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Real-Time Microchip PCR for Detecting Single-Base Differences in Viral and Human DNA

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This report describes real-time 5′ nuclease PCR assays to rapidly distinguish single-base polymorphism using a battery-powered miniature analytical thermal cycling instrument (MATCI). Orthopoxviruses and the human complement component C6 gene served as targets to demonstrate the feasibility of using the MATCI for diagnosis of infectious diseases and genetic disorders. In the *Orthopoxvirus* assay, consensus *Orthopoxvirus* PCR primers were designed to amplify 266−281 base-pair (bp) segments of the hemagglutinin (HA) gene in camelpox, cowpox, monkeypox, and vaccinia viruses. A vaccinia virus-specific fluorogenic (TaqMan) probe was designed to detect a single-base (A/G) substitution within the HA gene. In the C6 gene assay, a 73-bp segment of the C6 gene was PCR-amplified from human genomic DNA, and TaqMan probes were used to detect a single-base (A/C) polymorphism in the second position of codon 98. The MATCI correctly identified the nucleotide differences in both viral DNA and human genomic DNA. In addition, using a rapid DNA preparation method, it was possible to achieve sample, preparation of human genomic DNA, DNA amplification, and real-time detection in less than 1 h.

Recent advances in molecular techniques and microfabrication technology will have a significant impact on the future of medical diagnostics. Over the past few years, microchip-based devices have been developed and employed for the detection of infectious disease agents and genetic disorders and for drug discovery.[1,2] Although microchips associated with many existing devices are relatively small,[3] large accessory components for power, detection, and fluidics are still required. One exception is the miniature analytical thermal cycling instrument (MATCI) that can perform rapid thermal cycling and analysis by capillary electrophoresis[4] and 5′ nuclease PCR.[5] The integration of solid-state light sources and detectors in this instrument provides superior power efficiency and portability. Its rapid heating and cooling rates due to the low thermal mass of silicon reaction chambers and integration of thin-film heaters allow rapid PCR amplification.[5] These characteristics, when combined with a real-time detection technique such as the 5′ nuclease PCR (TaqMan), make such a device useful for rapid mobile diagnostic applications, e.g., assessment of biological threats in the field, investigation of disease outbreaks, and forensic and other medical diagnostic applications.

We evaluated the MATCI and 5′ nuclease PCR technique for the ability to detect single-base differences in real-time, using orthopoxviruses and the human complement component (C6) gene as model targets. The 5′ nuclease PCR technique was developed by Holland et al.[6] and refined by Lee et al.[7] The technique has been used for allelic discrimination of the human insulin gene[8] and for specific identification of *Listeria monocytogenes*[9] and orthopoxviruses.[10]

Orthopoxviruses include the variola virus that caused smallpox for centuries. Although the variola virus has not been implicated in human disease since the eradication of smallpox in 1979, other orthopoxviruses, including cowpox (CPV), monkeypox (MPV), and the vaccinia (VAC) subspecies buffalopox, can cause smallpox-like disease in humans.[11,12] Evidence exists that relatively minor changes in the *Orthopoxvirus* genome may be involved in virulence and pathogenicity.[13] Similarly, certain human genetic disorders

