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Rapid and Sensitive Human-Specific DNA Quantitation Using a Microfluidic Amplification Module at the Point-of-Care

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***Abstract*—A rapid microfluidic human-specific DNA quantitation assay module was developed for chip-based amplification of the human TH01 and Alu loci in the presence of PicoGreen. The method makes use of the thermal cycler and 488 nm Solid State laser-based optical train that are components of the fully-integrated, sample-in to results out, ANDE Rapid Nucleic Acid Analysis system. The assay was effective in quantitating human DNA from a variety of sample types, including blood, buccal, and forensic touch samples mixed with varying amounts of non-human DNA. The 28-cycle TH01 and 10-cycle Alu reactions were completed in 18 minutes and 7 minutes, respectively. The observed limit of detection (LOD) of the assay is approximately 0.3 ng, and the flexibility of assay design allows an LOD of as little as 0.005 femtograms.**

***Clinical Relevance*—We have developed a fully-integrated, sample-in to results-out, Rapid Nucleic Acid Analysis system that characterizes nucleic acid fragments (whether generated by PCR, rt-PCR, sequencing, or SNP reactions) by electrophoresis in plastic microfluidic channels. Here we describe the development, characterization, and validation of the microfluidic quantitation module. The quantitation module is the first that can be incorporated into integrated microfluidic workflows for the analysis of highly-multiplexed clinical diagnostic assays interrogating hundreds of genomic targets in a single sample. In particular, the use of a microfluidic quantitation module allows reaction volumes, thermal cycling conditions, and electrophoretic injection protocols to be determined based on nucleic acid content during and throughout fully-automated processing—dramatically enhancing the power of the fully-automated diagnostic system.**

I. Introduction

Point-of-care (POC) nucleic acid analysis tests are typically limited by the minimal DNA and RNA characterization that can be performed in the field. The ANDE Rapid Nucleic Acid Analysis system[1,2] is capable of performing a wide range of molecular biology reactions, including endpoint PCR, rt-PCR, sequencing, and SNP reactions and is also capable of identifying thousands of individual amplified peaks in a single channel, enabling highly-multiplexed reactions to be analyzed. The microfluidic system is designed for POC or laboratory operation and is ruggedized to MIL-STD-810G for shock and vibration, has low power consumption, and is CE marked under the Low Voltage Directive 73/23/EEC. The ANDE System has been utilized in field-based, high profile military operations, in thousands of criminal investigations around the world, and for disaster victim identification in the 2018 California wildfires[3], the 2019 Conception dive boat fire, the 2020 Calabasas helicopter crash, and Hurricane Ian. It is currently being used extensively in the Ukraine to identify war victims. Finally, the system has been used to perform highly-multiplexed clinical diagnostic assays, for example demonstrating in clinical trials sensitive and specific detection of SARS-CoV-2 and identification of all major strain types in less than two hours.

Accurate nucleic acid quantitation is critical in the diagnosis and evaluation of certain infectious diseases and forms of cancer and in the evaluation of forensic casework samples. More broadly, nucleic acid quantitation is critical to ensure optimal conditions for a wide variety of analytical techniques. To the extent that microfluidic systems in general and fully integrated chip-based analytic systems in particular are designed to perform such assays, the incorporation of nucleic acid quantitation into integrated biochip workflow would have widespread clinical diagnostic application. Furthermore, human-specific DNA quantitation is an essential step in the generation and accurate interpretation of short tandem repeat (STR) profiles for forensic identification. The quantity of DNA present in a forensic sample can vary over several orders of magnitude, but the range of input DNA required to generate STR profiles with balanced peak heights is quite narrow, generally between 0.5 and 2 ng of human DNA[4,5,6]. When insufficient template is used for the multiplex PCR, artifacts including allele peak height imbalance, allele drop-out, and electropherogram irreproducibility can occur. When excessive template is applied, artifacts include increased stutter, non-specific bands, incomplete non-template addition, and pull-up peaks resulting from incomplete color separation. These artifacts lead to difficulties in profile interpretation[7,8].

