Published in final edited form as:
Semin Perinatol. 2010 April ; 34(2): 163–169. doi:10.1053/j.semperi.2009.12.008.

Digital Microfluidics: A Future Technology in the Newborn Screening Laboratory?

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Abstract

Expansion of newborn screening for inherited metabolic disorders using tandem mass spectrometry has generated interest in screening for other treatable conditions, including lysosomal storage diseases. Limitations to expansion include labor and equipment costs. We describe a cost-effective new platform that reduces the time to result reporting and can perform multiplexing assays requiring different platforms. Immunoassays and enzyme activity assays currently used in newborn screening have been translated to a disposable microchip programmed to dispense, transport, mix, wash, and incubate individual microdroplets from specimens, including dried blood spot extracts, and reagents all under software control. The specimen and reagents consumed are approximately 1% of those required by equivalent bench assays. In addition to immunologic and enzymatic assays, DNA amplification, amplicon detection, and sequencing have been demonstrated using the same microchips and control equipment. Recently, the multiplexing of 4 different enzyme activities has also been demonstrated with negligible cross-contamination. We review assays relevant to newborn screening.

Keywords

newborn screening; lab-on-a-chip; lysosomal storage disease; severe combined immunodeficiency; digital microfluidics

Newborn screening for inherited metabolic disorders began in the early 1960s with a simple mission—to identify neonates at risk for phenylketonuria and treat them with a special diet to avoid the inevitable consequences of profound mental retardation that afflicts untreated individuals with phenylketonuria.[1] The specimens were collected as dried blood spots (DBS) on special filter paper, usually sent to a state public health laboratory, and analyzed for phenylalanine content by an inexpensive bacterial inhibition assay.[2,3] Neonates with presumptive positive screening tests were recalled for a diagnostic test, which, if positive, would enable treatment to begin before irreversible brain damage ensued. During the next several decades, additional tests were introduced to screen for more conditions that cause mental retardation, including congenital hypothyroidism and galactosemia. By the mid-1990s,

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newborn screening was mandated in the United States for at least these conditions, and in several states for as many as 3 or 4 additional conditions.

The addition of an expanded screening panel of approximately 30 heritable metabolic conditions occurred from 1997 to 2007 with the introduction of tandem mass spectrometry (MS/MS), a technology that detects multiple disease biomarkers simultaneously in a single specimen.[4-6] The sudden increased attention to newborn screening that occurred during this period has prompted widespread interest in the addition of more conditions to the newborn screening panel, especially those for which new treatment options have been developed.[7] Workshops, proposals, and pilot studies aimed at the expansion of newborn screening for lysosomal storage diseases,[8,9] severe combined immune deficiency (SCID),[10,11] fragile × syndrome,[12,13] and other conditions are rapidly proliferating. This situation is causing some concern because the additional costs of technology and manpower required to perform these assays is likely to place an additional burden on resources that are already stressed. Furthermore, the lack of resources in developing countries, even for basic newborn screening for feasibly treatable disorders, leaves most of these countries with no option but to continue to accept the burden of untreated genetic diseases exacerbated by consanguinity among many cultures.[14]

With advances in treatment for more of inherited diseases, the rationale to initiate screening programs is becoming clearer. But with that, it is also clear that new technological developments are needed to perform mass screening tests more cost-effectively than is possible with current methods. An additional problem is that the limited amount of blood obtained with current standard newborn screening sampling methods will ultimately limit the expansion of screening tests unless more efficient use can be made of the available specimen. One practical solution to both the cost and specimen limitation problems is to develop assays that can be translated onto a miniature testing platform, such as a microfluidic microchip, which uses smaller specimen volume at a reduced testing cost. The concept of a lab-on-a-chip is that it should enable fluid dispensing, transport, mixing, incubation, detection, and disposal within a self-contained unit. Lab-on-a-chip systems can be broadly categorized into continuous-flow and discrete-flow systems.

Continuous-flow microfluidic systems rely on liquid that is continually fed into fixed channels. An alternative system, referred to as digital microfluidics, manipulates liquids as discrete microdroplets under software control. This approach has several advantages over continuous-flow systems, the most important being reconfigurability and scalability, offering a truly generic microfluidic platform. This platform has already been used to develop several assays, including immunologic, enzymatic, and DNA-based tests of the type currently used in newborn screening laboratories. Here, we describe examples of these assays that indicate the potential of digital microfluidics to provide an attractive, low-cost, integrated alternative to technologies currently applied to newborn screening.