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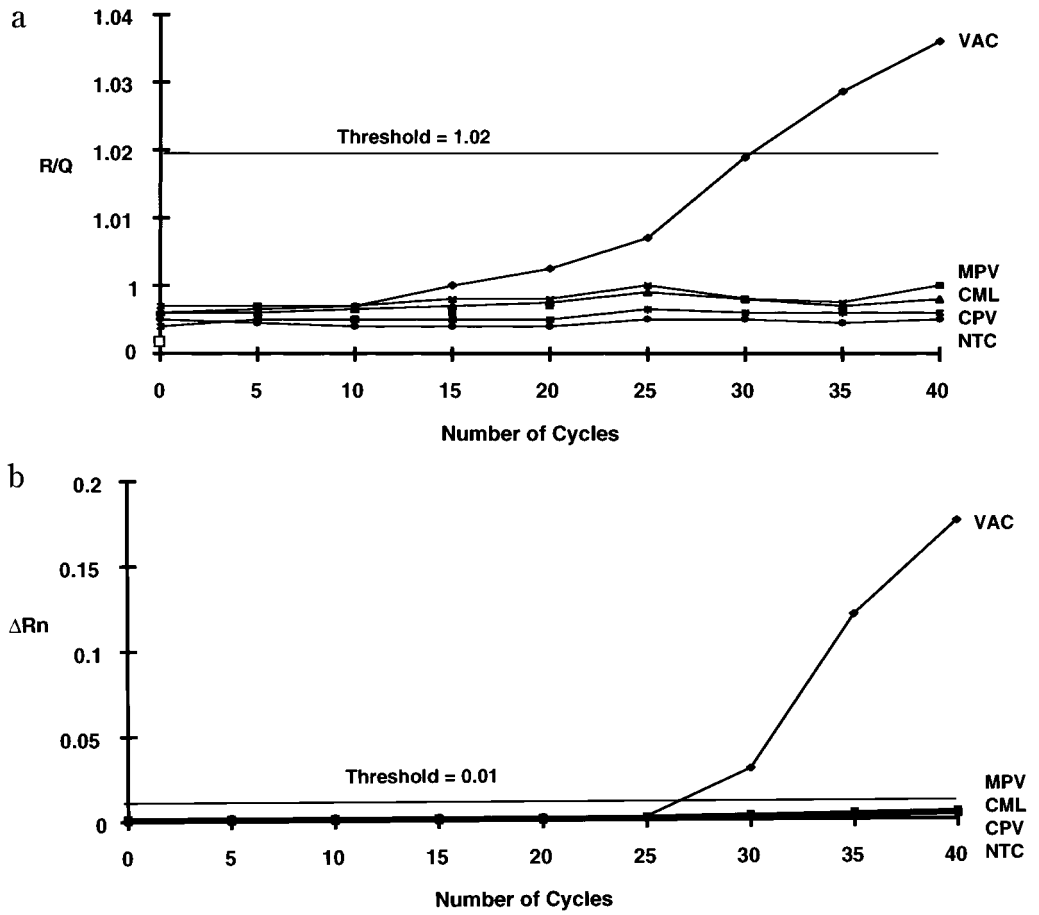

Figure 1. Real-time detection of vaccinia virus DNA using MATCI (a) and ABI Prism 7700 (b). DNA (1 ng) from VAC, CPV, MPV, and CML viruses and 1 ng of uninfected Vero cell DNA were tested with a VAC-specific TaqMan probe.

are caused by minor polymorphic changes. For example, hemochromatosis[14] and Factor V Leiden[15] are caused by a single-point mutation. The nucleotide substitution (C to A) in the second position of codon 98 of the C6 gene serves as a convenient model for point mutation detection in human genomic DNA.[16]

In this report, we demonstrate real-time detection of single-nucleotide polymorphism in orthopoxviruses and the human C6 gene using 5′ nuclease PCR technique and a portable, battery-powered miniature analytical system.

EXPERIMENTAL SECTION

Viruses. Copenhagen VAC, Zaire MPV, Brighton CPV, and Somalia camelpox (CML) viruses were propagated in Vero cell cultures as described by Ropp et al.[17] and Ibrahim et al.[10] Infectious viral titers were determined by plaque assay and were 4.4×10^6, 5.6×10^6, 1.4×10^6, and 1.1×10^6 plaque-forming units (PFU) for VAC, MPV, CML, and CPV viruses, respectively.

Virus and Human DNA Preparation. Virion DNA and virus-infected Vero cell DNA were extracted by the Easy DNA method (Invitrogen, La Jolla, CA). Human blood samples were collected from two volunteers with their consent, and they were informed of the assay according to a human use protocol. The blood was spotted on FTA cards (Fitzco, Minneapolis, MN) and stored at room temperature until needed. The cards were punched into $^1/_{16}$-in.-diameter punches. Each punch was immersed in 200 μL of proprietary purification reagent (provided by the manufacturer) in a plastic tube and incubated at room temperature for 5 min. The solution was discarded by aspiration, and then 200 μL of fresh solution was added and incubated for another 5 min at room temperature. The solution was discarded, and the punch was incubated with 200 μL of TLE buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) twice for 1 min each. The TLE buffer was discarded, 200 μL of 200-proof ethanol was added for 10 s and discarded, and the punch was dried at 95 °C in a heat block for 5−10 min and stored at room temperature until used for PCR.

