Application of advanced microfluidics and rapid PCR to analysis of microbial targets

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ABSTRACT

We are developing field-portable instruments and disposable fluid cartridges that enable the rapid and quantitative analysis of target bacteria or viruses in complex mixtures. Up to many milliliters of raw sample are processed automatically in a flow-through manner, and processing functions are sequentially effected. These include specimen dilution, coarse filtration, target microbe extraction/separation, microbe lysis with or without chemicals and/or heat, neutralization, DNA/RNA purification and concentration, PCR reagent mixing, and PCR or RT-PCR. As part of the development of this system, we have designed and tested a silicon-based fluidic chip that extracts DNA from 500 uL of fluid and dilutes into 25-50 uL at greater than 50% efficiency and resulting in a 10- to 20-fold increase in concentration. This operation can be completed in several minutes. We have also designed, built, and tested complex fluidic cartridges with large specimen volume capacity, integral waste regions, filters, heaters, integrated DNA chips, fluid divertors, and PCR reaction tube filling. Rapid PCR modules, capable of 7-10°C/sec and 2-5°C/sec heating and cooling, respectively for 100 uL reaction volumes and 2 or 4-color real-time fluorescence detection suitable for Taqman®, Molecular Beacons, and other homogeneous chemistry systems, have been built and demonstrated.

Introduction

Cepheid has assembled a diverse group of engineers and scientists to design and commercialize miniature bioanalytical instruments that will move the detection and analysis of microorganisms from the laboratory to the field. An array of conventional sample preparation techniques, microbiological methods, and molecular methods already exist, but in general are not amenable to automation and miniaturization. Increased speed and sensitivity are essential, along with low power requirements, and a toolbox of modular technologies are now being developed to replace the laboratory centrifuge, gel electrophoresis apparatus, fluid handling equipment, and large thermal cyclers.

Because of recent advances in rapid polymerase chain reaction (PCR) (5, 8, 11), in the design and construction of silicon microstructures (6), and in plastic microfluidic device design, it is now possible to design small, field-portable instruments and cartridges. We present device designs and performance data for several of the key component technologies needed for future field-portable instrument systems.

Microbial Biosystems: New Frontiers
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Bell CR, Brylinsky M, Johnson-Green P (eds)
Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.

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Instrumentation systems for the analysis of chemical and biochemical fluids can be achieved through the application of microfabricated methods based on integrated circuit technologies. Various terms for instruments made by these methods include MEMS (microelectromechanical systems), micro systems technology, and μ-TAS (micro Total Analytical Systems). In 1990, Manz proposed the term μ-TAS (7) and argued that based on the typical concentration range of most clinical chemistry analytes, appropriate confidence limits around having at least one analyte molecule in the reaction, and fluorescence as a detection mode, that it is possible to assay for these analytes in reaction volumes as small as a picoliter. Even for analytes for which immunoassays are utilized, it is still possible to contemplate detection in reaction volumes between 1 nanoliter and 1 microliter.

Unfortunately, for the detection of microbial targets, much larger volumes are required, up to many milliliters. This is due to (a) low intrinsic concentration and (b) sample processing where the volume of the sample increases during manipulation. Examples of low intrinsic concentration include: < 400 virions/mL of whole blood for human immunodeficiency virus (HIV); < 250 CFU/gm in paper products for food containment; < few genome copies per gallon in sources for drinking water; < 10 CFU/gm of food in food poisoning assessment; and < 1 CFU/mL in carcass washings for EHEC.

Figure 1 shows an adaptation of the figure originally conceived by Manz (7) showing the relationship of target analyte to sample volume in terms of detectability. Clearly, those target analytes in the examples above would occupy the lower right hand side of the graph. Steps in the sample processing itself, such as dilution, filtration, buffer exchange, and reagent mixing, can lead to further diluting of the target concentration and an increase in sample volume required for analysis.

Therefore, the challenge for fluidic device technologies is that DNA or RNA from target microorganisms must be extracted and concentrated from large (> 1 mL) volumes of sample fluids and other reagents. In PCR using fluorescent probes, this is important because the PCR reagents are expensive and it is desirable to keep the final reaction volume small. If conventional “micro-nano-pico” microfluidics, sometimes called “lab-on-a-chip”, are used they require a large surface of silicon and are thus very expensive. They are not suitable as disposable devices for field use. Alternatively, our devices will be comprised of plastic microfluidics that integrate filters, silicon devices, or other materials required for sample prep, and will also include DNA/RNA amplification reaction sites such as PCR. An example of such a system, called the MicroBE is shown in Figure 2. This hand-held instrument contains all of the processing hardware and software and accepts a single, disposable fluidic cartridge containing all reagents and that performs automated sample preparation followed by fluorescence-based PCR. Results are outputed to an LCD or can be transmitted by IR or other telemetric means.