Many amplification-based methods have been developed as alternatives to hybridization-based approaches to human DNA quantitation. These procedures can be used to measure the effective amount of amplifiable human DNA, (a matter of particular importance if the sample contains inhibitors or is highly degraded). In real-time polymerase chain reaction (RT-PCR) assays, amplification progress is monitored after each cycle as a fluorescent output signal from a specific probe. RT-PCR quantitation targets include Alu[9,10], X and Y chromosomal[11], and TH01 and CSF1PO[12] sequences. Chemistries applied for real time PCR detection include SYBR Green[13], fluorogenic probes (e.g., TaqMan probes[14]), and molecular beacons[15]. Based on the specific system employed, the dynamic range of RT-PCR quantitation typically spans only 3 orders of magnitude[16]. Limitations of

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RT-PCR include cost and the two to three hours required to conduct the assay. End-point PCR assays use similar targets, exhibit similar dynamic ranges, and are similarly time-consuming.

Microfluidics offers the potential to dramatically accelerate nucleic acid quantitation and to enable quantitation to be performed at the POC as a module in a fully-integrated, sample-in to results-out diagnostic system. In this study, we report the development of an amplification-based rapid human-specific quantitation assay that can be applied in both clinical and forensic settings. Critically, the quantification system utilizes the existing consumable and instrument, meaning that it can be readily incorporated as a process in fully-automated nucleic acid analysis.

II. Methods

Sample Sourcing and Preparation. Genomic DNAs were purified from blood samples from cattle (*Bos taurus*), chicken (*Gallus domesticus*), horse (*Equus caballus*), mouse (*Mus musculus*), and rhesus monkey (*Macaca mulatta*). Genomic DNA was also purified from bacterial cell pellets (*Bacillus megaterium, Bacillus cereus, and Bacillus subtilis*) and Canine (*Canis familiaris*) buccal swabs, and purchased from ATCC (Manassas, Virginia) for yeast.

Clinical and mock forensic casework samples were prepared from human buccal, palm, and touch swabs, and fresh human whole blood samples. Touch samples were prepared by using a pre-wet swab on a ceramic tile that was handled several times by a single donor. Liquid blood samples were prepared by pipetting 100 µl of whole blood onto a ceramic tile and collecting with cotton swabs. Dried blood samples were pipetted onto a tile and allowed to dry overnight at room temperature. Palm samples were collected by rubbing a pre-wetted swab head (with sterile water) on the palm of the subject. DNA purification from pelleted bacterial cells was performed as previously described[17]. DNA purification was performed using the QIAmp Kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

PCR probes and microfluidic amplification. Two loci were targeted in the end-point PCR assays: 1) the highly repetitive human Alu element (GenBank M57427.1/NC_000006.11) and 2) the single-copy human tyrosine hydroxylase gene, TH01 (GenBank D00269.2/NC_000011.9). An injection molded 16-lane PCR biochip (7 µL reaction mix volume per lane) with optically clear reaction chambers[18] was fabricated to enable microfluidic amplification and interrogation of the reaction products within the fully-integrated instrument. Microfluidic amplification was performed as previously described[18].

Microfluidic Electrophoresis. Separation of the DNA fragments takes place within a 16-sample plastic microfluidic biochip (Fig. 1) with channels loaded with a polyacrylamide-based sieving matrix. Samples for electrophoresis contain 2.7 µL of PCR product, 0.3 µL of GS500 sizing standard, and 10 µL of formamide.

Laser-based Detection. The fully integrated instrument[1] enables microfluidic analysis of nucleic acids (both RNA and DNA) including quantitation, amplicon sizing, indel detection, nucleic acid sequencing, and SNP typing. The instrument is ruggedized for field forward applications, is CE marked under the Low Voltage Directive 73/23/EEC, and has low power consumption. For the TH01 assay, the laser was set to output 20 mW, and an OD2 neutral density filter was inserted at the output aperture of the laser to attenuate the excitation power to 0.2 mW. For the Alu assay, the laser was set to output 200 mW, and an OD4 neutral density filter was applied to attenuate the excitation power to 0.02 mW. For both assays, the gain of blue PMT was set to 30% of full scale, gain of red, yellow, and green set to 0, and the scan rate was set to 5 Hz. Time-resolved signal was collected, and the signal strength 0.2 s from the initiation of excitation was recorded for all the data sets.