PCR Primers, Target Sequences, and Fluorogenic Probes. The primers were synthesized by using standard phosphoramidite chemistry with an ABI 394 DNA/RNA synthesizer (Perkin-Elmer Applied Biosystems Division, Foster City, CA). The *Orthopoxvirus* primers were OPXHAU1 (5′-ACCAATACTTTTGTTACTAAT-3′) and OPXHAL1 (5′-CAGCAGTCAATGATTTA-3′).[10] The C6 gene primers were C6U1 (5′-GTCTTGCGTCCCAGTCA-3′) and C6L1 (5′-ATGGAATGCATGGTTGAA-3′). The TaqMan probes were synthesized by Perkin-Elmer and contained 6-carboxyfluorescein (FAM) in the 5′ end and 6-carboxytetramethylrhodamine (TAMRA) and a phosphate in the 3′ end. The vaccinia virus probe

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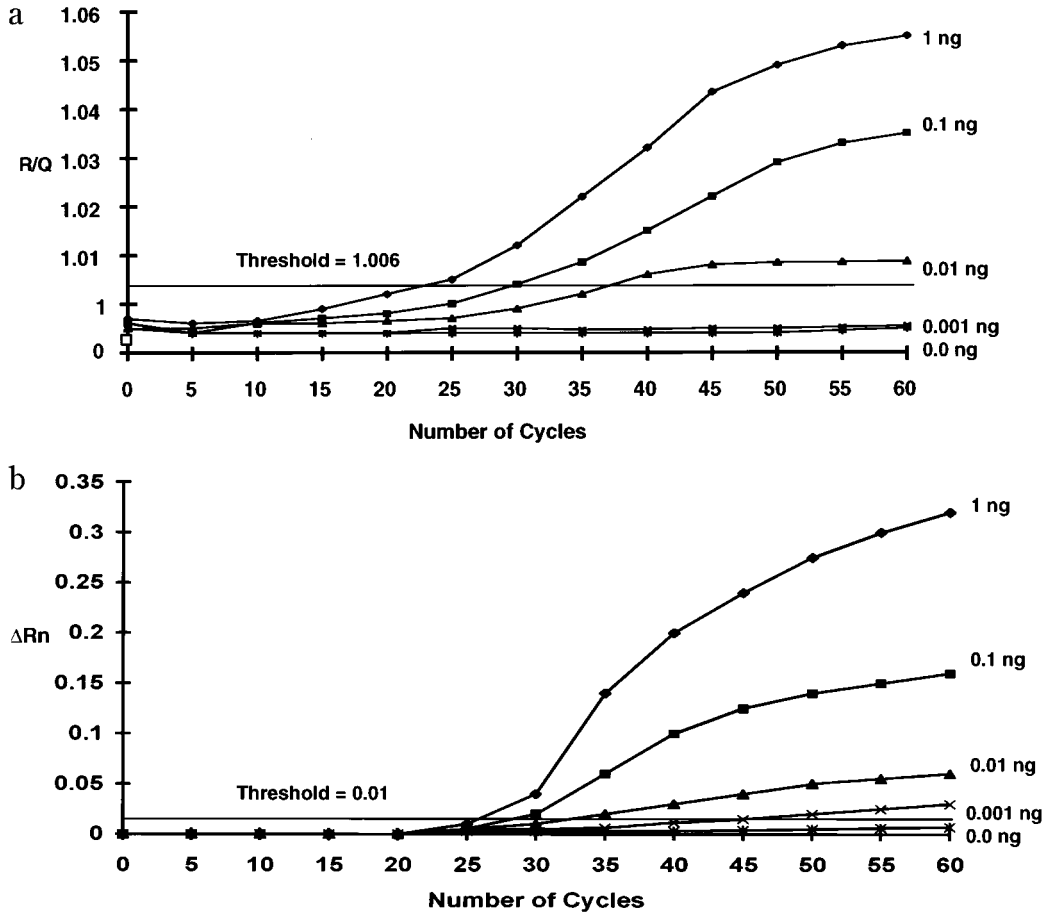

Figure 2. Detection limit for *Orthopxvirus* DNA of MATCI (a) and ABI Prism 7700 (b). Ten-fold serial dilutions of VAC virion DNA (1−1000 pg); 1000 pg of CML, CPV and MPV virion DNA; and 1000 pg of Vero cell DNA were tested with a VAC-specific TaqMan probe.

