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Rheonix CARD® Technology: An Innovative and Fully Automated Molecular Diagnostic Device

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Abstract

A versatile microfluidic platform for the evolving molecular diagnostics industry is described. It incorporates low cost Rheonix CARD® (Chemistry and Reagent Device) technology to analyze a variety of clinical specimens. A patented lamination process incorporates all pumps, valves, microchannels and reaction compartments into an inexpensive disposable plastic device. Once an untreated clinical specimen is introduced, all assay steps, including cell lysis, nucleic acid purification, multiplex PCR, and end-point analysis, are automatically performed. Three distinct CARD assays are described which utilize either a low density microarray for multiplex detection of amplicons or an integrated primer extension assay to detect single nucleotide polymorphisms of interest. The STI (Sexually Transmitted Infections) CARD® is able to simultaneously detect four sexually transmitted infectious agents (*N. gonorrhoeae*, *C. trachomatis*, *T. pallidum* and *T. vaginalis*). Human C33A cervical epithelial cells were spiked with different levels of genomic DNA from the four species of interest, singly or in combination, and applied to the CARD device. Using multiplex PCR amplification of the targets followed by microarray detection, the CARD device was able to correctly detect a minimum of 10 copies of each of the four pathogens. The HPV (Human Papillomavirus) CARD® was able to detect and distinguish 20 different clinically relevant HPV types using cloned HPV DNA. In addition, the HPV CARD could identify HPV types in vaginal specimens previously demonstrated to contain high or low risk HPV using a currently commercially available testing method. Finally, the detection of specific single nucleotide polymorphisms (SNP) associated with warfarin dosing sensitivity was achieved on the Warfarin Genotyping CARD® by analyzing human buccal swabs. Once multiplex PCR was completed, the SNPs were detected using a primer extension assay.

INTRODUCTION

The use of molecular diagnostics has expanded greatly since its inception in the early 1980s, particularly as a means to permit the detection of slow growing or fastidious bacteria responsible for infectious diseases (1–10). The detection of viral pathogens, including viral load testing (11–17) has also been significantly improved by molecular diagnostics. As more data have become available regarding the human genome, the use of molecular diagnostics in pharmacogenomic, companion diagnostics, and other personalized medicine applications continues to gain momentum (18–24). Despite its power and versatility, however, the need for highly trained personnel and expensive capital equipment has restricted the use of

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molecular diagnostics to specialized laboratories or central labs suitably equipped and staffed.

While many effective “point-of-care” (POC) diagnostics have been developed that rely upon immunological assays in a lateral flow assay format (e.g., pregnancy tests), the ability to perform the more complex molecular assays has not yet been fully achieved in an easy-to-use and inexpensive POC format. Currently, “bench top” molecular assays require significant effort by highly trained personnel to prepare the samples for analysis, starting with raw clinical specimens. Subsequently, the gene amplification and detection steps also require significant skill and expensive equipment. Moreover, while lateral flow POC assays frequently rely upon subjective interpretation of color intensity on test strips, results from more sophisticated molecular assays would be more meaningful if unambiguous and objective digital results can be provided. If all of these processes could be integrated in a seamless, fully automated manner, individuals of varying skill level could perform a range of POC molecular assays and achieve objective, clear-cut interpretation of results in an economical assay format.

The unique challenges posed by the molecular POC markets have led to the introduction of several “sample-to-results” platforms, but most still require either separate “sample preparation” steps and/or equipment or considerable “pre-preparation” of the sample prior to introduction into the system to achieve gene amplification and detection. In order to achieve true “sample-to-results” simplicity, we have expended considerable effort to develop a platform that integrates all required sample preparation, amplification, and detection steps into a single, inexpensive disposable plastic device capable of achieving fully automated molecular diagnostic testing. The design of the Rheonix CARD[®] (Chemistry And Reagent Device) platform requires only the introduction of an untreated specimen by the user, with all other subsequent steps performed automatically. The Rheonix CARD[®] platform’s low cost of both capital equipment and disposables, as well as the absence of any “hands on” efforts, will help make molecular diagnostics a reality in the entire spectrum of critical and point-of-care testing.

MATERIALS AND METHODS

Rheonix CARD[®] device

For all experiments, the Rheonix CARD[®] device was produced using a proprietary weak solvent lamination process previously described (25). Briefly, a 1.0 mm thick layer of polystyrene, containing discontinuous channels, was laminated to a 38 μ m layer of flexible polystyrene. To perform any of the assays described, the laminated CARD can be mounted onto the manifold of either the Rheonix EncompassMDx[™] workstation, capable of performing up to 24 simultaneous assays or Rheonix Portable CARD Controller, capable of performing up to 4 simultaneous assays. Both manifold assemblies contain a set of diaphragm pockets, constructed in a silicone rubber gasket that, in conjunction with the polystyrene layers mounted above it, create valves and pumps (Figure 1). When positive pressure is applied through the aperture located in the diaphragm pocket, the flexible layer of polystyrene is forced upward, blocking flow between the discontinuous channels and thereby acting as a closed valve. Similarly, if negative pressure is applied, the flexible layer of polystyrene is pulled into the diaphragm pocket, creating an open valve and permitting flow between the discontinuous channels. Furthermore, sequential actuation of multiple diaphragm pockets in series causes them to act as a pump, moving fluids as desired from one location on the CARD to another. Moreover, the fluid flow can be bi-directional, thereby providing added versatility and effectiveness for mixing. The actuation of all the various diaphragm pockets contained in the CARD is achieved pneumatically, under the control of software.

Optimum conditions for the various assays were first established using standard bench top methods. Then, the assays were migrated onto the CARD platform, which consists of four major steps, including cell lysis, DNA isolation, multiplex PCR amplification and detection by either reverse dot blot or primer extension assay. Each is more fully described below.