The Basic Concept of Digital Microfluidics

The promise of the lab-on-a-chip concept is the capability to handle all steps of analysis from sampling, sample preparation, sample-processing, mixing, incubation, and detection to waste handling on the chip. Due to the limitations in versatility and functionality in the dominant paradigm of continuous-flow fixed-channel-based microfluidic devices, the most logical alternative requires manipulation of discrete submicroliter droplets independently and directly under an electric field.[15] The droplets are sandwiched between 2 parallel plates where the top plate physically contains the droplets and the bottom plate contains an array of individually addressable control electrodes. The surrounding space is filled with immiscible oil to prevent evaporation of the droplet. An electric field, when applied to a control electrode on the array,

modulates the interfacial tension between the droplet and the hydrophobic surface on the bottom plate. This effect, called electrowetting, can be used to transport droplets using surface energy gradients established by activating a pattern of control electrodes on the bottom plate along any path of contiguous electrodes. In addition to transport, other operations including merging, splitting, mixing, and dispensing of droplets are accomplished in the same manner, simply by varying the patterns of voltage activation.[15,16] Large numbers of droplets can be simultaneously and independently manipulated, allowing complex protocols to be flexibly implemented directly through software control. There is no Joule heating within the droplets and near 100% utilization of sample or reagent is possible because there are no channels to be primed.

A chip designed to perform up to 8 assays on each of 12 samples is depicted in Figure 1, and is also shown schematically in Figure 2. The basic design includes 12 specimen wells at the left edge (Fig. 2) and 4 reagent wells on 2 other sides (top and bottom in the figure). It also includes wash and waste reservoirs and is the chip used for several of the preliminary results reported in this manuscript. These chips are compatible with the analyzer shown in Figure 1, which is a prototype of the model that will be supplied for newborn screening pilot studies. A full description of this analyzer is beyond the scope of this article. Essentially, it comprises an integrated electric switch array that is preprogrammed to electronically dispense and mix reagents with samples. It completes all fluid handling steps required for the assays, including detection, on the microchip under software control and without operator intervention.

The key characteristics that make digital microfluidic technology attractive to newborn screening include the following:

- **i.** Low volumes—each test consumes 0.2%-0.3% (300 nL) of the total aqueous extract (100 μL-150 μL) from a single 3-mm blood spot punch. Reagent volumes are similarly reduced to a small fraction of those used in current bench assays, which significantly lowers reagent and solvent costs.
- **ii.** Direct translation of existing assays—several of the methods used in current newborn screening assays, including immunologic and spectrophotometric enzyme assays, can be directly translated to the chip using existing assay kits with minor modifications.
- **iii.** Automation—reduces numerous manual steps to full automation on the chip. This improves accuracy and reliability of the tests and at the same time greatly reduces manual labor and equipment costs.
- **iv.** Portability—miniaturized, battery-powered instruments will include handheld devices for potential use at bedside, at birthing center or by midwives at home.
- **v.** Inexpensive Manufacturing—disposable chips are produced using widely available printed circuit board technology. The instrument consists of an integrated electronic switch array, a microprocessor, built-in heaters, magnets, and a detector with minimal to no electromechanical components, and therefore it is quite inexpensive.
- **vi.** Sample compatibility—droplet transport is relatively insensitive to pH, viscosity, ionic strength, and water content. Whole blood, serum, plasma, and several other biological fluids are compatible.[18]
- **vii.** Scalability—since there are no interconnected channels, each droplet is discretely manipulated, which affords scalability of number of droplet operations. Design and layout of chips is modular and facilitates scale-up for additional assays.
- **viii.** Multifunctionality—multiplexing of similar assays and integration of different methodologies, such as immunoassays with DNA amplification plus amplicon

detection and sequencing, for example, onto the same cartridge will allow more tests to be added, obviating the need for additional technology investment.

Example Newborn Screening Assays

Examples of assays already developed or under development and are of relevance to newborn screening are provided in the ensuing sections.