F-VACHA1 (5′-FAM-TATCATGTA**A**TCGAAATAATAC-TAMRA-3′) was used to discriminate an A/G substitution in orthopoxviruses. The C6 gene probes C6C (5′-FAM-CCATGCACTG**C**GCCTCTG-GTAG-TAMRA-3′) and C6A (5′-FAM-CCATGCACTG**A**GCCTCTG-GTAG-TAMRA-3′) were used to identify C or A polymorphism in human genomic DNA, respectively. The primers and probes were selected with the aid of Oligo 5.0 software (National Biosciences, Plymouth, MN).

5′ Nuclease PCR Assay. The reagents for 5′ nuclease PCR were from Perkin-Elmer. The assays were carried out in 25-μL volumes (50-μL volumes for the ABI Prism 7700). Each assay reaction contained template DNA, PCR buffer (final concentration of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 15 pmol of each primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, 10 pmol of probe (1 pmol for ABI Prism reactions), and 2.5 units of AmpliTaq DNA polymerase. Thermal cycling was performed for 40 or 60 cycles using different thermal cycling profiles as described in the Results and Discussion. Preliminary experiments to optimize primer concentration, $MgCl_2$ concentration, probe concentration, and annealing temperature were performed with PE 9600 (Perkin-Elmer) or PTC 225 (MJ Research, Watertown, MA) thermal cyclers, and the reactions were analyzed by agarose gel electrophoresis and a LS-50B luminescence spectrometer (Perkin-Elmer). Optimum thermal cycling and amplification conditions were adapted to TaqMan assays with the MATCI as described in the Results and Discussion, and the MATCI's single-base discrimination ability and its detection limit were compared with an ABI Prism 7700 instrument. Data acquisition and analysis were carried out with Igor software (Wavemetrics, Lake Oswego, OR) for the MATCI and with SDS 1.0.5d30 software (a beta version, Perkin-Elmer Applied Biosystems Division) for the ABI Prism 7700. The ABI Prism 7700 system uses an algorithm which calculates the mean R/Q ratio for cycles 3−15 and subtracts this value from each observation, and the results are denoted as ΔRn. The MATCI plots the observed R/Q ratios without adjustment. The threshold values for both instruments were calculated by multiplying the mean R/Q values of cycles 3−15 by 10 times the standard deviation.

Table 1. Mean Threshold Cycle and Time of the MATCI and ABI Prism 7700 Obtained with 1000, 100, 10, and 1 pg of Vac DNA Templates

	1000 pg	100 pg	10 pg	1 pg
MATCI	25.1 ± 2.2 (23 min)	34.1 ± 1.8 (31 min)	38.4 ± 0.5 (35 min)	not detected
ABI	26.7 ± 0.8 (65 min)	29.8 ± 0.4 (73 min)	32.6 ± 0.9 (80 min)	36.2 ± 1.2 (89 min)

RESULTS AND DISCUSSION

Our goal was to evaluate the utility of the MATCI and the 5′ nuclease PCR technique for real-time detection of single-nucleotide polymorphism. We used orthopoxviruses and the human C6 gene as model targets. PCR amplification of DNA from four *Orthopox-*