Rapid-PCR

PCR is finding increased acceptance and use in a wide range of applications. Amplification of target genetic material is accomplished by mixing target DNA with appropriate primers, DNA polymerase, and dNTP’s, and then cycling back and forth between the melt (95°C) and anneal (65°C) temperatures of the target DNA. Assuming perfect efficiency, each cycle

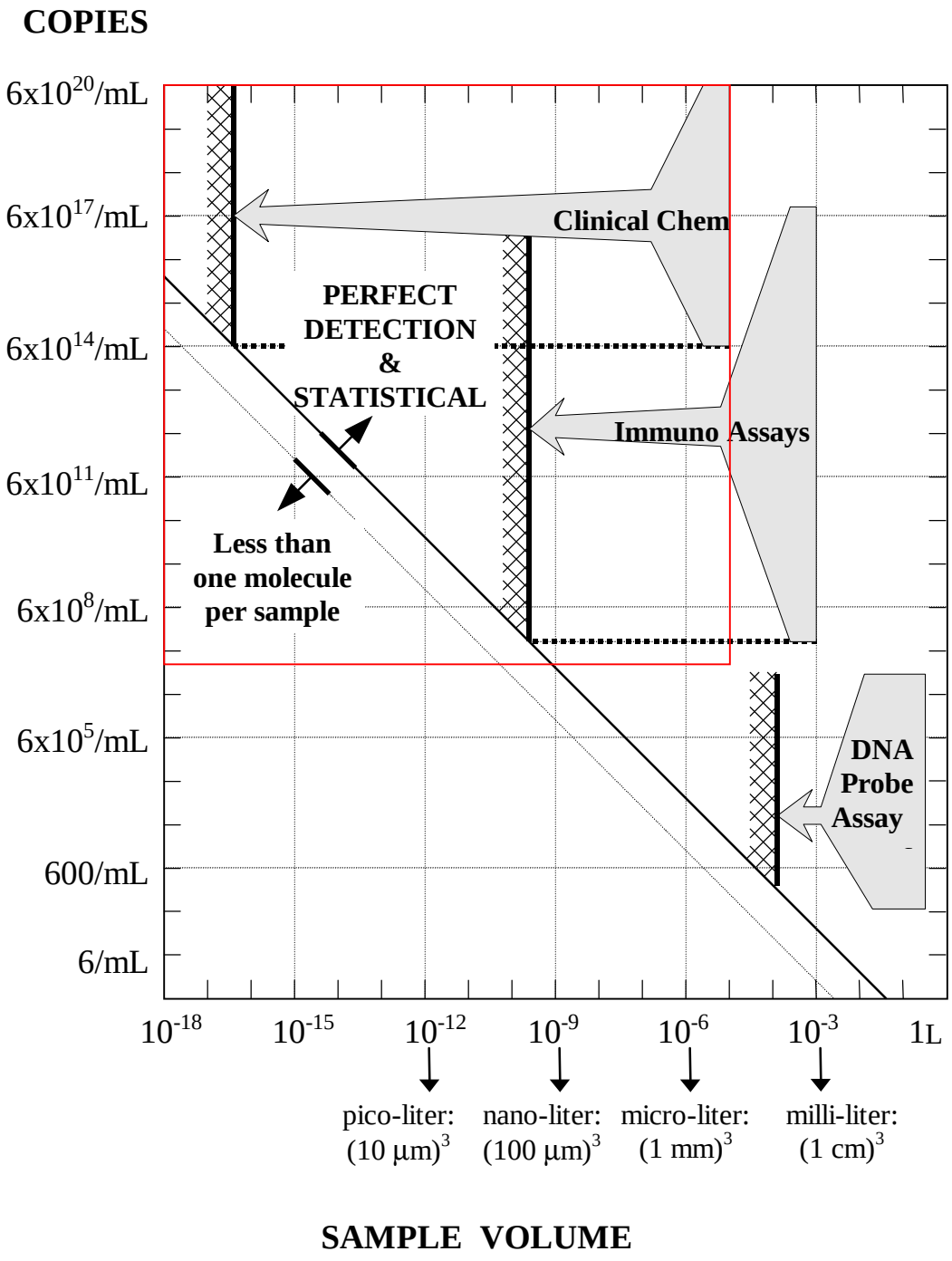


Figure 1. A graph created by Manz, et al. (xx) defines the relationship between analyte concentration and minimum sample volume needed to achieve detection. The concentration ranges for clinical chemistry assays and immunoassays are between about 10^{14} and 10^{21} copies/mL and between 10^{8} and 10^{18} copies/mL, respectively. The original graph has been expanded towards the lower left to encompass microbial targets where a range of 1 to 10^{7} copies/mL are typical. It is clear that a minimum sample volume of 100 uL to many milliliters must be analyzed to achieve reliable detection and measurement.

Figure 2. The MicroBE Instrument System. Disposable cartridges include reagents and all sample preparation steps are performed automatically. Calibration and quality control are built into each cartridge. The system eliminates sample carryover and amplicon contamination.

results in a doubling of the amplified product, and after a sufficient number of cycles, the product can be detected either by gel electrophoresis or, if a fluorescent-labeled probe or intercalating fluorescent dye is included in the reaction mixture, by fluorescence detection (3, 4). Typical procedures can take many hours and require a high level of operator expertise.