Immunoassays

Current immunoassays used in newborn screening laboratories include the 17-OH progesterone assay for congenital adrenal hyperplasia, thyroxine and thyroid-stimulating hormone for congenital hypothyroidism, galactose and galactose-1-phosphate uridyl transferase for galactosemia, and immunoreactive trypsinogen assay for cystic fibrosis. Although none of these particular assays has yet been developed on the microchip, Advanced Liquid Logic Inc. (ALL) has already developed several immunoassays on the digital microfluidics platform and performed thousands of tests to characterize the platform. For example, an assay for the cytokine interleukin (IL)-6 has been developed.[19] The basic principle of the assay is depicted in Figure 3. In this assay, 1 droplet (1 μL) each of magnetic beads labeled with anti-IL-6 antibody, blocking agent, and anti IL-6 antibody labeled with alkaline phosphatase were dispensed and merged in that order. One droplet of sample (1 μL) was dispensed and merged with the droplet containing the beads and the antibodies and was allowed to incubate for 2 minutes. The antibody-magnetic bead-analyte sandwich complex was transported to an electrode with a permanent magnet placed underneath the cartridge. The magnetic beads with the immune complex were attracted by the magnet and the supernatant removed as depicted in Figure 3. The beads were washed using a serial dilution protocol in which a fresh wash buffer droplet was added to the bead droplet while attracting and retaining the beads with the magnet underneath, and the supernatant was removed by energizing the adjacent electrodes and switching off the intermediate electrode. This step resulted in 2 droplets, one containing the beads resuspended in fresh wash buffer and the other containing the supernatant with excess, unbound material. This process was repeated until all the unbound material was removed. Lumigen APS-5 (chemiluminescence substrate) was added after washing was completed, and chemiluminescence measured over a period of 120 seconds using a photon-counting photomultiplier tube. The area under each kinetic curve of the CL measurement was calculated and plotted against the concentration of the analyte to obtain a standard curve as shown in Figure 4. A 4-parameter logistic fit was performed to model the standard curve data. The total assay time for an enzyme-linked immunosorbent assay on IL-6 was less than 8 minutes, including the detection step.

The device shown in Figure 2 facilitates multiplex assays by performing up to 8 immunoassays on each of 12 samples. All the required reservoirs for wash buffer, reagents, waste, substrates, and samples are built into the chip so the user needs only to load the sample and reagents into the respective reservoirs and an electronic program then performs all the immunoassays. Newborn screening tests use immunoassays that are modified for fluorescence and will simply rely on the same miniature fluorescence detector described in the next section.

Enzyme Assays

There is considerable interest in screening newborns for lysosomal diseases (LSDs), partly owing to the development of enzyme replacement and other therapies[7] and the development of enzyme assays suitable for DBS.[8] One of these screening methods uses MS/MS to quantify the products from reagents targeted to specific LSD enzymes.[20] These novel reagents more closely resemble the natural enzyme substrates than typical fluorogenic (4-methylumbelliferyl [4-MU]) substrates, and several of them, by design, also incorporate a fluorophore that permits their use with either MS/MS or fluorometric assay platforms.

