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A Battery-Powered Notebook Thermal Cycler for Rapid Multiplex Real-Time PCR Analysis

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A compact, real-time PCR instrument was developed for rapid, multiplex analysis of nucleic acids in an inexpensive, portable format. The instrument consists of a notebook computer, two reaction modules with integrated optics for four-color fluorescence detection, batteries, and a battery-charging system. The instrument weighs 3.3 kg, measures 26 × 22 × 7.5 cm, and can run continuously on the internal batteries for 4 h. Independent control of the modules allows differing temperature profiles and detection schemes to be run simultaneously. Results are presented that demonstrate rapid (1) detection and identification of *Bacillus subtilis* and *Bacillus thuringensis* spores and (2) characterization of a single nucleotide polymorphism for the hereditary hemochromatosis gene.

The introduction of homogeneous, fluorogenic PCR assays[1−4] and fluorometric thermal cyclers[5,6] had paved the way for quantitative, real-time detection of PCR products in a closed-tube format. Real-time PCR greatly simplifies the nucleic acid analysis process because post-PCR steps for amplicon detection and characterization are not required. The method is very robust and a wide variety of real-time PCR-based tests, including pathogenic microbe detection and single nucleotide polymorphism (SNP) discrimination, have been demonstrated. However, the execution of these tests has been limited to the laboratory because commercial fluorometric thermal cyclers are slow, large, or AC-powered.

The need for portable devices to detect and counter biological weapons of mass destruction[7,8] led to the development of a new class of small, efficient, battery-powered instruments to perform real-time PCR in the field.[9−11] This was made possible by utilizing microfabrication processes to replace the traditional components of the fluorometric thermal cycler. For example, the heating block, refrigeration unit, laser, and charged-couple device camera found in the ABI PRISM 7700 sequence detection system were substituted in the new, miniature instruments by a thin-film resistive heater, a fan, an LED, and silicon photodiode detectors, respectively. The result was a low power, efficient, and effective approach to perform real-time PCR. Microbial detection could be completed in a manner of minutes using these man-portable systems composed of inexpensive, miniature components.[12,13]

In the future, portable PCR instruments may have a big impact on the manner in which health care providers dispense medication. The consequences of relying on the slow central laboratory approach for infectious disease testing is that patients are administered antimicrobials before the lab results are made available. Unfortunately, the practice of indiscriminate use of antibiotics is contributing to the emergence of resistant pathogenic microorganisms.[14−17] One solution may be the implementation of rapid point-of-care testing to provide the patient with the most effective, specific treatment. In addition, rapid characterization of SNP markers for drug tolerance and efficacy could be applied for a wide range of medications,[18] which would allow therapy to be tailored with high precision at the molecular level.

This report describes the next generation portable, fluorometric thermal cycler. The new instrument is highly compact and battery-powered and performs rapid thermal cycling on 25- and 100-μL reaction volumes. New technologies are incorporated that dramatically reduce the size and weight of the instrument as compared to previously described portable devices. The sophisticated computer control and optical detection system establishes novel features such as four-color fluorescence detection and independent operation of each reaction site.

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EXPERIMENTAL SECTION

Samples. *Bacillus subtilis* and *Bacillus thuringensis* spores were provided by Lawrence Livermore National Laboratory (Livermore, CA). Spores suspended in water were quantitated by spreading serial dilutions of the suspensions on agar plates containing brain heart infusion (BHI) media (Difco, Mauston, WI). The plates were incubated overnight at 37 °C, and the colonies were counted. Prior to PCR analysis of the samples, the spores were lysed by sonication using a previously described method[19] with some modifications. Briefly, 100 μL of spore suspension was placed in a flat tube (Cepheid, Sunnyvale, CA) containing 40 mg of 106-μm glass beads (Sigma, St. Louis, MO). The tube was pressed against an ultrasonic horn having a 0.25-in. diameter (Sonics and Materials, Newton, CT). Sonication was performed for 10 s at 6 W and 47 kHz. A 10-μL aliquot of the lysed spore suspension was added to the PCR mix.

Real-Time PCR Analysis. PCR amplification and fluorescence detection using the 5′ nuclease assay[3,4] was accomplished using the notebook PCR instrument (see below). Oligonucleotides were synthesized by Synthegen (Houston, TX).