We recognized the need to decrease the time for amplification and detection for PCR down to as few as 20 minutes if the utility of field applications were to be widely accepted. We chose to focus on rapid thermal cycling and real-time homogeneous fluorescence detection, basing our development efforts on technology developed by M.A. Northrup of Lawrence Livermore National Laboratory (8, 9). Figure 3 shows an I-CORE thermally-controlled fluorometer module. A triangular-shaped ceramic heater assembly (thermal sleeve) interfaces with optics modules containing blue and green LED's, filtered solid-state

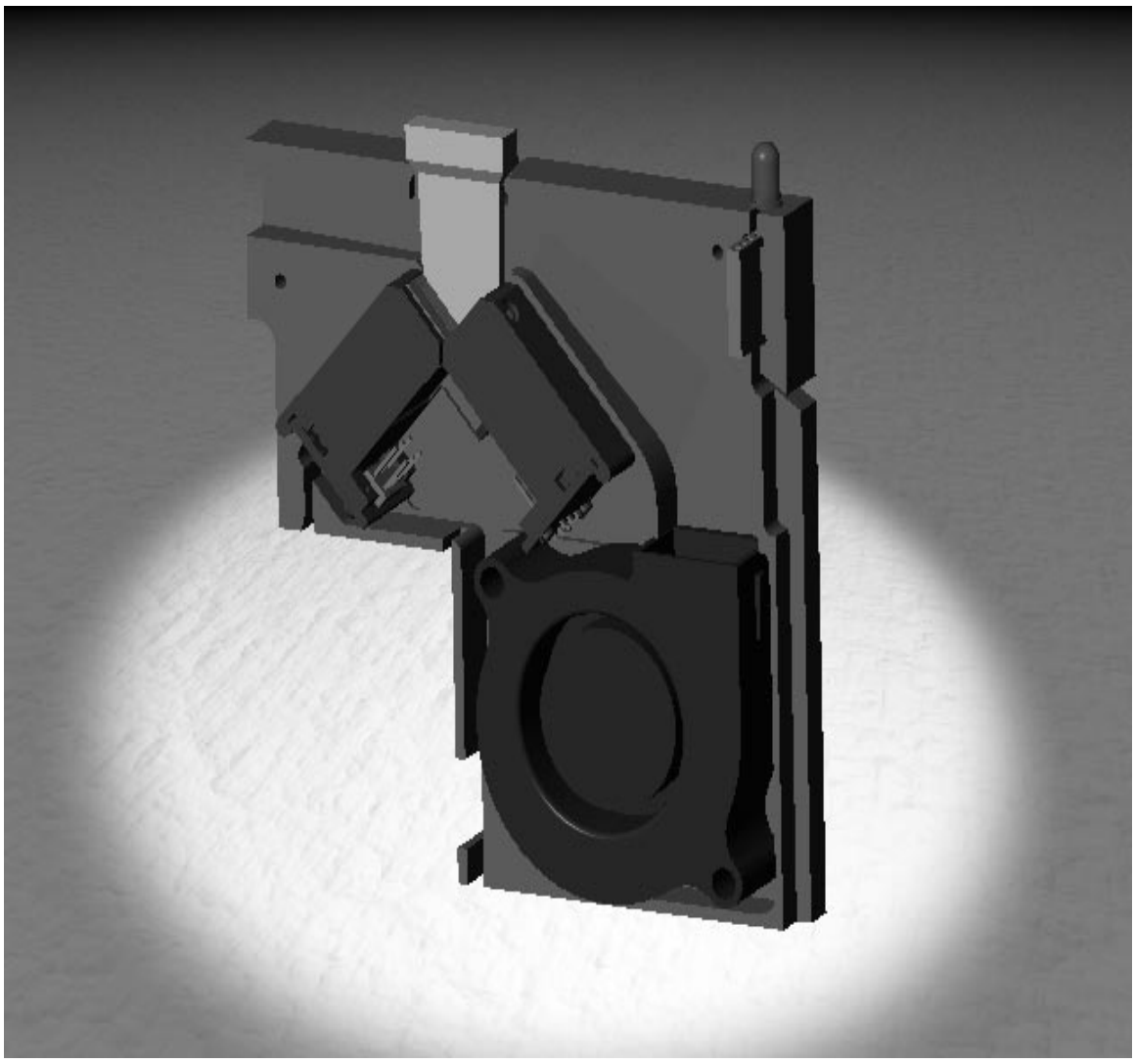


Figure 3. I-CORE Module. Functional capabilities include rapid and accurate thermal cycling; real-time optical detection of 4 different fluorescent dyes; inexpensive, all solid-state optical components; local microprocessing for flexibility in instrument design.

photodiodes, and molded plastic lenses. The thermal sleeve is composed of a very low thermal mass of aluminum nitride, has very high thermal conductivity, and achieves heating and cooling rates for 100 uL of water of 7-10°C/sec and 2-5°C/sec, respectively. Currently, cycle times as short as 30 seconds have been demonstrated, and even shorter times will be possible with improvements in software control. A disposable reaction tube, shown in Figure 4, is a completely new design and is simultaneously both optically and thermally efficient. I-CORE modules can be ganged together in groups of 8 or more to form multi-site instruments, and because each site is individually programmable, fully independent thermal cycling is possible. Optimization of PCR assays can be accomplished in a few runs in a single day, instead of the several days to weeks required with conventional thermal cyclers. We have successfully amplified cFTR, M13, Lamda phage, Venezuelan Equine Encephalitis Virus, Beta-Actin, *Bacillus subtilis*, and HCV (RNA) targets. Figure 5 shows a comparison of an I-CORE-based Smartcycler® instrument to the Perkin-Elmer ABI Prizm 7700 instrument using a FAM Taqman® beta-actin PCR assay.