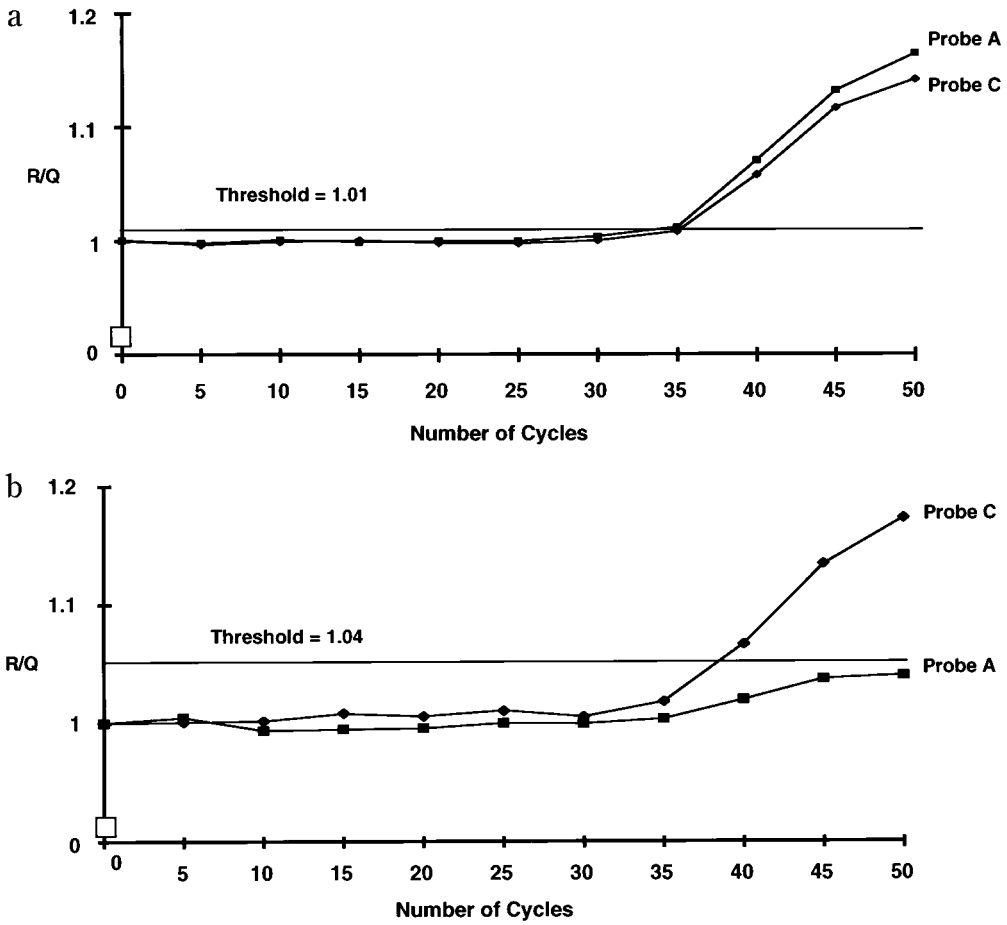

Figure 3. Single-nucleotide discrimination in heterozygous (a) and homozygous (b) individuals with C6A (probe A) and C6C (probe C). DNA was extracted with FTA cards, and the assay was performed on the MATCI as described in the Experimental Section.

virus species was achieved by using a consensus pair of primers designed from conserved regions of the hemagglutinin protein gene. VAC virus identification was achieved by a fluorogenic probe that contained A/G mismatch with CML, CPV, and MPV viruses. From a previous study,[10] using the 5′ nuclease PCR technique and an LS50-B luminescence spectrometer (Perkin-Elmer), we found that specific detection of VAC DNA could be achieved with F-VACHA1 probe concentrations of 0.65−20 pmol/reaction. Additional experiments with the ABI Prism 7700 indicated that 1.5 mM $MgCl_2$, 15 pmol of each primer, 1 pmol of probe, and 52 °C annealing temperature produced amplicons of the expected sizes (266−281 bp) from all four orthopoxviruses, but only VAC DNA was detected specifically and reproducibly with the F-VACHA1 probe. These conditions were used as general guidelines for adapting the assay to the MATCI. For the MATCI, we used the following thermal cycling profile: one cycle at 94 °C for 30 s, followed by 40 cycles of denaturing at 94 °C for 2 s, annealing at 50 °C for 10 s, and extension at 72 °C for 10 s.

In a blind experiment using coded samples that contained 1 ng of purified genomic DNA from each virus and 1 ng of Vero cell DNA, the MATCI correctly identified the VAC DNA sample after 30 cycles in approximately 30 min (Figure 1A). The R/Q ratio observed with the homologous VAC DNA was 1.04, clearly above the threshold value (1.02), while the R/Q values of heterologous DNAs from the other *Orthopoxvirus* species and Vero cell DNA (0.99−1.00) were lower than the threshold value. As a standard for comparison, the ABI Prism 7700 instrument was operated under the following conditions: one cycle at 94 °C for 30 s, followed by 40 cycles of denaturing at 94 °C for 15 s, annealing at 52 °C for 15 s, and extension at 72 °C for 30. As expected, the ABI Prism 7700 confirmed the results obtained with the MATCI, distinguishing the homologous DNA ($\Delta Rn = 0.175$) from the heterologous DNAs and the Vero cell DNA, whose ΔRn values were at or below the threshold (0.01) (Figure 1B). In another blind experiment, the assays were performed as described in Figure 1, except that 3 ng of DNA extracted from virus-infected Vero cells was used as template. Both the MATCI and ABI Prism 7700 correctly identified VAC DNA in the background of cellular DNA (data not shown).