Multiplex Bacillus Assay. 100-μL reactions consisted of 1× PCR Buffer (Life Technologies, Rockville, MD), 0.4 μM of each PCR primer, 0.2 μM of each fluorogenic probe, 6 mM $MgCl_2$, 0.2 mM each dNTP, 0.05 U/μL of Platinum Taq DNA polymerase (Life Technologies), and 10 μL of sample. Primers and probe sequences (BS215F, BS345R, BS238T, BT159F, BT238R, BT182T) for *B. subtilis* and *B. thuringensis* were obtained from the Naval Medical Research Center (Bethesda, MD). Probe BS238T for *B. subtilis* contained a 6-Fam dye conjugated to the 5′ end, and probe BT182T for *B. thuringensis* contained a Joe dye conjugated to the 5′ end. Both probes contained a Tamra quencher dye at the 3′ end. Thermal cycler settings consisted of an initial hold at 95 °C for 30 s, then 45 cycles of 95 °C for 1 s and 60 °C for 6 s.

Hereditary Hemochromatosis (HH) Assay: 25-μL reactions consisted of 1× PCR buffer, 0.4 μM of each PCR primer, 0.2 μM of each fluorogenic probe, 5 mM $MgCl_2$, 0.2 mM each dNTP, 0.05 U/μL of Platinum Taq, and 1 μL of sample. The following primer and probe sequences were used: The forward primer was 5′-CTG GAT AAC CTT GGC TGT ACC-3′, the reverse primer was 5′-CTC AGG CAC TCC TCT CAA CC-3′, the probe for the G allele was 5′-Fam-AGA GAT ATA CGT GCC AGG TGG AGC A-Tamra-3′, and the probe for the A allele was 5′-Tet-CAG AGA TAT ACG TAC CAG GTG GAG CA-Tamra-3′. The polymorphic site in the probe sequences is underlined. Samples were purified from blood using the QIAmp blood kit (Qiagen, Santa Clarita, CA). Blood was obtained from consenting individuals with known genotypes at position 845 of the HFE gene. Thermal cycler settings consisted of an initial hold at 95 °C for 30 s, then 45 cycles of 95 °C for 3 s and 60 °C for 20 s.

The Notebook PCR Instrument. The instrument consisted of two reaction modules, with each module containing two ceramic heaters forming a reaction chamber, four InGaN light-emitting diode (LED) excitation sources, miniature optical components, four standard silicon photodiode detectors, and a cooling fan. The ceramic heaters were fabricated from Al_2N_3 patterned with resistive thin film. The components were mounted on the lid of a Sony notebook computer (model PCG-505TR). Custom software allowed independent control of each reaction module and provided real-time displays of the reaction temperatures and fluorescence signals.

The thermistor associated with each reaction chamber was calibrated to ±5 °C using National Institute of Standards and Technology (NIST) traceable standards. The temperature of the heating system was measured at two temperature, 60 and 95 °C, using a thermocouple placed in a PCR tube containing water. Calibration coefficients that corrected for small errors in the raw thermistor readings of the heaters were stored in the memory of each module.

Reactions were accomplished in custom 25- or 100-μL flat polypropylene tubes. The tubes consisted of polypropylene thin-film walls (0.002-in. thickness) laser-welded to a polypropylene frame. The walls resulted in efficient transfer of heat from the heaters to the reaction solution. The frame also functioned as optical windows for the excitation and detection blocks. The optical system was calibrated using standard concentrations of each dye conjugated to an arbitrary oligonucleotide. For each optical channel, the signal produced by the PCR buffer alone was subtracted from the raw signal produced by a dye−oligonucleotide standard to determine the spectral characteristics. The limit of detection, defined as a level-3 sigma above the noise level, was determined to be 1.0 nM for a Fam dye−oligonucleotide standard.

RESULTS AND DISCUSSION

A compact, battery-powered fluorometric thermal cycler was built that consisted of two reaction modules, an electronics board, and batteries mounted on the lid of a small notebook computer. The instrument weighed only 3.3 kg (including the batteries) and measured 26 × 22 × 7.5 cm when the notebook computer was closed. Each reaction module contained a reaction chamber with dedicated low-power heaters, a cooling fan, and a fluorescence detection system (Figure 1a). The shape of the chamber was specifically designed for rapid and efficient heat transfer, precise temperature uniformity, and long optical paths for high sensitivity. A custom plastic tube[20] that held the reaction components (Figure 1b) became pressurized when the cap was closed. This expanded the thin polypropylene walls of the tube for snug contact with the heaters.