Sample Preparation

For each of the assays described, the only step required of the operator is the initial introduction of the untreated specimens directly onto the CARD. All other steps described are automatically performed under the control of software. For all assays, the introduced volume of starting material was 30 μ l collected in either a commercial storage/transfer buffer (e.g. Qiagen Digena STM) or a proprietary storage buffer developed in-house, both containing a chaotropic denaturant (e.g. GuHCl). For the STI and HPV CARD, C33 A (ATCC, Manassas, VA) cells were resuspended in storage buffer at 5×10^6 cells/ml followed by spiking with the genomic DNAs of the pathogens. Furthermore, for testing of the HPV CARD with authentic biological samples, vaginal swabs were collected in 1 ml commercial storage buffer and 30 μ l was applied to the device. For the Warfarin Genotyping CARD, buccal cells were collected in 1 ml of storage buffer from which a 30 μ l aliquot was used. Once the samples were introduced onto the various CARD devices, cell lysis was achieved by the addition of 30 μ l of proteinase K (~2 mAU) containing lysis buffer (50 mM MOPS buffer containing 2.5 M Guanidine HCl, 10 mM EDTA, 1% Triton X-100, pH 7,) pumped into the sample reservoir followed by the bi-directional pumping capabilities of the CARD. Once mixing was completed, 30 μ l of ethanol was added to the lysed material, mixed via pumping, and then allowed to flow through a silica membrane to bind the DNA. The silica membrane was washed with 70 μ l of buffer containing 15 mM MOPS, 4 M Guanidine HCl and 60% ethanol, pH 6.5, followed by wash with 15 mM MOPS buffer containing 0.5 M Guanidine HCl, pH 7. Finally, the DNA was eluted with 30 μ l of nuclease free water and a portion mixed at a ratio of 1:9 with 1.1 X PCR master mix containing reaction buffer, dNTPs, MgCl₂, primers, heat stable uracil-DNA glycosylase, UDG, (USB Corp., Cleveland, OH), and Go Taq Hot Start DNA Polymerase (Promega Corp., Madison WI). The final concentrations of the individual reaction components used were: 0.2 mM each dATP, dGTP, and dCTP, 0.075 mM dTTP, 0.125 mM dUTP, 50 mM KCl, 0.1 mg/ml BSA, 10 mM Tris-HCl, pH 9, 0.002 u/ μ l UNG, and 0.025–0.05 u/ μ l polymerase. Primers were at a final concentration of 0.2 μ M– 0.5 μ M and MgCl₂ at 1.5–3 mM depending on the specific assay. The final reaction mixture was introduced via pumping into the onboard thermocycling chambers.

PCR Thermocycling

Thermocycling was performed for 40 cycles for each of the individual assays. All assays were initiated with 10 min incubation at 37°C to activate the UDG followed by 2 min incubation at 95°C to inactivate the UDG and denature the template DNA. All thermocycling protocols were completed with a 2 min final extension at 72°C. Cycling conditions for each individual assay were as follows. Warfarin: 40 cycles of 95°C, 30 sec; 45 °C, 30 sec; 72 °C, 30 sec. STI: 40 cycles of 95°C, 30 sec; 60 °C, 30 sec; 72 °C, 30 sec. HPV: 10 cycles of 95°C 30 sec, 46°C 30 sec, 72°C 30 sec, 30 cycles of 95°C 30 sec, 49°C 30 sec, 72°C 30 sec.

Following completion of the thermocycling program, detection of the amplicons was accomplished via reverse dot blot assay for the STI and HPV assays. In the Warfarin Genotyping CARD assay, the SNPs of interest were detected via an integrated primer extension assay.

Reverse Dot Blot (RDB) Assay

To detect the amplicons generated in the STI or HPV assays, membrane filters were prepared as described by Zhang et al (26). Briefly, negatively charged nylon, 0.45 μm , Biotodyne C membranes (Pall Corporation, Port Washington, NY) were pre-wet in a solution of 0.1 N HCl followed by activation in a 20% solution of N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride for 15 min. Membranes were rinsed in water and air-dried. Nucleic acid probes modified on the 5' end with an amino group were resuspended at 200 μM in a solution of 0.5 M Sodium Bicarbonate containing 0.1 % Tween and spotted on the membranes using a BioRobotics Biogrid (Boston, MA). Membranes were air-dried and desiccated until inserted into the CARD. To initiate the RDB assay, membranes were incubated with 80 μl 0.1 N NaOH with cycling for 3 min in order to quench any remaining un-bound activated sites followed by 3 rinses with 80 μl dH₂O. Membranes were incubated with 80 μl pre-hybridization/hybridization buffer containing 450 mM sodium chloride, 30 mM sodium phosphate, pH 7.4, 3 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) and 25% formamide for 5 min. When PCR was complete, the thermocycler was set to 95 °C and 80 μl hybridization buffer pumped into the PCR reaction mix. Following denaturation for 5 min, amplicon and hybridization mix was pumped onto the membrane and hybridization performed at room temperature for 15 min. The solution was pumped to waste and the membranes washed 3 times with 80 μl of 0.1% SDS via pumping and circulation. The membranes were then incubated with 80 μl of a 1:500 dilution of horseradish peroxidase conjugated streptavidin (Thermo Fisher Scientific, Inc., Waltham, MA) in a solution containing 150 mM sodium chloride, 10 mM Sodium phosphate, pH 7.4, 1 mM EDTA, and 0.1% SDS followed by washing 3 times with 80 μl of a solution containing 75 mM sodium chloride, 5 mM Sodium phosphate, pH 7.4, 0.5 mM EDTA and 0.3% SDS. For color detection, membranes were incubated for 5 min with 80 μl of One-Step TMB-Blotting Solution (Thermo Fisher Scientific, Inc.). TMB was removed and membranes washed 3 times with 80 μl water. Developed membranes were scanned using a Hewlett Packard Scanjet 4850 and/or photographed using a USB CMOS Monochromatic camera (The Imaging Source, Charlotte, NC) for documentation.