Because the MATCI's thermal cycling speed (1.1 cycles/min) is approximately 2.8 times faster than that of the ABI Prism 7700 (0.4 cycles/min) for the temperature range used in these experiments, we reasoned that the annealing and extension times of the MATCI should not be lower than one-third of those used for ABI, i.e., 5 s for the annealing temperature and 10 s for the extension temperature. However, we used 10 s at 50 °C instead of 5 s at 52 °C for the annealing temperature in order to compensate for any differences in thermal response and uniformity, which are often associated with integrating heaters into the bulk of silicon.[5] Faster cycling intervals (<10 s) for the annealing and extension segments resulted in poor detection.

The detection limit of the MATCI was evaluated with 10-fold serial dilutions of VAC DNA, ranging from 1 to 1000 pg. The assays were performed as described in Figure 1, except that the

number of cycles was increased to 60. As shown in Figure 2A, the MATCI was capable of detecting 10 pg (about 370 PFU) of VAC DNA compared with a minimum detection of 1 pg of VAC DNA (about 37 PFU) on the ABI Prism 7700. The mean C_t values were determined from three experiments for 1000, 100, 10, and 1 pg of DNA (Table 1). For 1000, 100, and 10 pg of VAC DNA, the C_t values were 25.1, 34.1, and 38.4 and were obtained within about 23, 31, and 35 min, respectively. The 1 pg of VAC DNA could not be detected, even after 60 cycles (Figure 2A). The C_t values of the same DNA concentrations were determined with the ABI Prism 7700 using both standard and rapid thermal cycling parameters. The standard cycling profile (15 s at 94 °C, 15 s at 52 °C, 30 s at 72 °C) resulted in C_t values of 26.7, 29.8, 32.6, and 36.2 and were obtained within 65, 73, 80, and 89 min, respectively (Figure 2B). The rapid cycling profile (2 s at 94 °C, 10 s at 50 °C, 10 s at 72 °C) resulted in C_t values of 34.2, 35.1, 51.9, and 60, which were reached within 63, 65, 96, and 111 min, respectively (data not shown). Apparently, the rapid cycling profile only slightly enhanced the detection speed of the ABI Prism 7700 but adversely affected its detection limit. The detection limit dropped by 1 order of magnitude over 60 cycles. With conventional 40−50-cycle assays, the detection limit dropped by 2 orders of magnitude when using the rapid cycling profile. It is noteworthy that, with the ABI Prism 7700, the C_t value and time were determined retrospectively after the reactions had been completed (approximately 111 min).

The potential for using the MATCI to detect point (C/A) mutations in the human C6 gene was evaluated. Human genomic DNA was characterized with probes C6A and C6C under the same cycling conditions as described above. In addition, we used a rapid method to prepare blood samples that required no centrifugation or long incubations and enabled sample processing to be completed in 20 min. As shown in Figure 3, both the A and C alleles were detected for the first individual, but only the C allele was detected for the second individual, indicating that individual 1 was heterozygous and individual 2 was homozygous with respect to the genotype at this position. The results for individual 2 clearly demonstrated single-nucleotide discrimination. The initial increase in the relative fluorescence emission ($R/Q = 1.05$) was detected after 34 min, reaching a peak R/Q value of 1.17 after 50 min. The threshold value observed with the homologous reactions was approximately 1.01 and was higher (1.04) in the heterologous reaction. This slight increase in the R/Q value of the heterologous reaction is not unusual and may be attributed to partial hybridization of probe A. However, as shown in Figure 3B, probe C clearly identified the C/A mismatch.

CONCLUSIONS

This report demonstrates for the first time that real-time discrimination of single-base polymorphisms can be achieved by using a portable battery-powered microchip instrument. The entire instrument is housed in a briefcase and can be hand-carried.[5] The use of rapid sample processing, such as the method used herein to prepare human genomic DNA from blood in 20 min, indicates that sensitive and specific molecular diagnosis can be achieved in 1 h in a portable, battery-powered device. The assay's and instrument's precision and reproducibility were demonstrated by the ability to distinguish single-base mismatches in two different targets, representing viral and human DNAs. The standard deviations of the C_t values obtained with orthopoxvirus experiments varied from 0.5 to 2.2, with coefficient of variations from 1 to 9%. However, the detection limit of the MATCI was 10-fold lower than that of the ABI Prism 7700 system. This is because the couple-charged device (CCD) optical system used with the ABI Prism 7700 is more sensitive than the MATCI's simple photodiode optical system. Further improvements to this device will include multiple reaction chambers, integration of advanced microprocessor control, parallel channels to allow multisample and multiprobe detection, and enhanced optical detection system.

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