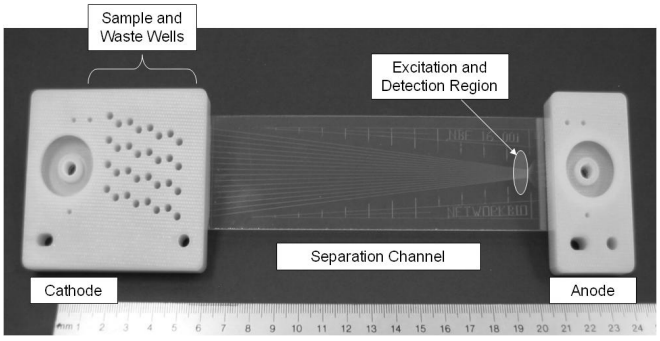

Figure 1. 16-sample microfluidic chip for electrophoretic separation and detection. The anode and cathode have been modified (from the module in the fully-integrated chip) to enable manual sample loading.

III. Results and Discussion

Determination of excitation and detection parameters for quantitation. A master mix of DNA and PicoGreen intercalating dye was prepared and loaded into the quantitation biochip and used for optimization of the excitation and detection parameters. Fig. 2a show that signal strength increases with increasing PMT gain, and Fig. 2b illustrates that signal-to-noise ratio approaches saturation at approximately 22 and at 30% PMT gain. The effect of laser excitation on signal strength is shown in Fig. 2c. Data indicate that incorporation of neutral density optical filters to reduce the excitation power at the sample generate linear response in signal strength with increase in laser power. Fig. 2d shows that high levels of excitation rapidly degrade the fluorophores by photobleaching, resulting in the irreversible loss of fluorescence activity in the sample. Taken together, these

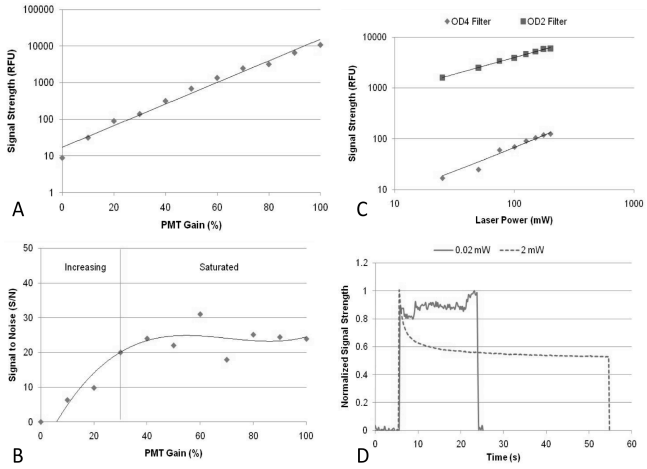

Figure 2. (a) Signal strength as a function of PMT gain. Fluorescence signal increases with increasing PMT gain from 10 relative fluorescence units (RFU) to 10,000 RFU, demonstrating the wide dynamic range of the optical detection system. **(b)** Signal-to-Noise ratio as a function of PMT gain. Noise contribution associated with the detection electronics is negligible compared with the background fluorescence of the chip and saturates at approximately 22 for PMT gains of 30% and higher. **(c)** Signal strength as a function excitation power. The two datasets were generated using neutral density optical filters, with optical densities of 4 and 2, to reduce the excitation power at the sample by 10000 and 100 times that of the laser output, respectively. Incorporation of these filters shows no effect on linearity of detected signal response with laser power. **(d)** Photobleaching of fluorescent dye with excitation power. A moderate excitation power (0.02 mW) incident on the sample generated relatively constant signal strength over time, consistent with that of the fluorophores that are excited within the sample. A high level of incident excitation (2 mW) exhibited an exponential decay of the signal to a level that is consistent with that of the background.