A multiplex assay has recently been developed on the digital microfluidics platform for 4 lysosomal enzymes: acid α-glucosidase (Pompe), α-galactosidase (Fabry), α-L-iduronidase (Hurler), and β-glucuronidase (MPS VII) and applied successfully to newborn screening specimens.[21] The test was developed with conventional 4-MU substrates following literature methods and required only 0.3 μL per assay from a total aqueous extract of 150 μL from a single 3-mm punch from a dried blood spot. Four assays were simultaneously performed from the same extract, consuming a total volume of only 1.2 μL, equivalent to approximately 50 nL blood. The substrates for each enzyme assay were the 4-MU derivatives of α-L-iduronic acid, α-D-galactose, α-D-glucose, and β-D-glucuronic acid that were required to target Hurler, Fabry, Pompe diseases, and MPS VII, respectively. These reagents, prepared in appropriate buffers at the required optimum pH for the assay (3.5, 4.5, 3.8, 4.8) plus appropriate inhibitors were loaded into reagent wells (Fig. 2). In the experiment reported here, the chip was programmed to perform 4 enzymatic assays simultaneously on each of 4 different DBS extracts. Each extract was loaded into a separate sample reservoir on the chip, which was programmed to dispense 4 droplets of 0.3 μL each. Each droplet was then distributed on the chip in its reaction area and mixed with the substrate droplets of 0.3 μL for Pompe disease, Fabry, disease, Hurler disease, and MPS VII. After mixing on-chip for about 1 minute, each mixed droplet was split into 2 sets of droplets of equal volume, 0.3 μL each. Then, each of the first set of droplets was mixed with 1 droplet of a quench buffer ($NaHCO_3$ at pH 10) and fluorescence was measured, which was recorded as time t = 0 measurement. The second set of droplets from each reaction was allowed to incubate at 22°C for up to 8 hours and then quenched with the same buffer. The fluorescence from each droplet (time t = 0 and *t* = 2, 4 or 8 h, respectively) was read at 350-μm excitation and 420-μm emission wavelength by a fluorometer. The fluorescence obtained from each of these reactions from a normal DBS are displayed in Figure 5. The increase in signal above background was easily detected after only 2-hour incubation, and increased linearly for about 4-8 hours. It should be noted that although all the droplets were sequentially transported to the same electrode on the chip positioned below a miniature, custom-built fluorometer, there was no discernible carry-over from droplet to droplet.[21]

These results are preliminary and are given here only to demonstrate proof-of-principle. Nevertheless, there is reason to be encouraged by the ease with which the device handles these assays and the considerable reduction in time to result compared with current end-point assays. It is also worth mentioning that several existing enzyme assays used in newborn screening, such as for biotinidase[22] and immunoreactive trypsinogen for cystic fibrosis screening[23] are fluorescence-based and could therefore readily be adapted to the digital microfluidic platform.

DNA-based Assays

Although DNA-based assays are not currently routinely used in newborn screening, pilot studies have already been initiated in a few programs in the United States to screen for SCID using a DNA-based method that targets T-cell receptor excision circles (TREC), which are normal byproducts of T-cell maturation.[10,11] The absence of a DNA product after polymerase chain reaction (PCR) of the target using TREC-specific primers is strongly correlated with T-cell deficiency conditions, including SCID and complete DiGeorge syndrome. ALL has also developed a digital microfluidic lab-on-a-chip for performing real-time PCR. It has been successfully tested on several targets, particularly DNA from various infectious agents, including *Candida albicans, Bacillus anthracis, Francisella tularensis*, methicillin-resistant *Staphylococcus aureus*, *Mycoplasma pneumoniae*, as well as human genomic DNA targets.

DNA amplification on-chip is performed by shuttling a droplet containing the specimen plus primers and reagents between 2 thermal zones set up with built-in heaters that provide both accurate temperature and rapid heating and cooling for thermal cycling (Fig. 2). Because only a submicroliter-sized droplet is subject to thermal cycling, extremely rapid thermal cycling is

achieved. Detection of amplification products is performed by measuring the fluorescence levels at each cycle, thereby providing real-time data for quantification of the DNA. After each thermal cycle, the droplets are routed to a spot where fluorescence detection is performed by a miniature, custom-built fluorometer. For example, the following protocol describes PCR amplification of DNA on-chip to amplify a 273-bp fragment from the 18S ribosomal RNA gene (131-383 bp) of *C. albicans* strain ATCC (American Type Culture Collection) 10231. The final reagent concentrations in the PCR reaction mix were 50 m_M KCl, 20 mM Tris-HCl pH 8.4, 200 μ_M each dNTP, 25 U/mL iTaq polymerase, 3 m_M $MgCl_2$, and 1 μ_M oligonucleotide primers. The target DNA concentrations in the PCR reaction were chosen to provide 1, 10, or 100 copies of the *C. albicans* genome. After PCR on-chip, the amplicons were detected by Eva Green fluorescence. The samples displayed C_t values of 21, 24, and 27 after amplification with a total time to result of about 5 minutes (Fig. 6). The specificity of the PCR reaction on-chip was also tested by agarose gel electrophoresis. Only 1 band for each sample was observed corresponding to the expected 273-bp amplicon of *C. albicans* (Fig. 6).

