TECHVIEW: INFECTIOUS DISEASE

PCR Detection of Bacteria in Seven Minutes

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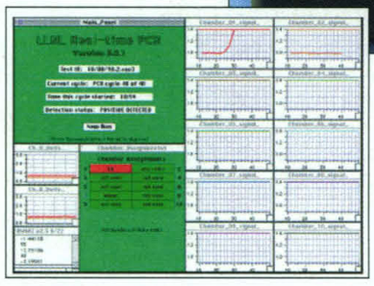

Fig. 1. The portable ANAA, which consists of an array of ten reaction modules and a laptop computer. (**Inset**) Screen of the laptop computer after running a reaction containing 500 *Erwinia* cells, based on colony forming units, in chamber 1 and a reaction containing no cells in chamber 2. A positive signal is observed only in the real-time graph panel for chamber 1. The main panel displays the run number, current cycle, elapsed time, and detection status. The positive signal in chamber 1 caused the color of the box corresponding to chamber 1 in the chamber assignments panel to change from green to red and the detection status line in the main panel to display "POSITIVE DETECTED."

Identifying, monitoring, and isolating the source of an outbreak of infectious disease can sometimes be difficult. The process would be greatly facilitated by a portable instrument that could be used in any suspect location and was capable of rapidly detecting and characterizing microbial pathogens. Nucleic acid analysis is preferable to immunoassays for such a test because detailed genetic information can be obtained about a particular microbe's virulence, antibiotic resistance, and epidemiology. A growing number of these bacterial tests use polymerase chain reaction (PCR) because of its demonstrated effectiveness, sensitivity, and reliability (*1*, *2*). For field use, a variation of standard PCR called real-time PCR is the most practical, because it does not require time-consuming post-PCR manipulation and processing of the reaction with slab gel and capillary electrophoresis, hybridization to immobilized oligonucleotides, or mass spectrometry.

Real-time PCR can be accomplished with the fluorogenic 5′-nuclease assay called TaqMan (*3*, *4*) and a spectrofluorometric thermal cycler (*4*). TaqMan is a homogenous PCR test that uses a fluorescence resonance energy transfer (FRET) probe typically consisting of a green fluorescent "reporter" dye at the 5′-end and an orange "quencher" dye at the 3′-end. When the probe anneals to a complementary strand of an amplicon during PCR, Taq polymerase cleaves the probe during extension of one of the primers, and the dye molecules are displaced and separated. The electronically excited reporter dye is no longer suppressed by the quencher dye, and the significant increase in green emission can be monitored by a fluorescence detector. The intensity of the green fluorescence directly correlates with the concentration of PCR product in the reaction.

Commercial spectrofluorometric thermal cyclers are not ideally suited for on-the-go testing of samples because of a number of limitations, particularly with respect to size, weight, power usage, speed, and ruggedness. We have been developing, building, and testing silicon chip–based spectrofluorometric thermal cyclers that overcome these limitations (*5*–*8*). The Advanced Nucleic Acid Analyzer (ANAA) (Fig. 1), which has been recently described (*8*), can be used in the field and offers real-time monitoring, low power usage (for battery operation), no moving optical components, custom plastic sample tubes with caps, and software tailored for first responders, such as emergency medical technicians (Fig. 1, inset). Ten reaction modules and a laptop computer are housed in a protective casing, with each reaction module comprising a silicon reaction chamber with highly efficient integral thin-film heaters and a dedicated low-power optical system. The TaqMan probe is monitored with a blue light-emitting diode as an excitation source and two photodiode detectors with bandpass filters centered at 530 and 590 nm wavelength. When a real-time analysis algorithm determines that a signal is positive, the software will automatically inform the user via an audible alert and green-to-red indicator (Fig. 1, inset).

Recent modifications made on the ANAA to enhance its performance led us to examine the time that is required to perform the TaqMan assay. *Erwinia herbicola*, a vegetative bacterium that serves as a surrogate for a pathogenic microbe such as *Yersinia pestis* (plague), was used in this study. Reactions containing 500 colony forming units (cfu) of *Erwinia* cells were

Cycle Time (s)	Denature Time (s)	Anneal/Extend Time (s)	C_T (cycle number)	Detection Time (min)
38	4	19	23	14.6
28	4	10	23	10.7
24	4	5	24	9.6
17	1	1	25	7.0

Table 1. Thermal cycling settings and detection times for analyzing bacteria cells with the ANAA. Denature time and anneal and extend time represent the setpoint values at 96° and 56°C, respectively. The threshold value, C_T, is the initial cycle in which significant fluorescence signal is detected. Detection times were obtained from the reactions shown in Fig. 2A.

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subjected to a series of thermal cycle settings (Table 1) ranging from our standard cycle time of 38 s to a reduced time of 17 s. Each run on the ANAA consisted of an initial heating at 96°C for 15 s to lyse the cells, followed by two-temperature cycling between 96° and 56°C. The detection profile (Fig. 2A) acquired with the 38-s cycle (Table 1) was characteristic for 500 *Erwinia* cells based on extensive quantitation studies and blind field tests performed at the Joint Field Trials at Dugway Proving Grounds in Utah (*9*). After 24 cycles, or 15 min, a positive signal was clearly evident and was reported by the software. The displayed signal profile represented the collected raw data that was neither processed nor smoothed. The uniform baseline of the profile was attributed to the high signal-to-noise ratio.