The instrument contained unique optical detection features (Figure 1c). An excitation block of 4 LEDs and a detection block of four photodiodes flanked the two bottom edges of the tube. These edges were perpendicular and served as optical windows for the series of focusing objectives, mirrors, and band-pass filters associated with the blocks. During a PCR reaction, the LEDs would flash sequentially over a 5-s period at the 60 °C step of each cycle. This provided excitation wavelengths at 470, 510, 540, and 574 nm. As the LEDs flashed, emission signals were collected at 520, 550, 585, and 628 nm.

The software contained a deconvolution algorithm, derived from the individual spectral characteristics of pure dye−oligonucleotide conjugates, to quantitatively discriminate up to four fluorescent dyes in a mixture. Figure 2 depicts processed signals acquired in the four detection channels from known mixtures of

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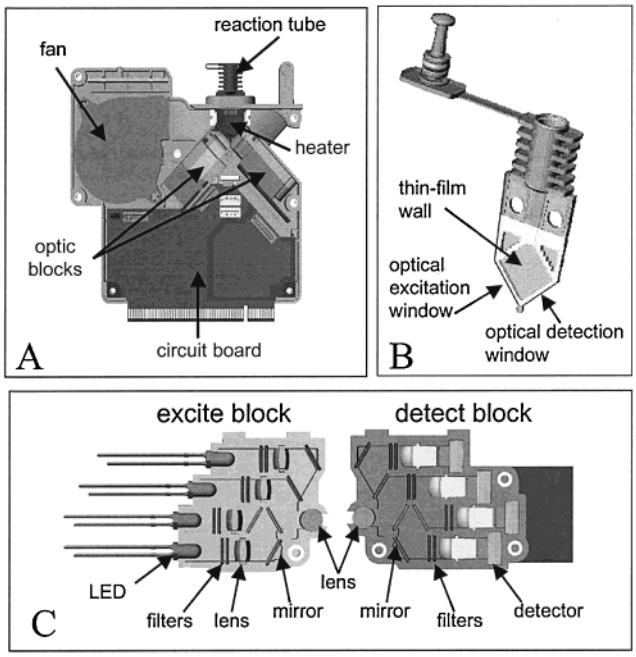

Figure 1. The reaction module is the basic unit of the notebook PCR instrument. Diagrams of (A) the complete reaction module, (B) the disposable 100-μL reaction tube, and (C) the four-color optical detection system.