For many analyses, such as those for the diagnosis of potential infectious disease syndromes, more than one candidate microorganism can cause the disease or there may be antibiotic resistance factors that must be detected before the patient is treated. In

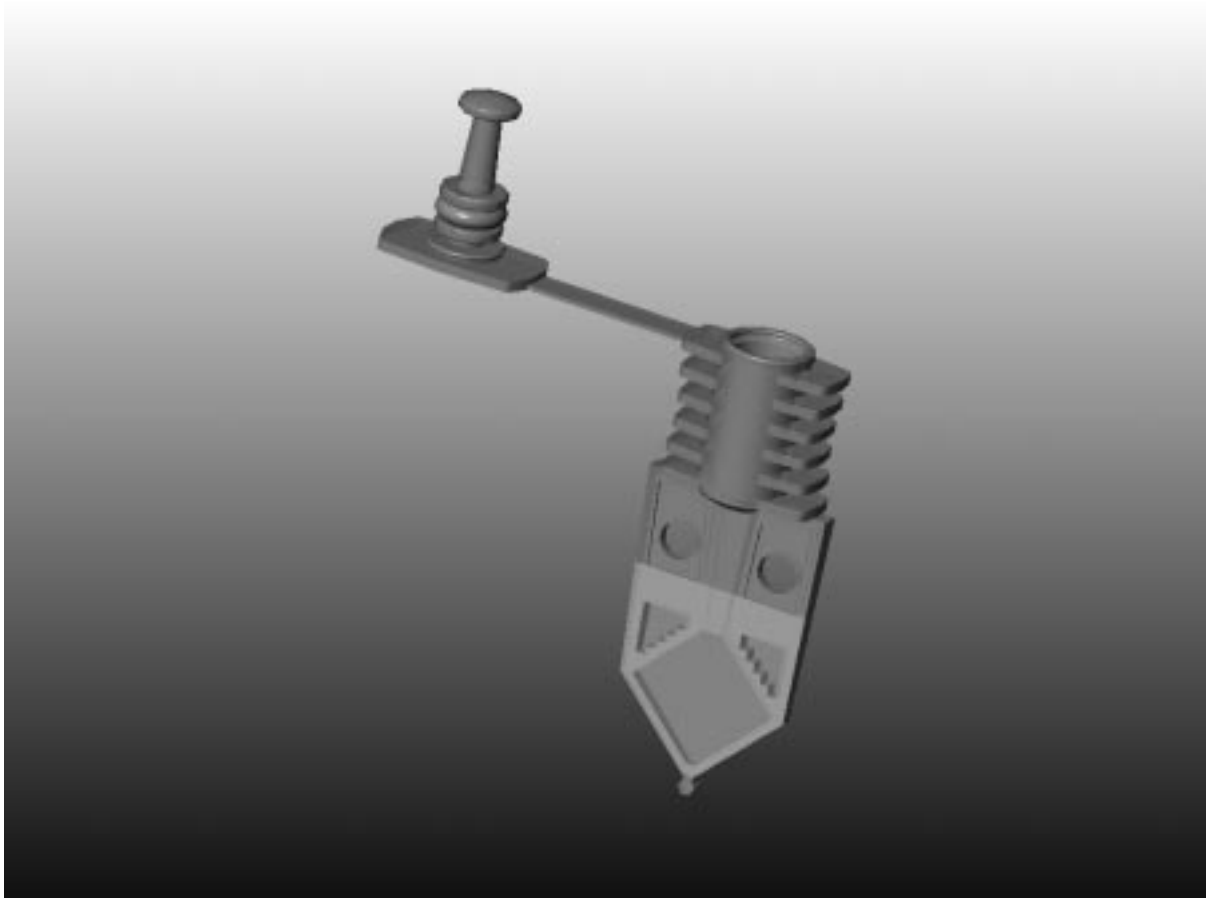
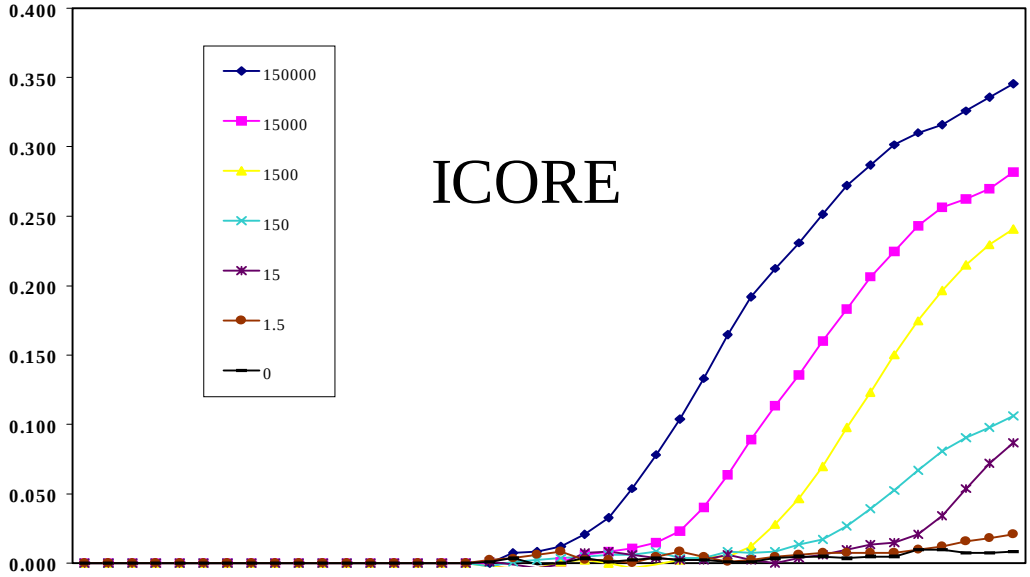


Figure 4. I-CORE Reaction Tube. Thin, diamond-shaped geometry simultaneously optimizes thermal and optical responses.