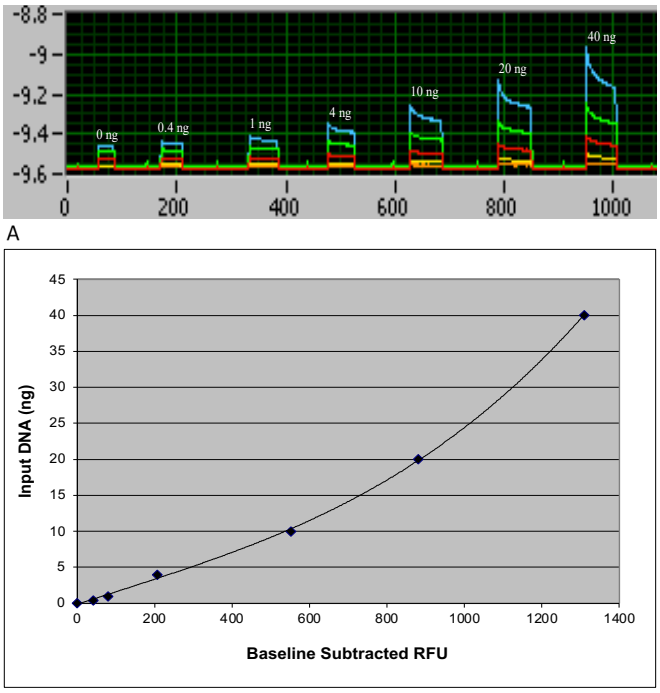

Figure 3. (a) Top panel. Raw data output signal from laser excitation for each input template concentration versus lane displacement. Seven lanes of the chip, each containing 0, 0.4, 1, 4, 10, 20, or 40 ng of 9947A into PCR using TH01 primers were studied. Increase in signal is observed with increase in template DNA. **(b)** Bottom panel**.** Standard curve for TH01 quantitation primers with 28 PCR cycles. A plot of input DNA versus baseline subtracted signal strength gave a polynomial third order fit with R^2=0.999 from which signal strengths of unknown samples were extrapolated to determine presence and quantity of human DNA.

results suggest that the optimal sample excitation power for quantitation is achieved—with respect to both signal-to-noise and dynamic range--when set to between 0.02 to 0.2mW and PMT gain is set to 30% of full scale.

Generation of standard curve and evaluation of assay specificity using TH01 primers. As an initial evaluation of the TH01 and Alu primer pairs, rapid biochip amplification of 10 and 40 ng human genomic DNA samples was performed using the rapid microfluidic thermal cycler[18]. 28 cycles were completed in less than 18 minutes, and fragment mobilities were consistent with the expected amplicon sizes of 186 and 123 bases, respectively.

Human genomic DNA (9947A) was amplified microfluidically using the TH01 primers to generate a standard curve. Fig. 3a shows the raw output from the instrument as a function of lane displacement. An increase in fluorescence signal was observed with increasing input DNA as plotted in Fig. 3b. This standard curve is used to extrapolate values of unknown human DNA in purified samples.

Human genomic DNA and non-human genomic DNA (bacterial, yeast, or canine) were then mixed to evaluate the specificity of the assay. The quantity of DNA in a given sample was determined by fitting the observed signal strengths (RFU) to the standard curve. DNA in the samples was also quantified by UV absorbance using a Nanodrop spectrophotometer for comparison. For the non-human samples, no DNA was detected in the microfluidic TH01 amplification assay, whereas DNA was detected in the UV absorbance assay. For mixed samples (human and non-human), the microfluidic amplification assay detected only the human specific component of the DNA in the sample, whereas the UV absorbance assay reported the total DNA present within the sample. Taken together, these results (Table 1) show that the microfluidic amplification assay is specific for human DNA.