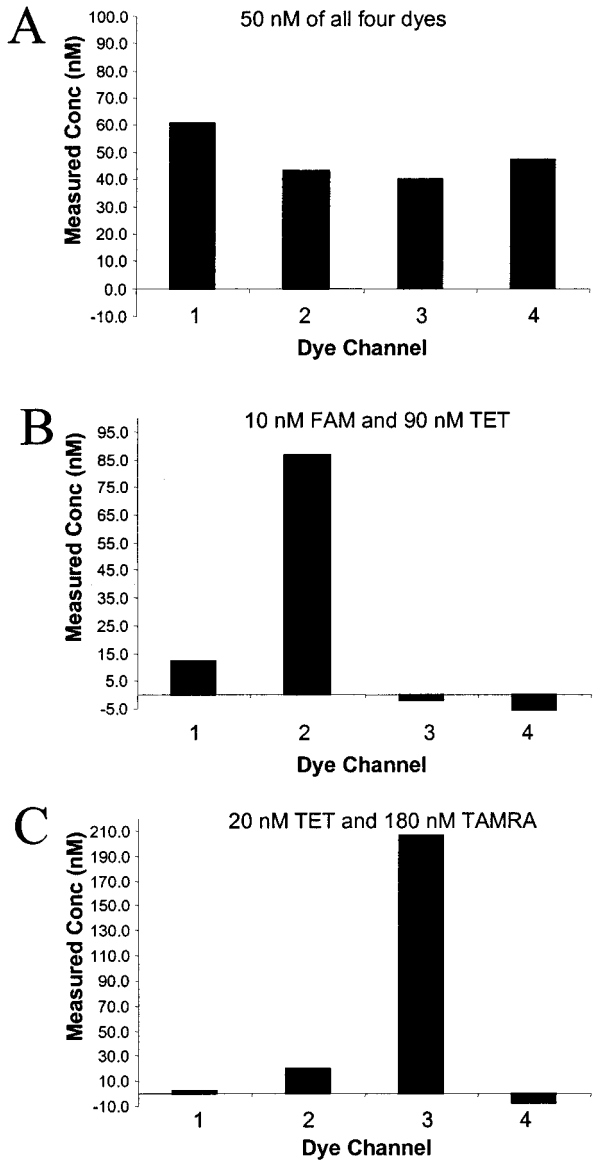

Figure 2. Spectral resolution of differing combinations of fluorescent dyes: (A) an equimolar mixture of Fam, Tet, Tamra, and Rox; (B) an unequimolar mixture of Fam and Tet; and (C) an unequimolar mixture of Tet and Tamra. The raw optical data was deconvoluted to isolate the respective fluoresence signals. The deconvoluted signals correlated with the actual concentrations of the dye standards.

dye−oligonucleotides. This provided the capability for real-time monitoring of multiple fluorogenic probes, such as those specific for microbial, allelic, or internal positive control[21] templates, in the same PCR reaction.

Because each module is a complete fluorometric thermal cycler, the modules can be ganged in a variety of configurations, depending on the user's requirements. This two-module instrument represented a platform for easy man-portable transportation and minimal power consumption. The modules could be controlled to operate together or independently. The latter allowed assays requiring differing temperature profiles to be run simultaneously on the same instrument (Figure 3). In addition, if a positive signal was observed in one module during a run, the site could be stopped and then started with a new reaction without affecting the other module.

The notebook PCR instrument was used to demonstrate discrimination of microorganisms at the species level. A multiplex assay was developed to distinguish *B. subtilis* and *B. thuringensis* spores. The assay contained two pairs of primers and two fluorogenic probes. The probe specific for *B. subtilis* was tagged with a Fam reporter dye and the probe specific for *B. thuringensis* was tagged with a Joe reporter dye. The reaction consisted of 1000 cfu of either *B. subtilis* or *B. thuringensis* spores. Prior to PCR, the spores had been subjected to 10 s of sonication treatment using a miniature device to break the spore cortex and release the DNA.[19] The signals from both probes were monitored in real-time during the course of the reaction. The signal profiles displayed positive detection curves in 10 min (cycle 25) (Figure 4). The *B. subtilis* sample displayed a positive Fam signal in channel 1 (Figure 4a), whereas the *B. thuringensis* displayed a positive Joe signal in channel 2 (Figure 4b). Thus, each probe was clearly species-specific with no indication of cross-talk between the detection channels.

The example above shows how rapid PCR analysis using a portable platform could enhance defense capability against bioterrorism. Anthrax hoaxes have become a serious problem, triggering expensive emergency responses by police, fire, and public health officials.[22] Incidents have occurred in which thousands of individuals were potentially at risk for exposure. The notebook PCR instrument could be deployed to an area containing a suspect sample, and swabs taken from nasal passages and a wide variety of surfaces could be rapidly analyzed.

Another application of the instrument is the characterization of single nucleotide polymorphisms (SNPs). Human hereditary

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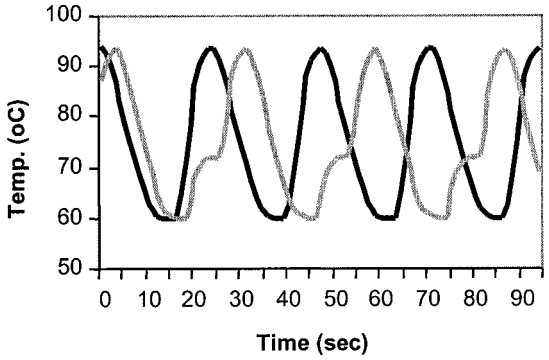

Figure 3. Independent control of the reaction modules permits differing temperature profiles to be run simultaneously. Shown are real-time temperature recordings for both modules running together. Module 1 was set for a two-step thermal profile (95 °C for 1 s and 60 °C for 6 s; black line) and module 2 was set for a three-step thermal profile (95 °C for 1 s, 60 °C for 6 s, 72 °C for 5 s; gray line). The two-step PCR setting was used for the *Bacillus* assay in Figures 4.