Primer Extension Assay

For the Warfarin Genotyping CARD assay, the single nucleotide polymorphisms of interest were detected by an integrated primer extension assay. The assay relies on DNA polymerase lacking the 3'-5' exonuclease proof-reading function. The immobilized probes, linked to the filters in a similar fashion as to the RDB filters, are approximately 20 nucleotides long and contain the informative nucleotide at their 3' termini. Under these conditions the denatured and annealed amplicon strand behaves as the template, while the solid-phase probe represents the "primer" to be extended. When an exact match is present between the template and the primer, DNA synthesis proceeds incorporating dNTPs including biotinylated dUTP. However, if there is a single mismatch between the terminal base of the immobilized "primer" and the template, elongation, and thus biotin incorporation does not occur. After multiplex PCR, 2 μl of amplicons were mixed with 75 μl of primer extension reaction mix composed of 20 mM Tris-HCl, pH 8.8, 10 mM (NH₄)₂SO₄, 10 mM KCl, 5 mM MgSO₄, 0.1 % Triton X-100, 5 μM each dATP, dCTP, dGTP, and Biotin-20-dUTP (Biotium, Hayward, CA) and 0.05 u/ μl Vent_R (exo-) DNA Polymerase (New England Biolabs, Inc., Ipswich, MA). Following wetting of the probe conjugated membrane by pumping 100 μl water over it, the reaction mix was pumped and cycled over the membrane and the chamber subjected to one cycle of 95°C, 2 min, 45 °C, 5 min, and 72 °C, 15 min. The reaction mix was then pumped out and replaced with wash buffer and the temperature returned to ambient. The filters were washed and processed similar to the protocol described above for color detection of the RDB membranes. Developed membranes were scanned or photographed as described above for RDB. The results of the primer extension assay were

independently confirmed via bi-directional sequencing performed at the Cornell University Life Sciences Core Laboratories Center (Cornell University, Ithaca, NY) using an Applied Biosystems Automated 3730DNA Analyzer with Big Dye Terminator chemistry (27–28) and Ampli-Taq-FS DNA Polymerase (Applied Biosystems, Inc., Foster City, CA).

Primer and Probe Sequences

All target DNA sequences were obtained from the National Center for Biotechnology (NCBI) information (<http://www.ncbi.nlm.nih.gov/>) and PCR primer pairs and probes were designed using CLC Sequence Viewer (www.clcbio.com), Integrated DNA Technologies SciTools, (www.idtdna.com/scitools/) and NCBI Primer Blast (www.ncbi.nlm.nih.gov/tools/primer-blast). The primer pairs and probe sequences used for each of the various assays, reported in Tables 1–4, were synthesized by Integrated DNA Technologies (Coralville, IA). In the case of RDB, the primer complementary to the probe strand was modified on the 5' end with a biotin molecule. All capture probes were modified on the 5' end with an amino modification to allow covalent linkage to the membrane filter used for the endpoint detection.

All microbial and viral DNA with the exception of *Chlamydia trachomatis*, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). *C. trachomatis* serovar L2DNA was kindly provided by Dr. Marci Scidmore, Cornell University College of Veterinary Medicine, Ithaca, NY. Additional HPV types were cloned out of HPV positive clinical samples.

Clinical Specimens

The buccal swabs used in the Warfarin Genotyping CARD were obtained from volunteer donors after obtaining informed consent. After all samples were de-identified, they were provided to the laboratory for analysis.

Deidentified cervical brush samples were collected in Digene Storage Transport Medium following informed consent from women attending the Haidian Maternal and Children Health Hospital in Beijing, China.

RESULTS

In order to demonstrate the versatility of the fully automated Rheonix CARD platform, three different assays, using two different detection schemes, are reported. In general a “quad CARD” configuration (Figure 2), whereby four simultaneous assays can be performed was used in the experiments. The reproducibility of DNA isolation and PCR amplification in the CARD format was investigated by first introducing 30 µl of C33 A cells resuspended in storage buffer at 5×10^6 cells/ml into each of the four sample wells in the CARD device. Once the cells were lysed and DNA isolated, the process was temporarily stopped and 1.5 µl of purified DNA was removed and analyzed in a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). As noted in Figure 3A, each of the four sets of assay channels yielded qualitatively and quantitatively similar DNA. Additionally, since each quadrant of the CARD can perform two separate PCR reactions, the reproducibility of the PCR reactions was compared by allowing three of the four sample channels to continue the assay. DNA from the fourth channel was removed and amplified on a bench top thermocycler (BioRad, Hercules, CA). Comparison of the amplicon generated on the bench top with the duplicate PCR reactions achieved in each of the three other CARD channels revealed similar PCR amplified products (Figure 3B).

STI CARD® Assay

In order to develop the STI CARD® assay that will analyze raw clinical specimens, initial efforts were undertaken using microbial DNA-spiked human cells. Based on our experience with several other assays, including the other assays described here, it is anticipated that authentic biological specimens will behave similarly to the model system. The STI CARD assay was configured to simultaneously detect four sexually-transmitted infectious agents (*N. gonorrhoeae*, *C. trachomatis*, *T. pallidum* and *T. vaginalis*). In order to increase the sensitivity of the assay, multi-copy genes were chosen as the targets for detection. Respective rRNA genes were chosen as targets for *N. gonorrhoeae* (4 copies/cell), *T. pallidum* (2 copies/cell) and *T. vaginalis* (6 copies/cell). A region contained within its extrachromosomal plasmid was chosen as the target sequence for *C. trachomatis* (~4–8 copies/cell). For these initial studies, *T. denticola* was chosen as a surrogate for *T. pallidum* due to the lack of an available source of *T. pallidum* DNA. An alignment of the targeted region from both organisms was performed demonstrating 90% identity, including 100% identity in the reverse primer and one of the probes (data not shown). The forward primer and other probe contain a single base pair mismatch between *T. pallidum*, and *T. denticola*. Since ultimately the target is *T. pallidum*, the sequences were chosen identical to its sequences. However, our experiments demonstrated that these primers and probes worked as well for *T. denticola*, thereby confirming the suitability of *T. denticola* for these initial studies.