Table 1. Quantitation of DNA in background, spiked, and mock forensic samples by microfluidic amplification using TH01 primers. Two replicates measured using the well-characterized UV absorbance technique were included as the comparator. Values are taken from the average of n replicates ± 1 STD.

Samples Tested	[DNA] Microfluidic Amplification	[DNA] UV Absorbance
Non-human Samples Only		
Bacillus cereus	0 ng (n=2)	9.5 ± 0.7 ng (n=2)
Saccharomyces cerevisiae	0 ng (n=2)	16.0 ± 1.0 ng (n=2)
Canis familiaris	0 ng (n=2)	9.5 ± 0.5 ng (n=2)
Non-human Samples Spiked with 10 ng Human DNA		
Bacillus cereus + 9947A DNA	11.0 ± 1.0 ng (n=6)	19.5 ± 0.7 ng (n=2)
Saccharomyces cerevisiae + 9947A DNA	10.8 ± 0.9 ng (n=4)	26.0 ± 1.0 ng (n=2)
Canis familiaris + 9947A DNA	10.7 ± 2.0 ng (n=2)	19.5 ± 0.5 ng (n=2)
Human DNA Samples		
Human Buccal Swab DNA	10.5 ± 2.3 ng (n=10)	9.8 ± 0.8 ng (n=2)
Human Whole Blood DNA	37.8 ± 4.0 ng (n=10)	39.0 ± 1.0 ng (n=2)

Improved LOD using Alu primers and assay repeatability. A 15-cycle microfluidic amplification of 9947 human genomic DNA using Alu primers was performed in approximately 10 minutes. Data was recorded and analyzed as in the TH01 reaction, and as expected, increase in fluorescence signal with increasing input DNA was observed (Fig. 4a). Photobleaching was minimized by placing an OD4 filter with laser power set to 200mW. This resulted in an effective laser power that was 10% of that used in the TH01 assay. At reduced laser power, a similar curve was noted as compared to the TH01 experiment, consistent with the multi-copy nature of the Alu target. The data suggested that further reduction in cycle number during amplification is feasible. To test this possibility, 3 sets of samples were quantified; each set with input template DNA of 1, 5, 10 and 30 ng and amplified using 10 cycles in 7 minutes. Fig. 4b shows that signal strength increases directly with input DNA.

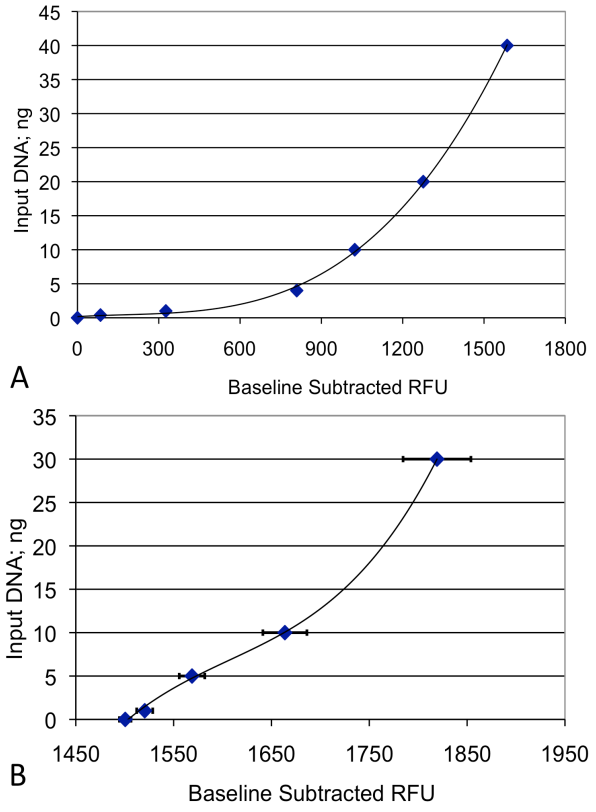

Figure. 4 (a) Plot of input DNA versus baseline subtracted signal strength with 15-cycle Alu primer amplification. **(b)** Plot of input DNA versus baseline subtracted signal strength with 10-cycle Alu primer amplification. This standard curve (R^2=0.999) was generated from average of 15 measurements (5 replicates for each of the 3 data sets) and used to quantify human DNA content in samples. Error bars represent one STD.