ALL has also recently performed on-chip PCR on human genomic DNA to amplify the TREC region (data not shown). Because there are several possible diagnoses from a positive screen, a second-tier test or tests will be required to provide more definitive results. These assays, which may include immunoassays for IL-7, CD3, and CD4 as well as additional tests, can be multiplexed onto a single cartridge designed for this purpose. It should be noted that the cartridge depicted in Figure 2 can be configured in software to run PCR reactions or immunoassays. More recently, the same cartridge was programmed to perform enzymatic assays also. The only difference in running different types of assays is that the chips are programmed differently to suit the droplet operations required for the assay and assay-specific reagents are loaded. Otherwise, the instrument and the cartridge remain the same between enzymatic assays, immunoassays, PCR, and even DNA sequencing.

Test Development in the Newborn Screening Laboratory and at the Point of Care

ALL, in collaboration with Duke University Biochemical Genetics Laboratory, the Jean and George Brumley Jr. Neonatal-Perinatal Research Institute at Duke, and a state newborn screening laboratory, is committed to the development of new products for screening newborns. One project is to develop a product that will permit multiplexing of 5 LSD enzyme assays on 48 specimens on a single cartridge. Funding for this development under a Phase II Small Business Innovation Research grant from the Eunice Kennedy Shriver National Institute of Child Health and Human Development has been secured, and the high-throughput device is expected to be developed, tested, and deployed in pilot newborn screening studies within the next 12 months. Ongoing studies with the existing microchip include more complete pilot experiments with DBS samples from known affected patients with Fabry, Pompe, Hurler, and other LSDs. Collecting sufficient numbers of specimens from untreated patients to perform such studies is challenging, but necessary to establish feasibility for population-wide screening in a newborn screening laboratory. In addition to offering an attractive new platform for high-throughput newborn screening, the digital microfluidics system is eminently suitable for point-of-care testing.[17] Thus, an alternative strategy to screen newborns in the newborn nursery using a small quantity of whole blood can be considered. Instead of collecting and shipping dried blood specimens to a central laboratory and waiting days to weeks for results, screening could occur in the birth hospital with results readily available to the primary, and possibly subspecialty, caregiver.

Another successfully funded research project will target hyperbilirubinemia (HBR), a common and potentially serious condition in developed and developing countries, for which a rapid turn-around point of care test for bilirubin level and potential underlying causes such as

glucose-6-phosphate dehydrogenase (G6PD) deficiency is urgently required.[24-26] The aim of this research is to translate the current clinical test for HBR onto the digital microfluidics platform and evaluate its performance alongside the current testing method. Additionally, chip-based assays will be developed for congenital hypothyroidism and G6PD deficiency, the 2 most serious underlying conditions that can present with HBR, possibly multiplexed with the HBR assay. If successful, this will provide a basis for scaling-up to high-throughput inexpensive newborn screening assays for congenital hypothyroidism and G6PD deficiency.

Acknowledgments

Partially supported by Award Number R44HD057713 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Funding was also provided by the Jean and George Brumley Jr, Neonatal-Perinatal Research Institute.