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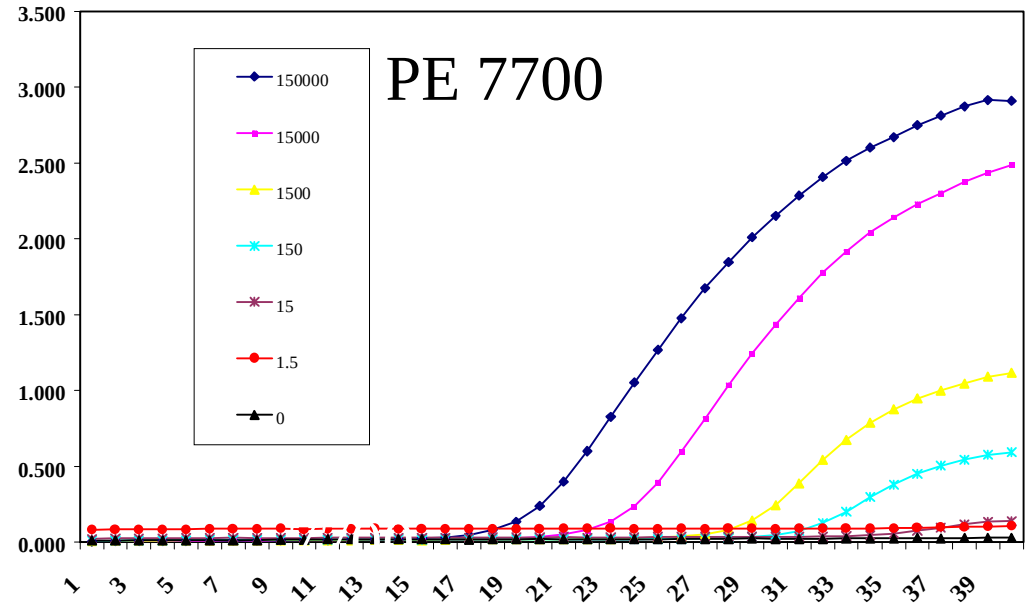

Figure 5. Comparison of Smartcycler and PE/ABI Prizm 7700. Titration of beta-actin targets using a FAM Taqman® beta-actin PCR assay. The Smartcycler signal are raw voltages without any normalization or curve smoothing.

environmental samples, one may wish to detect scant multiple genotypes out of a preponderance of irrelevant flora, or include calibrators or competitive mimics for quantitation of single targets. In order to preserve the individual detection limit for each target or to make the use of internal calibrators possible, it is preferable to be capable of detecting all targets in a single reaction mixture to avoid splitting of the sample. Multi-color fluorescence affords such as approach. In Taqman® and Molecular Beacons-based PCR methods, 4 or more different fluorescent probes, such as FAM, TET, TAMRA, and ROX, can be simultaneously detected during thermal cycling using a 4-color I-CORE module.

Silicon and Plastic Microfluidics

Plastics, such as polypropylene, polycarbonate, acrylic (PMMA), and polyesters, are ideal materials with which to fabricate fluidic devices because they are inexpensive; easy to mold; simple to assemble, anneal, bond, and cut; and are compatible with biochemical reagents required for sample preparation and PCR. Silicon chips, filters, and other devices can be encapsulated into molded plastic assemblies, and conventional packaging and integrated circuit assembly methods can also be employed. Because of the cost of silicon wafer processing and packaging, it is important to utilize plastic as much as possible in the design and fabrication of sample prep cartridges. However, for some functions, silicon chips provide a preferable material.

New micromachining techniques, including deep reactive ion etching (DRIE) (1, 10), have been used to fabricate high surface area structures capable of controllable capture and release of DNA from flowing test solutions. Figure 6 shows an SEM of one chip that consists of 200 um high columns, each about 18 um in diameter and a pitch of 34 um. After anodic bonding to a glass top cover, the chip has a total internal SiO_2 surface area of 36 mm^2 in an 'active' area of 3.5 mm^2. To demonstrate the functionality of the chip for low concentration targets, lambda stock was diluted into chaotropic salt solutions (6 M guanidine isothiocyanate) to a starting copy of 5×10^4 copies in 500 uL and flowed continuously through the chip at 0.5 uL/sec, followed by 250 uL of wash solution. The DNA was eluted from the chip in 25 uL increments. We show in Figure 7 that > 50% capture efficiency and 10x concentration was achieved. Newer chips containing microstructures that improve the contact time between the fluid and the surface have resulted in extraction times as fast as 90 seconds at greater than 75% capture efficiency.

Other chips have been designed and fabricated for a variety of tasks, including cell disruption, reagent mixing, liquid phase separation, flow cytometry, cell sorting, and filtering. For the latter, the SiO_2 surface of the chip can also be derivitized with biospecific moieties using conventional chemistries to produce devices capable of extracting intact bacteria or virus from complex mixtures. Work is underway to evaluate these schemes.