Figure 4 shows the results of a fully integrated Rheonix STI CARD. Figure 4A shows the results of spiking 5×10^6 C-33 A cervical carcinoma cells/ml with 10,000 copies/ml each of genomic DNA from *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, and *T. denticola*. Thirty μ l of spiked cells were applied to the STI CARD and subjected to a fully automated CARD assay. Under these conditions, and if full recovery of DNA extraction is achieved, the equivalent of 1 μ l starting material, or 5000 C33A cells with 10 copies of each of the microbial DNA samples, was analyzed via amplification and microarray. Figure 4B shows the RDB results following processing of 30 μ l of cells spiked with various combinations of the STI genomic DNAs. For the experiment shown, 40,000 copies/ml of genomic DNA were spiked into 5×10^6 cells/ml. The images shown in this figure demonstrate that the assay is able to distinguish the absence (Figure 4B-1) or presence (Figure 4B 2–4) of different combinations of microbial genomes.

HPV CARD® Assay

Figure 5 shows the results of the HPV CARD assay of cells spiked with individual cloned DNAs from 14 different HPV types. For these experiments 5×10^6 cells/ml were spiked with 1×10^5 copies/ml of HPV containing plasmid DNA. Thirty μ l was processed via the HPV CARD assay as described above. In addition to amplifying HPV DNA, a region of the human beta-globin gene was also amplified. The images in Figure 5A demonstrate the ability of the HPV CARD assay to detect both globin DNA and each individual HPV type tested.

In order to demonstrate compatibility with actual biological specimens, 69 cervical brush samples, originally evaluated by an outside clinical reference laboratory using Hybrid Capture (HC2, Qiagen, Valencia, CA) to detect the presence of low or high risk HPV types, was subjected to the HPV CARD assay. Of a total 54 samples found to be negative on the HC2 test, 48 were also found to be negative using the HPV CARD® test. Of the remaining six samples scored as negative on the comparator test, each was found to be positive for one or more HPV types using the HPV CARD® assay (Figure 6 and Table 5). Of a total of 15 clinical specimens found to be HPV positive by the HC2 test, all but one were also found to be positive for HPV on the HPV CARD™. The result on the comparator test (ID 9015,

Figure 6A), was noted to be at the cutoff threshold, while multiple replicates of the same sample on the HPV CARD were all found to be negative. PCR amplified HPV DNA from 12 clinical samples shown to be positive by HPV CARD assay were cloned and sequenced, and confirmed the HPV CARD[®] assay result.

Warfarin Genotyping CARD[®] Assay

Deidentified buccal swabs obtained from volunteers following informed consent were analyzed as described in Materials and Methods for three single nucleotide polymorphisms (CYP2C9*2, CYP2C9*3, and VKORC1*2) known to be predictive for warfarin sensitivity (27–32). The CYP2C9*2 and CYP2C9*3 SNPs correspond to mutations in the cytochrome P450, subfamily IIC, polypeptide 9 gene, and the VKORC1*2 corresponds to a mutation in the vitamin K epoxide reductase complex subunit 1 gene. Taking advantage of the prototype CARDS developed for both the HPV and STI assays (see above) which incorporate two PCR reaction chambers, the regions surrounding the two CYP2C9 SNPs were amplified in a single reaction in one chamber, and the region surrounding the VKORC1 SNP was amplified in the other chamber. At the completion of PCR, the amplicons from both reaction chambers were combined, denatured, and then moved to a chamber containing probes covalently linked to the membrane filters and the primer extension assay performed exactly as described in Materials and Methods. Buccal swabs from a total of 19 de-identified volunteers were analyzed on the Warfarin Genotyping CARD[®] and subjected to confirmatory testing using bi-directional DNA sequencing. For each SNP of interest, three possible genotypes are possible: Homozygous wildtype, homozygous mutant and heterozygous. Each of these three possible genotypes for the SNPs of interest can be detected by eye and/or established by determining the ratio of signal intensity of the individual spots. When each of the buccal swab samples from the 19 volunteers was evaluated on the Rheonix Warfarin Sensitivity CARD[®] assay, the genotypes were originally read by at least two different individuals. These results were confirmed with bi-directional sequencing. Representative results of seven individuals are shown in Figure 7. Table 6 shows the genotyping results of the 19 individuals obtained using the Warfarin Sensitivity CARD assay and confirmed with bi-directional sequencing. The more rare combinations of genotypes were not expected in this small sample set. Additional experiments are currently underway to validate the assay with the more rare genotypes.

Discussion

Molecular diagnostics will continue to play an ever increasing and important role in the detection and management of human diseases. Furthermore, because of its potential predictive capabilities, molecular diagnostics will also provide a basis for the management of *potential* disease states based upon an individual's genomic background. Moreover, many studies support the use of genetic testing as the basis for companion diagnostics and/or personalized medicine applications (18–24). Despite the utility and multiple applications of molecular diagnostics, the technology will remain primarily in specialized laboratories or well-equipped central labs with sufficient training and proficiency to perform the various complex preparative, analytical and detection steps required. In order to be of value in point-of-care settings, significant improvements in automation and reduction in costs will be required. Although there are considerable efforts in the IVD industry to develop “sample-to-results” performance, most products still require either substantial investment in capital equipment and/or significant “pre-analytical preparation” of the raw samples prior to analysis.

These requirements also need to be considered against a growing need to reduce overall healthcare costs as well as achieving acceptance from the ultimate payers of the services. Unless effective cost reimbursement can be delivered by the payers, considerable pressure

will continue to exist to avoid the inherently higher costs of capital equipment, training of personnel and the costs of the test kits themselves required to perform molecular diagnostics.

The Rheonix CARD® platform can provide solutions to many of these challenges. The unique design of the plastic device permits the incorporation of all necessary microfluidic networks, valves, pumps and reservoirs on a simple, inexpensive disposable CARD device. Since all assay functions (i.e., flow and mixing rates, temperature control, including thermocycling, resident times, etc.) are easily controlled by software, sophisticated multiplex PCR assays can be performed by individuals that might otherwise lack the experience and training required to perform the individual manual steps required for such assays. Since the CARD platform can automatically perform all necessary steps such as cell lysis, DNA isolation and purification, PCR amplification and detection, virtually no user intervention is required once the initial untreated specimen is introduced. In addition to reducing the training requirements, the expense of individual pieces of equipment can also be eliminated since one instrument is capable of performing all preparative, analytical and readout functions.

