques were used to prevent adsorption of reagents, notably the polymerase. Although dynamic passivation [3] by the addition of bovine serum albumen (BSA) was effective, we devised an improved approach in which the reaction volume was loaded between plugs of mineral oil. All reactions were prepared from a commercial master mix, AccuPower PCR PreMix (Bioneer, South Korea), consisting of a lyophilized pellet of 2.5 U Top DNA polymerase, 250 μM dNTPs, 10 mM Tris-HCl, 30 mM KCl, 1.5 mM $MgCl_2$, a tracking dye, and a stabilizer. First, 43 μL of nuclease-free water was added to the premix tube, which was vortexed and spun down. Next, 1 μL each of 50 μM forward and reverse primers were added. For conventional reactions, the mixture was divided into 4.5 μL aliquots and 0.5 μL of Epstein Barr virus template DNA was added at starting concentrations ranging from 1.25×10^{-3} ng/μL (1.4×10^{6} copies) down to 1.25×10^{-7} ng/μL (1.4×10^{2} copies) in 10× dilution increments. For microchip reactions, 0.5 μL aliquots were used with 0.5 μL of template at the same concentrations. Microchips were thermocycled in a water bath PCR system and detection was performed with an Agilent Bioanalyzer.

Results and Discussion: Successful amplification was demonstrated down to the limit of detection of conventional thermocycling with only ~100 starting copies. Although signal strength of 5 μL samples run on a conventional instrument is better than with our microchip approach, improvements to the sample loading protocol and refinements to recipe could further improve sensitivity and repeatability.

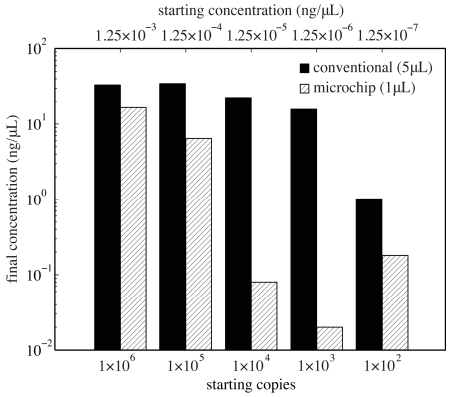

Figure 1. Comparison of conventional (*black*) and microchip-based (*gray*) amplifications for detection of herpesvirus (Epstein Barr virus). Comparable sensitivity was demonstrated down to hundreds of starting copies.

Conclusions: In order for the molecular biology community to embrace technological advancements to tools such as microfluidic PCR instrumentation, we must assess performance with the most challenging and relevant reactions. Our demonstration of a viable microchip-based alternative to conventional techniques using CDC virus discovery reactions is a step closer to massive reductions in the cost and labor involved in pathogen screening and countless other PCR applications.

References:

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