Integrated Sample Preparation Cartridges

Currently, the preparation of complex biological samples prior to analysis is quite complex, laborious, and time-consuming. In fact, it is the rate limiting step in molecular analysis because it involves many manual operations including reagent preparation and calibration, pipetting, vortexing, centrifugation, phase separations, and the employment of multiple

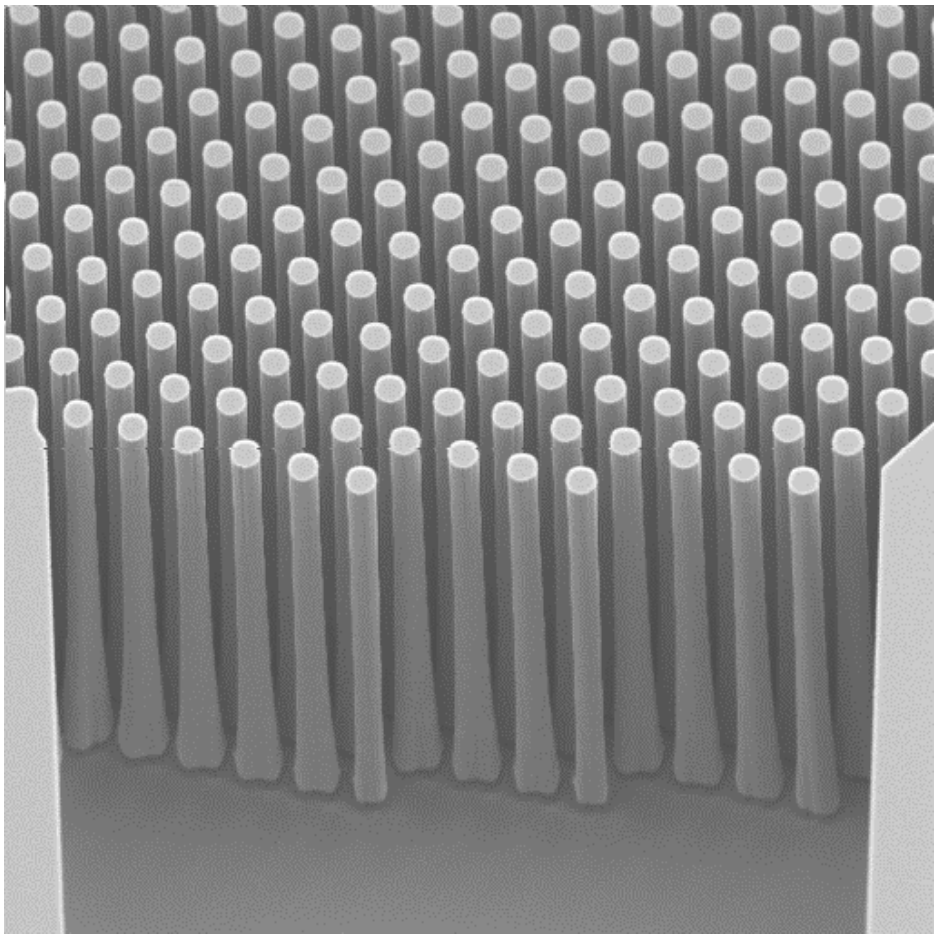

Figure 6. SEM of micromachined silicon chip for DNA extraction. that consists of 200 um high columns, each about 18 um in diameter and a pitch of 34 um.

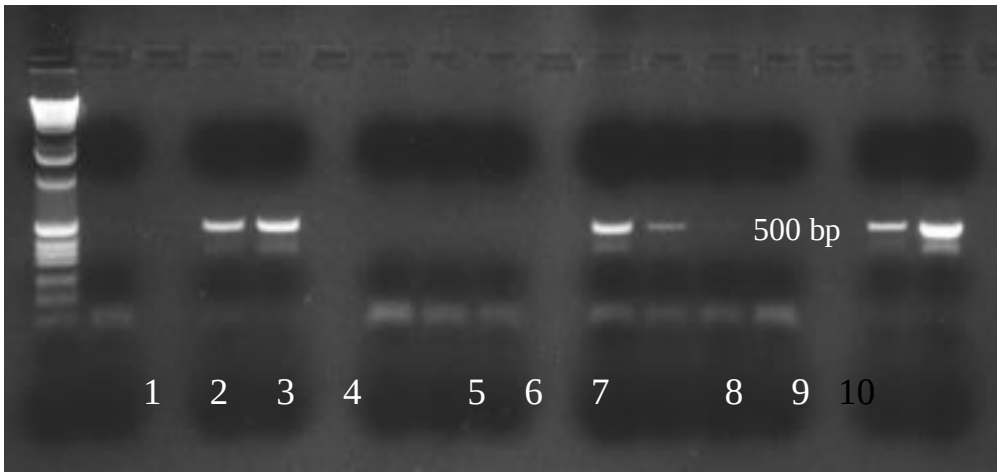

Figure 7. Photograph of electrophoretic gel showing DNA chip capture. Lanes: 1: Neg Ctl; 2: 10^4 standard in system water; 3: 5 x 10^4 standard in system water; 4-6: control run with no DNA; 7-10: chip elutions from DNA run; 11: 10^4 standard in pure water; 12: 5 x 10^4 standard in pure water. By comparing lane 7, obtained from the first 25 uL chip elution, to standard lanes 2 and 3, it is estimated that a capture efficiency of 50% was achieved, with a concentration factor of about 10x in the first elution.

instruments. A key focus of our efforts is the design of microfluidic-based cartridges that perform all of the sample preparation steps automatically on large input volume specimens. The manual methods listed above are all bolus-oriented, i.e., the entire sample is subjected to a single operation until it is completed before moving on to the next operation. Our designs are such that after adding the sample multiple operations can be conducted simultaneously on different parts of the sample as it flows through the device to an integrated waste region. This is analogous to how modern chemical processing plants routinely operate. Additionally, in microfluidic schemes, advantage is taken of splitting the fluids into smaller streams or regions because it can be assured that every fluid molecule is subjected to the same micro-environment.