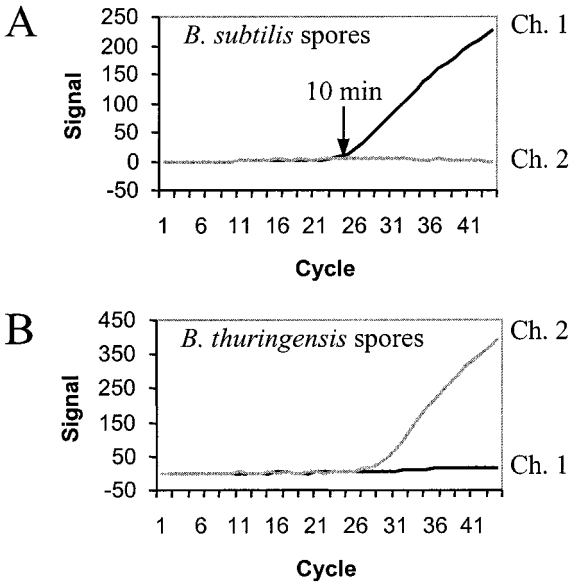

Figure 4. Multiplex PCR analysis of *B. subtilis* and *B. thuringensis* spores. The reactions contained the primers and probes for both spore species and either 1000 spore cfu of *B. subtilis* or 1000 spore cfu of *B. thuringensis*. The probe that was specific for *B. subtilis* contained a Fam reporter dye, and the probe specific for *B. thuringensis* contained a Joe reporter dye. (A) *B. subtilis* spores produced a positive Fam signal in channel 1 and a negative Joe signal in channel 2. (B) *B. thurnigensis* spores produced a positive Joe signal in channel 2 and a negative Fam signal in channel 1.

hemochromatosis (HH) was selected as a model system for testing the effectiveness of the instrument to rapidly characterize a SNP. HH is a common genetic disease that manifests as an iron metabolism disorder in individuals homozygous for a G-to-A transition at position 845 of the HFE gene.[23] A genetic test for

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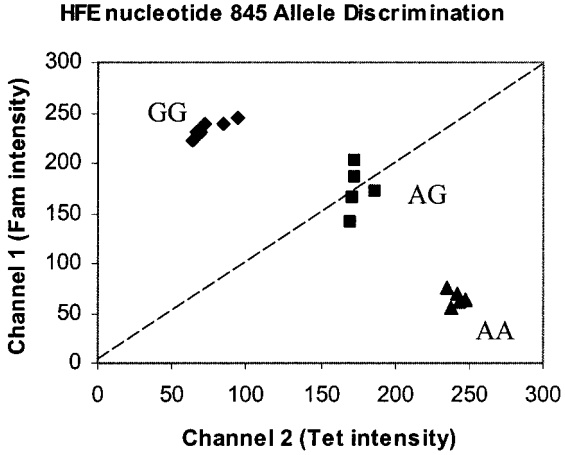

Figure 5. Characterizing the SNP at position 845 of the HFE gene. Samples from three individuals, representing the genotypes GG, AG, and AA, respectively, were characterized by a dual probe SNP assay. The probe specific for the G allele had a Fam reporter and the probe specific for the A allele had a Tet reporter. Each sample was analyzed 5 times. The scatter plot depicts the 5 endpoint fluorescence values acquired for each sample. The hemochromatosis patient had the AA genotype.

HH using the instrument may be appropriate to perform in a physician's office because HH is treatable and early diagnosis can lead to a reduction in morbidity and mortality.

The HH assay contained two allele-specific probes for position 845. The probe for the G allele contained a Fam reporter, and the probe for the A allele contained a Tet reporter. Samples from homozygous dominant (AA), heterozygous (AG), and homozygous recessive (GG) individuals were subjected to analysis. Endpoint values of the signal curves from replicate tests were presented as a scatter plot in Figure 5. The results indicated that the three genotypes were distinctly ascertained on the basis of the relative fluorescence of the two probes in the reaction.

The notebook PCR instrument offers the capability to perform rapid PCR analysis with high sensitivity and specificity where and when it is needed. It also establishes the concept of the personal PCR instrument, in which first responders, soldiers, clinicians, epidemiologists, and even students could be assigned their own instrument. This technology demonstrates how the merging of microelectronics with microbiology and genomics is resulting in smaller, less expensive bioinstrumentation with more flexibility and functionality.

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