Two of the assays described used an integrated reverse dot blot detection method to detect the gene targets of interest, while one used an integrated primer extension assay, further supporting the versatility of the Rheonix CARD® platform. Of the three assays described, two were compared in a head-to-head manner against either an FDA-cleared diagnostic (HPV testing) or bi-directional DNA sequencing, an accepted “gold standard” for SNP-based tests (Warfarin sensitivity testing).

Side by side comparison of the HPV CARD assay with a currently FDA-approved product demonstrated that the CARD assay performed as well as, if not better than, the FDA-approved product. Of 54 samples scored as “negative” on the FDA-approved product, all but six were also scored as negative on the CARD assay. The other six samples were found to be singly or doubly infected with HPV. These results were confirmed by DNA sequence analysis, indicating that the sensitivity of the CARD assay compared to the existing FDA-approved test. Of 15 samples found to be “positive” by the FDA-approved test, all but one were also found to be positive using the CARD assay. The remaining sample was repeated several times on the CARD assay and continuously found to be negative. Although it is possible that the CARD assay lacked the sensitivity to detect the presence of HPV, it is more likely that since the results of the comparator test were at the cutoff borderline between positive from negative results that this equivocal sample was probably negative. Since the samples were provided by an outside laboratory that also performed the FDA-approved test, it was not possible to perform any additional analysis on the sample. Another important distinction between the FDA-approved test and the CARD assay for HPV is that in contrast to the comparator test which was designed to distinguish “high risk” from “low risk” HPV types, the CARD assay was designed to detect and distinguish 20 separate clinically relevant HPV types as well as to detect simultaneous infection by multiple HPV types.

The same experimental approach of first developing a “bench top” assay and then migrating it to the CARD platform that was used for the HPV CARD assay is being employed for the STI CARD assay. Although the STI CARD assay has not yet progressed to the point where untreated clinical specimens are introduced, the preliminary data reported demonstrate that samples spiked with genomic DNA from the target organisms can be detected in the CARD assays at a sensitivity of 10,000 copies/ml in a 30 µl starting volume. Current efforts are underway to validate this assay with actual clinical samples.

While the Rheonix Warfarin Sensitivity CARD also performs a fully automated molecular assay, the terminal low density DNA microarray used for the reverse dot blot assay in the HPV and STI CARD assays was replaced with an array consisting of primers used in an integrated primer extension assay. Software currently being developed for the EncompassMDx workstation will be capable of evaluating the intensity of the spots on the primer extension array to automatically score the three SNPs of interest as either homozygous wildtype, homozygous mutant, or heterozygous. The goal is to provide clinicians with sufficient genomic data that will permit them, along with other clinical information, to determine appropriate therapeutic doses of warfarin. Therefore instead of relying upon time consuming and potentially dangerous repetitive “trial and error” PT/INR testing to finally achieve proper therapeutic doses of warfarin, physicians could initially start warfarin at more appropriate doses, based upon several current warfarin dosing algorithms (30–33).

Taken together, these assays provide a convenient, cost-effective means to perform sophisticated molecular assays in a completely “hands off” manner. Furthermore, the low capital costs of the equipment required to run the assays and the low disposable costs allows this platform to bring true “sample-to-results” molecular testing the point-of-care settings.

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References

1. Wagar EA. Direct hybridization and amplification applications for the diagnosis of infectious diseases [Review]. *J Clin Lab Anal.* 1996; 10:312–25. [PubMed: 8951597]
2. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science.* 1989; 244:359–62. [PubMed: 2523562]
3. Williams WJ, Radulovic S, Dasch GA, Lindstrom J, Kelly DJ, Oster CN, Walker DH. Identification of *Rickettsia conorii* infection by polymerase reaction in a soldier returning from Somalia. *Clin Infect Dis.* 1994; 19:93–9. [PubMed: 7948564]
4. Yamashita Y, Kohno S, Koga H, Tomono K, Kaku M. Detection of *Bacteroides fragilis* in clinical specimens by PCR. *J Clin Microbiol.* 1994; 32:679–83. [PubMed: 8195378]
5. Anderson JR, Smith MD, Kibbler CC, Holton J, Scott GM. A nosocomial outbreak due to non-encapsulated *Haemophilus influenzae*: analysis of plasmids coding for antibiotic resistance. *J Hosp Infect.* 1994; 27:17–27. [PubMed: 7916359]
6. Cormican MG, Glennon M, Riain UN, Smith T, Flynn J, Gannon F. Evaluation of a PCR assay for detection of *Mycobacterium tuberculosis* in clinical specimens. *Diag Microbiol Infect Dis.* 1995; 22:357–60.
7. Enroth H, Engstrand L. Immunomagnetic separation and PCR for detection of *Helicobacter pylori* in water and stool specimens. *J Clin Microbiol.* 1995; 33:2162–5. [PubMed: 7559969]
8. Mathis A, Deplazes P. PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. *J Clin Microbiol.* 1995; 33:1145–9. [PubMed: 7615719]
9. Whelen AC, Felmlee TA, Hunt JM, Williams DL, Roberts GD, Stockman L, Persing DH. Direct genotypic detection of *Mycobacterium tuberculosis* rifampin resistance in clinical specimens by using single-tube heminested PCR. *J Clin Microbiol.* 1995; 33:556–61. [PubMed: 7751357]