Figure 8 shows a microfluidic cartridge that accepts blood or other complex starting material. A flow-thru design, one or more layers of plastic encapsulate filters and a DNA chip in fluid communication with reagent input regions, waste region(s), a PCR reaction tube, and vents. In operation, the raw sample is mixed with a lysis solution and pipetted into the sample chamber, and wash and elution reagents or PCR reagent would be pipetted into the other chambers. A cap containing pneumatic fluid drive ports is used to close the cartridge and the device is placed into a processing instrument. The lysed sampled is pumped through a coarse filter to remove particulates that might clog the fluid channels, continue through the DNA chip, and eventually into the waste area. Then wash and elution/PCR reagent fluids are sequentially pumped through the DNA chip, the latter then being diverted to the PCR reaction tube.

Applications

Currently, the standard method (T449om-90) for measuring the bacterial spore content in paper intended for food containment is based on spore germination and growth in culture media, and results are obtained no sooner than 48 hours after the start of the analysis (2). Newer methods based on PCR are promising to shorten the length to time to a result in 8 hours, but still requires spore germination, PCR, and post-PCR analysis by gel electrophoresis. It is not yet clear if these newer approaches will have adequate sensitivity and precision of quantitation. With Cepheid microfluidic technologies and fast PCR, it is now possible to contemplate results in less than 30 minutes which would dramatically reduce the inventory carrying costs associated with holding lots of paper for one or more days. For example, the following protocol could be adapted to a fluidic scheme:

1. Add 0.5 gm paperboard sample to 10 mL water (5%w/vol)
2. Disintegrate for 5 minutes
3. Separate paper fibers from water and spores (10-25 um cutoff filter)
4. Capture spores on 0.22 or 0.45 um filter
5. Mechanically or chemically disrupt spores (2 seconds to 3 minutes)
6. Flow through fine filter
7. Flow through DNA chip
8. Wash and elute target DNA from chip
9. Flow into PCR reaction region containing dried-down PCR mastermix (using fluorescence probes)
10. Commence thermal cycling and detection

Figure 9 shows an isotropic shade drawing of a type of prototype cartridge Cepheid is evaluating that be could adapted the above protocol. This cartridge coupled with the MicroBE instrument would provide a system capable of the field-testing of paperboard samples in less than 30 minutes.