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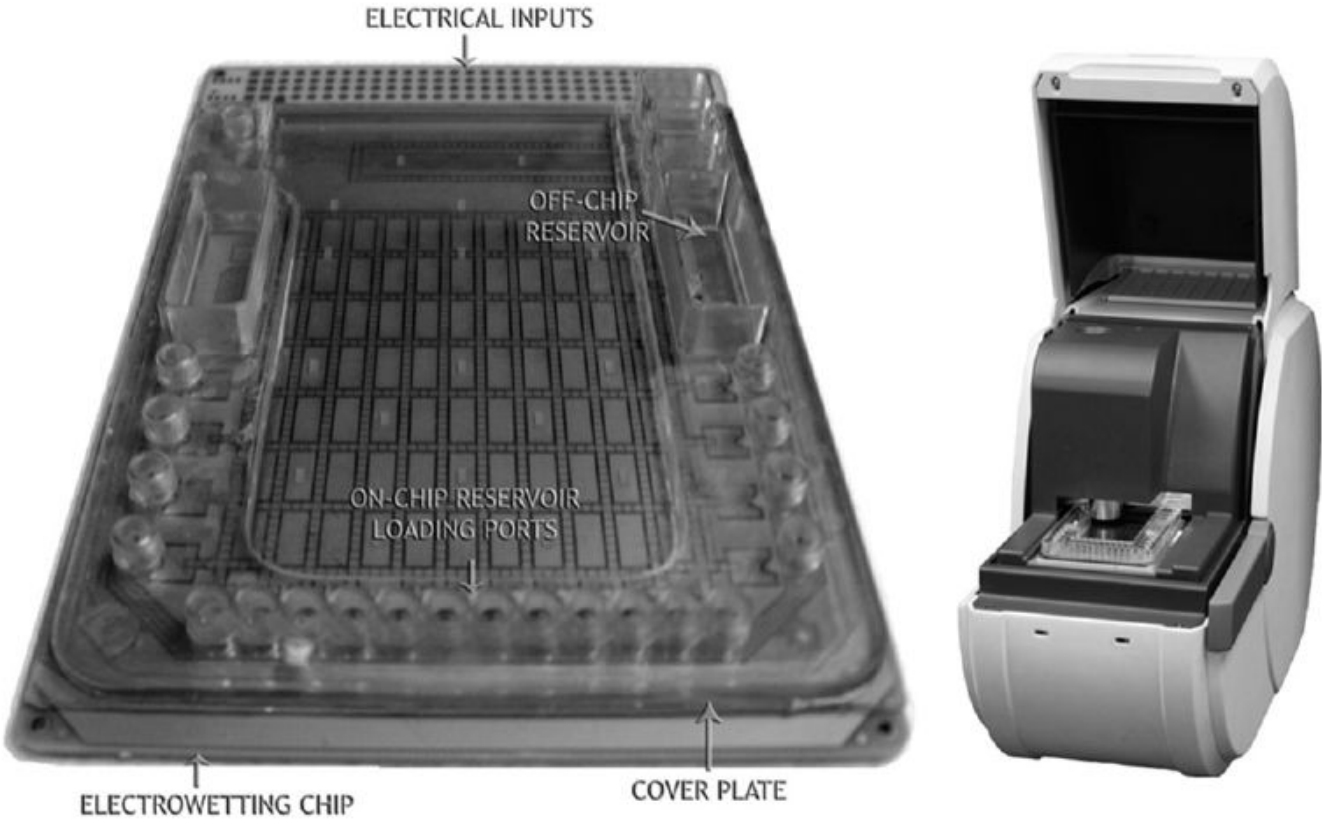

Figure 1.
(Left) A fully assembled digital microfluidic multiwell plate cartridge (8.55 × 12.78 cm); (Right) the control instrument (20.32 cm W ×33.02 cm H ×53.34 cm L). (Reprinted with permission.[17])

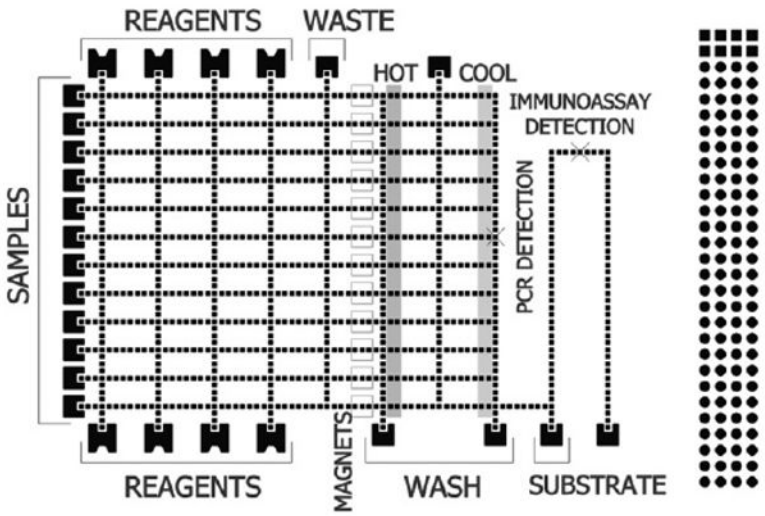

Figure 2.
Schematic of the digital microfluidic chip used for immunoassays and PCR. (Reprinted with permission.[17])