10. Jackson KM, Edwards RM, Bowden DS, Leslie DE. Evaluation of the Roche Amplicor polymerase chain reaction system for detection of *Mycobacterium tuberculosis* complex in specimens. *Pathology*. 1996; 28:65–7. [PubMed: 8714275]
11. Pacht C, Todd JA, Kern DG, Sheridan PJ, Fong SJ, Stempien M, et al. Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA signal amplification assay. *J AIDS*. 1995; 8:446–54.
12. Detmer J, Lagier R, Flynn J, Zayati C, Kolberg J, Collins M, et al. Accurate quantification of HCV RNA from all HCV genotypes using branched DNA (bDNA) technology. *J Clin Microbiol*. 1996; 34:901–7. [PubMed: 8815105]
13. Saksela K, Stevens CE, Rubinstein P, Taylor PE, Baltimore D. HIV-1 messenger RNA in peripheral blood mononuclear cells as an early marker of risk for progression to AIDS. *Ann Intern Med*. 1995; 123:641–8. [PubMed: 7574218]
14. Revets H, Marissens D, de Wit S, Lacor P, Clumeck N, Lauwers S, Zissis G. Comparative evaluation of NASBA HIV-1 RNA QT, Amplicor-HIV monitor, and Quantiplex HIV RNA assay, three methods for quantification of human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol*. 1996; 34:1058–64. [PubMed: 8727875]
15. Mellors JW, Kingsley LA, Rinaldo CR, Todd JA, Hoo BS, Kokka RP, Gupta P. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann Intern Med*. 1995; 122:573–9. [PubMed: 7887550]
16. O'Brien TR, Blattner WA, Waters D, Eyster, et al. Serum HIV-1 RNA levels and time to development of AIDS in the multicenter hemophilia cohort study. *JAMA*. 1996; 276:105–10. [PubMed: 8656501]
17. Mellors JM, Rinaldo CR Jr, Gupta P, et al. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*. 1996; 272:1167–70. [PubMed: 8638160]
18. Cichon S, Nöthen MM, Rietschel M, et al. Pharmacogenetics of schizophrenia. *Am J Med Genet*. 200; 97(1):98–106. [PubMed: 10813809]
19. Schwarz UI. Clinical relevance of genetic polymorphism in the human CYP2C9 gene. *Eur J Clin Invest*. 2003; 33(Suppl 2):23–30. [PubMed: 14641553]
20. Clayton TA, Lindon JC, Cloarec O, et al. Pharmacometabonomic phenotyping and personalized drug treatment. *Nature*. 2006:440.
21. Shastry BS. Pharmacogenetics and the concept of individualized medicine. *Pharmacogenomics J*. 2006; 6(1):16–21. [PubMed: 16302022]
22. Oldenburg J, Watzka M, Rost S, et al. VKORC1: molecular target of coumarins. *J Thromb Haemost*. 2007; 5(Suppl 1):1–6. [PubMed: 17635701]
23. Mansour JC, Schwarz RE. Molecular mechanisms for individualized cancer care. *J Am Coll Surg*. 2008; 207(2):250–8. [PubMed: 18656055]
24. van't Veer LJ, Bernards R. Enabling personalized cancer medicine through analysis of gene-expression patterns. *Nature*. 2008; 452(7187):564–70. [PubMed: 18385730]
25. Zhou, Peng; Young, Lincoln; Chen, Zongyuan. Weak solvent based chip lamination and characterization of on-chip valve and pump. *Biomedical Microdevices*. 2010; 12:821–832. [PubMed: 20526680]
26. Zhang Y, Coyne MY, Will SG, Levenson CH, Kawasaki ES. Single-base mutational analysis of cancer and genetic diseases using membrane bound modified oligonucleotides. *Nucleic Acids Res*. 1991; 19:3929–33. [PubMed: 1861985]
27. Rosenblum BB, Lee LG, Spurgeon SL, et al. New dye-labeled terminators for improved DNA sequencing patterns. *Nucleic Acid Res*. 1997; 25:4500–4504. [PubMed: 9358158]
28. Heiner CR, Kunkapiller KL, Chen S-M, et al. Sequencing Multimegabase-Template DNA with BigDye Terminator Chemistry. *Genome Research*. 1998; 8:557–561. [PubMed: 9582199]
29. Jeney C, Takacs T, Sebe A, et al. Detection and typing of 46 genital human papillomaviruses by the L1F/L1R primer system based multiplex PCR and hybridization. *J Virol Methods*. 2007; 140:32–42. [PubMed: 17169438]
30. Daly AK, King BP. Pharmacogenetics of oral anticoagulants. *Pharmacogenetics*. 2003; 13:247–252. [PubMed: 12724615]

31. Takahashi H, Eschizen H. Pharmacogenetics of warfarin elimination and its clinical implications. *Clin Pharmacokinet.* 2001; 40:587–603. [PubMed: 11523725]
32. Schwarz UI, Ritchie MD, Bradford Y, et al. Genetic determinants of response to warfarin during initial anticoagulation. *N Eng J Med.* 2008; 358:999–1008.
33. Osinbowale O, Al Malki M, Schade A, et al. An algorithm for managing warfarin resistance. *Clev Clinic J of Med.* 2009; 76:724–730.

Figure 1. Pump/Valve Assembly on Rheonix CARD® device

The polystyrene CARD® consists of a 1 mm upper layer and a 1 mm lower layer that are laminated together, with a 38 μm polystyrene layer between. Positive pressure through the aperture in the diaphragm pocket of the lower layer causes the deformable membrane to be driven upward, thereby preventing flow of fluids from one discontinuous channel to the next. Conversely, negative pressure causes the deformable membrane to be drawn down into the diaphragm pocket, thus allowing fluid to flow from one discontinuous channel to the next. In addition to serving as either open or closed valves as depicted, the same diaphragm pockets can serve as pumps by sequential application of positive and negative pressure.

Figure 2. Rheonix Quad CARD® Configuration

A Rheonix Quad CARD® is shown that is capable of performing up to four simultaneous molecular assays. Once the biological sample is applied where noted, the cells are lysed and DNA purified through an onboard silica membrane. The isolated DNA obtained from each individual sample can then be delivered to two separate PCR thermocycling chambers to overcome potential inhibition from multiple primer pairs required to PCR amplify multiple targets. Once PCR has been completed, the amplicons generated in each of the two PCR thermocycling chambers can be combined and delivered to the terminal portion of the CARD where the reverse dot blot or primer extension assays are performed.