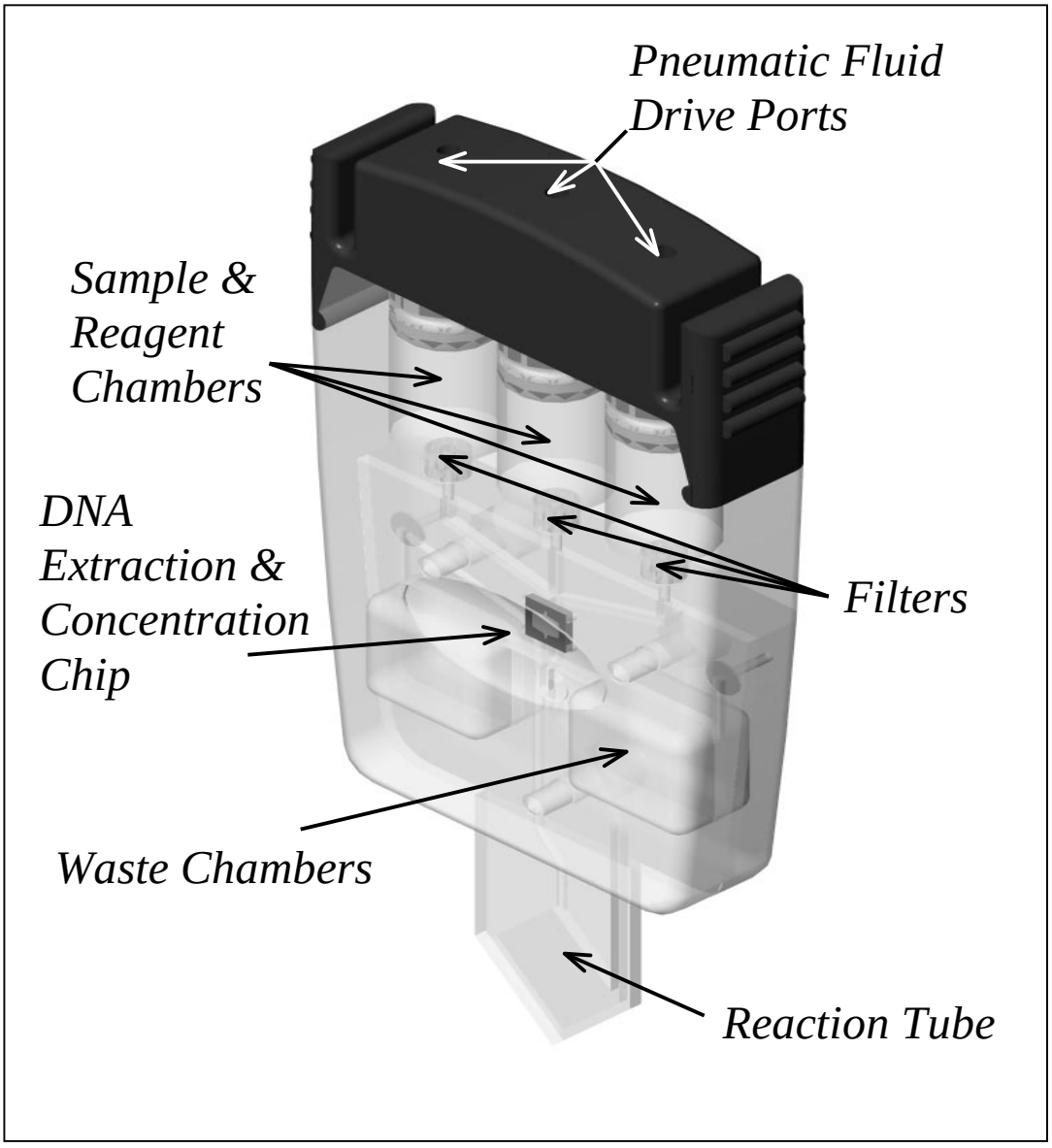

Figure 8. Integrated Fluidic Cartridge. This plastic device contains a DNA chip, sample and reagent ports, filters, integrated waste, and PCR tube.

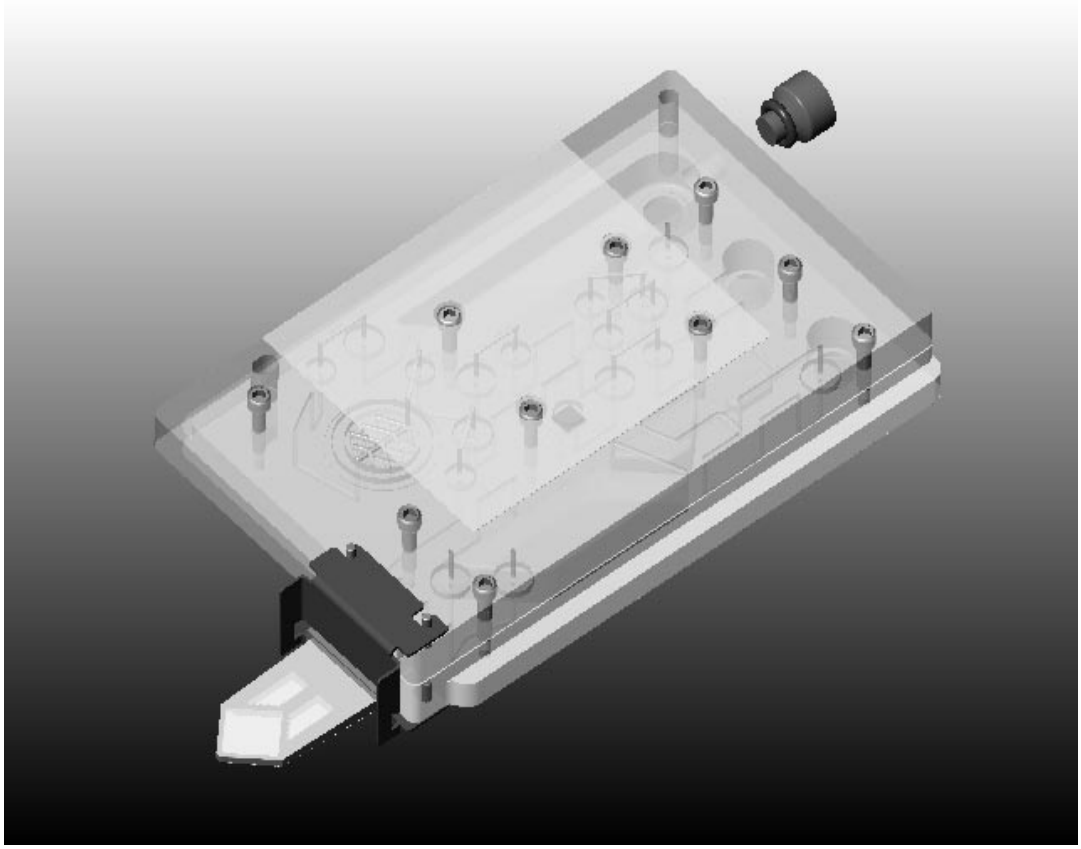

Figure 9. Prototype Cartridge Suitable for Adaptation to the Analysis of Spores in Paper Products. The device is pneumatic pressure driven, and includes a DNA purification chip.

Conclusion

Microfluidic technologies and rapid-PCR, when integrated into miniature, portable instruments and inexpensive, disposable cartridges, will for the first time enable the rapid and quantitative analysis of microbial targets from complex mixtures. No single technical approach is sufficient to meet this objective; however, plastic and silicon microfluidics provide a critical, universal technology base for these future systems. Cepheid has demonstrated significant progress towards this goal (1) by developing and demonstrating rapid PCR with real-time fluorescence detection, (2) by showing highly efficient extraction of DNA in silicon microstructures, and (3) by designing and fabricating continuous-flow prototype cartridges with high sample volume capacity adaptable to the analysis of low copy number targets in complex specimens.

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