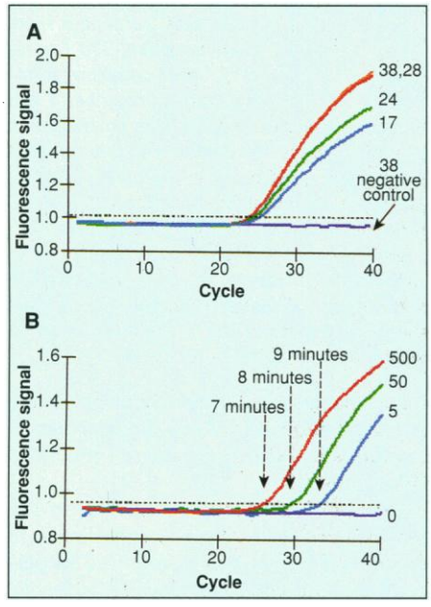

Fig. 2. Detection profiles obtained by rapid, real-time PCR analysis of *Erwinia* cells. **(A)** Effect of decreasing the cycle time for the analysis of 500 cells. The number to the right of each detection profile is the cycle time listed in Table 1. **(B)** Quantitative PCR performed with the 17-s cycle time. The number of bacteria cells and the time that a positive detection was automatically alerted by the software is indicated for each profile. Each 25-μl reaction consisted of 10× PCR II buffer (Perkin-Elmer), 0.4 mM of each PCR primer and probe, 5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 0.25 U of AmpliTaq polymerase (Perkin-Elmer), and 5 μl of sample. The probe contained a 6-carboxyfluorescein (6-FAM) fluorescent dye molecule at the 5′ end and a 6-carboxytetramethylrhodamine (TAMRA) fluorescent dye molecule at the 3′ end. Thermal cycle settings were 96°C for 15 s, and 40 cycles of 96° and 56°C at the times indicated in Table 1. Fluorescence signal is the ratio of the 6-FAM emission signal to the TAMRA emission signal. Dotted line indicates the positive signal threshold.

Although 15 min was considered to be rapid detection, the limits of the real-time PCR assay had not been reached. Therefore, runs were performed with decreasing step times until a thermal cycling time of 17 s was attained (Table 1). At this short cycle time, the rate of thermal cycling was limited by the sample heating and cooling rates of 6.5° and 4.3°C per s, respectively. The temperature profile of the solution remained very consistent from cycle to cycle, and temperature control was maintained within 0.5°C at each step temperature (Fig. 3). The faster cycling produced a strong positive signal, which was automatically alerted by the ANAA after just 7 min had elapsed (Fig. 2A). Only a small deleterious effect on the signal profile was observed, with the threshold cycle delayed by two cycles and the signal amplitude reduced by 16% compared to the 38-s cycle time.

To show that the assay was still quantitative under our most rapid conditions, reactions containing 500, 50, and 5 bacteria cells were tested with the 17-s cycle (Fig. 2B). Each 10-fold dilution resulted in an additional three to four thermal cycles or 1 min to detect the positive signal. This is exactly what was expected under conditions of very efficient PCR amplification where three to four cycles of exponential amplification would theoretically produce an 8- to 16-fold increase in the number of amplicons. The ability to perform quantitative assays under these conditions was due to (i) the efficient and uniform heating and cooling of the solution, (ii) the precision with which the target temperatures were reached and maintained (Fig. 3), and (iii) the sensitive optical system that only required 0.1 s to obtain a measurement at each cycle.

The results demonstrate that the total time to perform DNA analysis on a sample containing realistic concentrations of bacteria can be as little as 7 min with a highly efficient instrument consisting of inexpensive, low-power components. Total analysis time includes cell lysis, PCR, detection of the PCR product with a target-specific FRET probe, and automated alerting of a positive signal. Recently, a flow-through PCR system has been described in which 20 cycles of PCR were performed in 90 s (*10*). Although this is an intriguing concept, the low efficiency of the reaction required the starting sample to contain 10^8 molecules of PCR product that had been obtained with a commercial air thermal cycler. The test actually represented a nested PCR, and the total time of analysis, including detection by agarose gels, was more than 1 hour.

Ultimately, the reaction kinetics of primer and probe hybridization combined with enzymatic chemistry must limit how short the cycle time can be while still maintaining good productivity per cycle. Until such a kinetic limit is reached, however, it is conceivable that the analysis time could be further reduced by developing more-efficient silicon chambers for faster ramping while maintaining precise

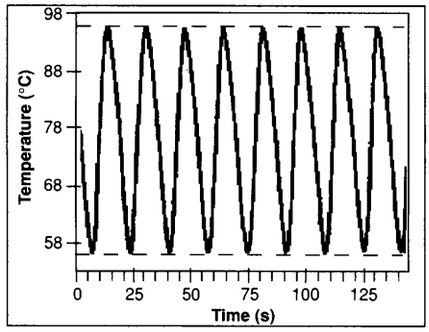

Fig. 3. Temperature profile of a reaction performed with a 17-s cycle time. A thermocouple connected to a strip-chart recorder was placed in a reaction tube in a chamber during thermal cycling. The dotted lines indicate the set temperatures of 96° and 56°C. Although the set time at each temperature was 1 s, the actual time that the solution was at each set temperature was less than 1 s because of the lag in heat transfer from the silicon chamber walls to the solution in the plastic tube.

temperature control, increasing the sensitivity of the detectors, and enhancing the thermal-stable polymerase activity by protein engineering. Silicon microstructures are also proving useful to rapidly purify and concentrate microbes and microbial DNA from complex mixtures (*11*, *12*). This will permit ultrafast DNA testing for a wide variety of environmental and clinical samples.

References

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