Figure 3. Reproducibility of Rheonix Quad CARD®

As described in the text, 30 μ l aliquots of a cell suspension were added to each of the four sample reservoirs of a Quad CARD® and DNA automatically isolated. (A) The spectrophotometric profile of each of the four isolated DNA samples was analyzed. An aliquot of the isolated DNA from one channel was used to PCR amplify the target sequence in a conventional bench top thermocycler while DNA from each of the other three quadrants was automatically PCR amplified on the CARD®. (B) Agarose gel electrophoresis of the amplicons generated using a bench top thermocycler (BT) and the amplicons generated automatically in each of the two thermocycling chambers of the three remaining quadrants on the CARD® (CARD®)

Figure 4. Rheonix STI CARD® Assay

Five million C33A cells/ml were spiked with 10,000 copies/ml each of genomic DNA from *N. gonorrhoeae*, *C. trachomatis*, *T. denticola*, and *T. vaginalis*, and subjected to Rheonix STI CARD® assay as described. Left panel: Image shows the colored precipitate formed from HRP activity following the capture of biotinylated amplicons incubated with streptavidinylated HRP and substrate. Right panel: Filter key; SC: Spotting Control- biotin labeled poly-A; CT: *C. trachomatis* capture probe; TP: *T. pallidum* capture probe; TV: *T. vaginalis* capture probe; and NG: *N. gonorrhoeae* capture probe. Note that *T. denticola* is recognized by the *T. pallidum* probe in this assay. (B) Five million C33A cells/ml were spiked with (1) no STI DNA, or 40,000 copies/ml of genomic DNA from (2) *T. denticola*, (3) *N. gonorrhoeae*, *C. trachomatis*, and *T. vaginalis*, or (4) all 4 microbial DNAs and subjected to Rheonix STI CARD® assay as described. Filter key is the same as shown in Figure 4A

A. **B**

SP	51	33	56	39	SP
45	31	52	35	58	59
16H	66	16M		16L	
18L		18M	68	18H	53
GbH	11	GbM	43	GbL	
6		42		44	SP

Figure 5. HPV CARD® Analysis of cloned plasmid DNA

Reverse dot blot results obtained from processing C33A human cells spiked with various HPV plasmids as described in text. (B) Filter key for probe spots. SP refers to a spotting control consisting of biotinylated poly A to demonstrate effective spotting of probes and performance of the HRP color generating reaction. Gb corresponds to human beta-globin capture probe. All numbers pertain to the HPV type of the individual probes. All probes except Gb, HPV 16, and 18 were spotted at 50 μ M. Gb, 16, and 18 were spotted at 2 (L), 20 (M), and 50 (H) μ M. SC spotted at 2.5 μ M. Note, the presence of HPV 56 detected on HPV 66 hybridized membrane is due to mixed HPV 56/66 clone, and not cross-hybridization.

Figure 6. HPV CARD® Results of authentic clinical specimens

Reverse dot blot results obtained from evaluating 15 clinical specimens previously shown to be positive using the HC2 Hybrid Capture assay. (B) Reverse dot blot results obtained from evaluating 6 clinical specimens previously shown to be negative using the HC2 Hybrid Capture assay. Both sets of experiments were performed as described in the text. C) Membrane key for *9015*, *8992*, *9016*, *8987*, *9003*, *8988*, and *9025* (*italics*). D) Membrane key for all others. Membrane keys. SP: Spotting Control; biotin labeled poly-A. All probes spotted at 20 μ M; Globin (Gb), 16 and 18: spotted at 0.2 (L), 2 (M), and 20 (H) μ M.

A.

Gene	Strand	
	na	na
Spotting Control	na	na
VKORC1-Mut	NC	C
VKORC1-WT	NC	C
CYP2C9*2-Mut	C	NC
CYP2C9*2-WT	C	NC
CYP2C9*3-WT	C	NC
CYP2C9*3-Mut	C	NC

B.

VKORC1	TT	CT	CC	CT	TT	CT	CT
CYP2C9*2	CC	CC	CC	CC	CT	CT	CT
CYP2C9*3	AA	AA	AA	AC	AA	AA	AC
Spotting Control							
VKORC1-Mut							
VKORC1-WT							
CYP2C9*2-Mut							
CYP2C9*2-WT							
CYP2C9*3-WT							
CYP2C9*3-Mut							

Figure 7. Representative image derived from the primer extension component of the Warfarin Genotyping CARD®

Warfarin Genotyping was performed as described in the text. (A) The key indicates the allele targeted by the capture probe (NC = noncoding strand, C = coding strand, na = not applicable). (B) Results of seven individual genotypes obtained using the Rheonix Warfarin Genotyping CARD®. Filter results from buccal swabs of seven individuals are shown in the lower portion of the (B). The upper portion of (B) indicates the genotypes read from these filters. In addition, all genotypes were verified via bi-directional sequencing. Note these genotypes were represented within the 20 individuals tested. The more rare combinations of genotypes were not expected in this small sample set. Current experiments are underway to validate the assay with the more rare genotypes.

Table 1**Amplification Primers**

Forward and reverse PCR primers are shown in the 5' to 3' direction for each target sequence. Terminal nucleotides were modified as described in the text.