For both TH01 and Alu assays, with filters in place, the LOD is approximately 0.3 ng. The assay design is flexible: with laser detection, only approximately 0.353 nL of the total volume in the chip was actually excited and detected based on the laser excitation beam diameter of 30 µm and a 0.5 mm chamber depth. Accordingly, in this configuration, for a 1 ng DNA input into a 7 µL PCR chip reaction, the theoretical limit of detection (LOD) of the system is 0.005 picogram. The minimum laser power setting that the instrument accepts is 20 mW and therefore, an OD4 filter with laser power set to 200mW is an OD3-equivalent laser filter. Without placement of this filter, the resulting theoretical LOD would be 0.005 femtogram. This LOD (with a significant reduction in cycle number) is an order of magnitude better than the use of TH01 probes. In the unlikely event that LODs of less than 0.005 femtograms are required, PCR product volume, amplification conditions including cycle number, primer sequences and concentrations, and optical parameters can be modified as desired.

Species-mixing assay – Human DNA titration with non-human DNA. To assess the specificity of the Alu-based quantitation assay in the presence of varying levels of non-human DNAs, samples were prepared by varying the ratio of human to canine DNA (0%, 25%, 50%, 75% and 100%) while keeping the total DNA of the samples constant, that is, 30 ng. Each sample was quantified (n=10) using the 10-cycle Alu-based microfluidic amplification assay described above. Figure 5a shows that signal strength increases with increasing human DNA content in the mixture. Figure 5b presents the same data, indicating that the quantity of human DNA as determined by the assay closely correlates with the known quantity of human DNA present in each mixture. Similarly, in the presence of varying levels of *Saccharomyces cerevisiae* DNA mixed with human DNA, signal strength increases with increasing human DNA content in the mixture. The measured quantity of human DNA closely correlates with the input human DNA levels (Figs. 5c and 5d).

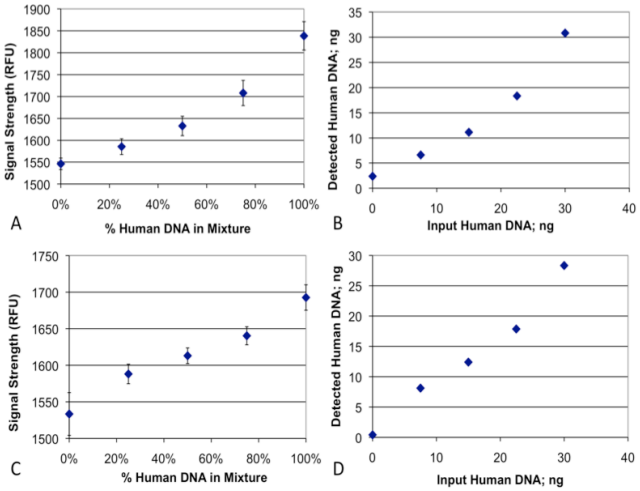

Figure 5 (a) Signal strength plot analysis from quantitation of mixed samples consisting of human and canine DNA. Error bars represent one STD from 10 replicate measurements. **(b)** Correlation plot of detected and input human DNA levels for mixed human-canine samples. Input human and canine DNA concentrations were determined by absorbance method. **(c)** Signal strength plot analysis from quantitation of mixed samples consisting of human and yeast DNA. Error bars represent one STD from 10 replicate measurements. **(d)** Correlation plot of detected and input human DNA levels for mixed human-yeast samples. Input yeast DNA concentrations were determined by absorbance.

Results of the human/canine and human/yeast mixture assays were used to determine the appropriate quantity of

human DNA to be incorporated in human STR amplification reactions. Mixed samples containing 0, 25, 50, 75, and 100% human DNA were subjected to microfluidic quantitation, and based on the assay result, a volume expected to contain 1 ng of human genomic DNA was subjected to STR analysis. Each STR reaction was performed in approximately 7 μL and rapid microfluidic amplification using 28 cycles[30] was performed. PCR reaction products were separated and detected microfluidically. All 27 expected amplicons were observed for all human/canine and human/yeast mixture samples, and the signal strengths of the human STR amplicons were similar regardless of the original concentration of human DNA in the mixtures. These data show that the microfluidic amplification assay provides accurate human DNA quantitation for use in multiplexed amplification reactions.

IV. Conclusion

This work combines rapid microfluidic amplification, a laser-based excitation and fluorescence detection system developed for microfluidic electrophoresis, and Alu and TH01 probes developed for human-specific quantitation of forensic samples. Both TH01 and Alu primer pairs allow effective human-specific DNA quantitation in the microfluidic assay. The Alu amplification has the advantage of approximately several hundred thousand-fold more targets per human genome as compared to TH01 amplification which, if amplification were 100% efficient, would translate to a reduction of approximately 9 amplification cycles compared to that of the TH01 primer pairs. The 10-fold reduced laser power (secondary to the OD4 filter) utilized in the Alu experiments represents approximately 3 additional required cycles. Taken together, under these conditions, the Alu reaction should require 12 cycles to generate signals comparable to those of the 28-cycle TH01 reaction. The observed results at 10 cycles of Alu amplification are consistent with these theoretical considerations.

The use of microfluidic cycling and Alu primers allowed quantitation to be completed accurately and within 7 minutes, significantly faster than conventional approaches and well-suited for rapid generation of diagnostic results at the POC. The flexibility of the laser-based excitation and detection system allows an LOD of 0.005 femtograms of DNA, much less than would be required for single cell detection and that can be achieved using conventional laboratory instruments. The advantages of the microfluidic approach over conventional assays include substantial reduction in both sample volume and amplification time. Plate readers are relatively crude detection tools based on their bulk and optical excitation properties and not particularly sensitive. Commercial quantitation kits based on hybridization and fluorescence detection, although specific for human DNA, are also labor-intensive and time-consuming. Real-time PCR is a sensitive tool for human-specific DNA quantitation but is cost-prohibitive and time-consuming.

The availability of a fully-integrated instrument that combines nucleic purification, reactions including reverse transcription, PCR amplification, SNP analysis, and sequencing, and separation and detection into a single, easy to operate instrument represents a substantial advance in clinical diagnostic and forensic DNA analysis. Human-specific DNA quantitation is required for certain clinical and forensic applications. The quantity of DNA in a blood sample may vary by more than three orders of magnitude, particularly in cases of infectious disease and hematologic malignancies. If total DNA is purified in a chip to perform an amplification or sequencing reaction to identify a pathogen, the use of a fixed volume of purified DNA may well lead to samples that fall outside of the dynamic range of the assay (e.g., excessive DNA concentration itself inhibits PCR reactions). Microfluidic quantitation of human or nucleic acid following purification allows an appropriate amount of purified material to be processed. Many other clinical and environmental sample types have wide ranges of DNA content and represent candidates for microfluidic quantitation using the approach resented herein. Similarly, these methods can be applied to the quantitation of pathogen, livestock, agricultural, industrial, and other nucleic acid-containing samples.

An additional advantage of the microfluidic quantification approach is that it makes use of the existing modules of the commercially-available ANDE Rapid DNA instrument[1,2]. In particular, the 16-lane PCR biochip with optically clear reaction chambers utilized in the experiments presented here has essentially the same dimensions and construction as that in the fully-integrated ANDE instrument. Accordingly, the addition of a rapid, highly-sensitive quantification step to the sample-in to results-out system leads to enhanced capabilities with minimal increase in per sample testing cost. With the quantity of human DNA present in a given sample available during a fully-integrated run, reaction volumes and cycle number can be automatically adjusted to optimize results—such in process modifications dramatically expand the complexity of reactions possible in sample-in to results-out microfluidic systems.

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