Assay	Target	Forward Primer(s)	Reverse Primers
HPV	HPV	CGTAAACGTATWCCCTATTTTTTT	ACTCTAAACACYCTATACTG
		CGTAAACGTTTWCCATATTTTTTT	ACYCTAAAYACTCTGTATTG
		CGTAAACGTATTCCCTATTTTTTT	ACCYTAAATACCCTGTATTG
		CGTAAACGTGTCCCTATTTTTTT	ACCCTAAAHACYCTATATTG
		CGTAAACGYGTWTCATATTTTTTT	ACSCKAAATACCCTATATTG
		CGTAAAGCTATCCCATATTTTTTT	ATTCTAAATACCCTGTATTG
		CGTAAACACCTTCCTTATTTTTTT	ACTCTAAATACTCTGTACTG
		CGTAAACGTATTCCCTATTTTCTT	ACCCTAAAGACCCTATACTG
	Beta-Globin	GAATAACAGTGATAATTTCTGGG	GAAGATAAGAGGTATGAACATGA
STI	N. gonorrhoeae 16S rRNA	CGGACGGCAGCACAGGGAAG	CTCGGATAGCGCAAGGCCCG
	C. trachomatis plasmid	TGCCCGGGATTGGTTGATCGC	ACGTGCGGGCGATTTGCCTT
	T. pallidum 16S rRNA	ACGGCCCAGACTCCTACGGG	TTCCGAACAACGCTCGCCCC
	T. vaginalis 16S rRNA	TGGTGGTGCATGGCCGTTGG	GCACCCTCTCAGGCTCGCAG
Warfarin	CYP2C9*2	CTAAAGTCCAGGAAGAGATTGA	AGTCCAGTAAGGTCAGTGATATG
	CYP2C9*3	CTAAAGTCCAGGAAGAGATTGA	GATACTATGAATTTGGGGACTTC
	VKORC1	ATAGGGTCAGTGACATGGAATC	TTGGATTGATTGAGGATGCTGTC

Table 2**STI Capture Probes**

Sequences of DNA capture probes used to detect the sexually transmitted infections in the reverse dot blot arrays are shown in the 5' to 3' direction. 5' termini were modified as described in the text.

Target	Probe 1	Probe 2
N. gonorrhoeae	GGGTAGCGGGGATAACTGA	CGAAAGATCAGCTAATACCG
C. trachomatis	ATCCTCAGAAGTTTATGCAC	TTTCTACAAGAGTACATCGGT
T. pallidum	TAGTCGTGCGAATAAGCC	GCTAATTACGTGCCAGCA
T. vaginalis	TTTAGCAGGTGGAAGAGG	GTAGCAATAACAGGTCCGTG

Table 3**HPV Capture Probes**

Sequences of DNA capture probes used to detect the various HPV types in the reverse dot blot arrays are shown in the 5' to 3' direction. 5' termini were modified as described in the text.

Nucleotide sequences of amino-terminated capture probes¹

HPV Type	Probe Sequence (5'-3')
6	TTC CAT AAA ACG GGC TAA CAA A
11	ACT CTA TCA AAA AAG TTA ACA A
16	AAA CCT AAC AAT AAC AAA ATA TTA
18	GGT GGC AAT AAG CAG GAT A
31	AAA TCT GAC AAT CCT AAA AA
33	AAA AAT CCT ACT AAC GCT AAA AAA
35	AAA ACA AGA TTC TAA TAA AAT AGC A
39	TAA AGT GGG TAT GAA TGG TGG T
42	CAA AAA GGC CAA ATA AGA CA
43	CCT TAA AAA TTC CTC TGG TAA AA
44	ATA CGA CCA GCA AAC AAG AC
45	ACC TAA TGG TGC AGG TAA TA
51	TAA AAC CTC AAC GCG TGC T
52	AAA ACA CCA GTA GTG GTA ATG G
53	CAT TTC TAA ATC TGG TAA AGC A
56	AAG GAC AAT ACC AAA ACA AAC A
58	CCA TCA AAA GTC CCA ATA AC
59	GGT GGT AAT GGT AGA CAG GA
66	CAA ATC TGG TAC CAA AAC AAA
68	TTA AGG TTC CTA TGT CTG GG
Globin	ATC GAG CTG AAG GGC ATC GAC TTC AA

¹Probe sequences except HPV 6, 11, 16, & 18 are from Jeney et al (29).

Table 4**Warfarin Capture Probes for Primer Extension**

Sequences of DNA capture probes used to detect the various SNPs associated with warfarin sensitivity in the primer extension assay are shown in the 5' to 3' direction. Where noted, sequences directed at either coding or non-coding strands for both wild type (WT) or mutant (mut) nucleotides are presented.

Gene	Strand	Sequence
CYP2C9*2	Coding-Mut	GGGAAGAGGAGCATTGAGGACT
CYP2C9*2	Coding-WT	GGGAAGAGGAGCATTGAGGACC
CYP2C9*2	Non-coding-Mut	GCGGGCTTCCTCTTGAACACA
CYP2C9*2	Non-coding-WT	GCGGGCTTCCTCTTGAACACG
CYP2C9*3	Coding-Mut	GTGCACGAGGTCCAGAGATACC
CYP2C9*3	Coding-WT	GTGCACGAGGTCCAGAGATACA
CYP2C9*3	Non-coding-Mut	GCTGGTGGGGAGAAGGTCAAG
CYP2C9*3	Non-coding-WT	GCTGGTGGGGAGAAGGTCAAT
VKORC1	Coding-Mut	GGTGCCAGGAGATCATCGACC
VKORC1	Coding-WT	GGTGCCAGGAGATCATCGACT
VKORC1	Non-coding-Mut	GACCTCCCATCCTAGTCCAAGA
VKORC1	Non-coding-WT	GACCTCCCATCCTAGTCCAAGG

Table 5**Discrepancy with HC2 Results**

HC2 and CARD[®] results are shown for six samples that were discrepant. The signal-to-noise ratio of the HC2 test (1.0 is the cutoff) and the reverse dot blot results on the CARD[®] assay are reported.

Sample #	HC2 Result	HPV type (RDB)
9040	0.12	18
9032	0.16	18
9014	0.20	58
9053	0.28	66
9029	0.48	51,42
9055	0.50	31

Table 6

Genotypes of 19 individuals with respect to the VKORC1, CYP2C9*2 and CYP2C9*3 SNPs are reported. For the VKORC1 SNP, the wildtype is a “C”, while a mutant is a “T”. For CYP2C9*2 SNP, the wildtype is a “C” and a mutant is a “T”. For the CYP2C9*3 SNP, the wildtype is an “A” and the mutant is a “C”.

Gene	Genotypes							
VKORC1	TT	CT	CC	CT	TT	CT	CT	
CYP2C9*2	CC	CC	CC	CC	CT	CT	CT	
CYP2C9*3	AA	AA	AA	AC	AA	AA	AC	
<hr/>								
# individuals	2	9	3	2	1	1	1	