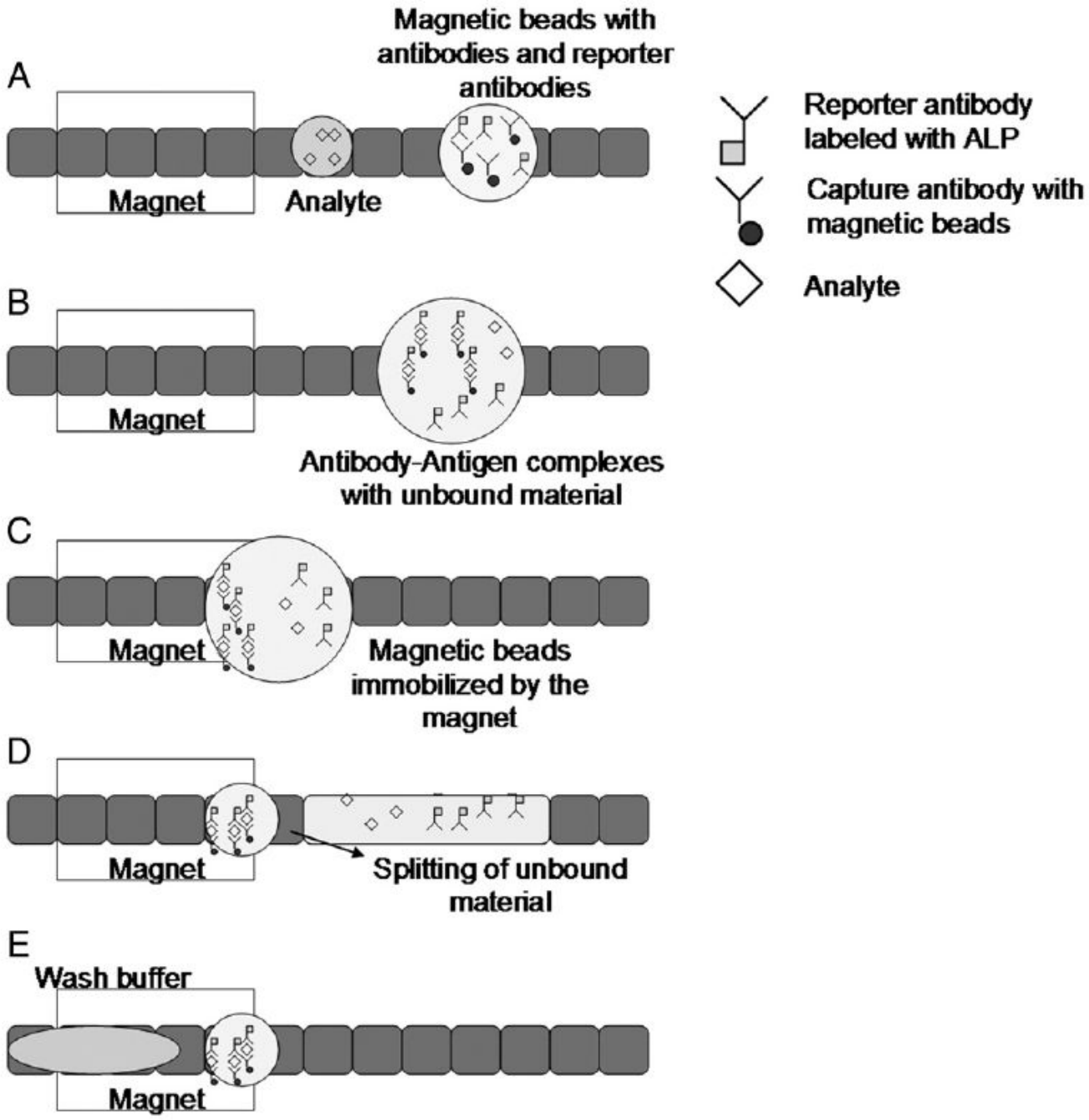

Figure 3.
Protocol for heterogeneous immunoassay on a digital microfluidics platform. (A) Dispensing of reagents, (B) incubation, (C) immobilization of magnetic beads, (D) removal of supernatant and washing, and (E) adding fresh wash buffer. (Reprinted with permission.[19])

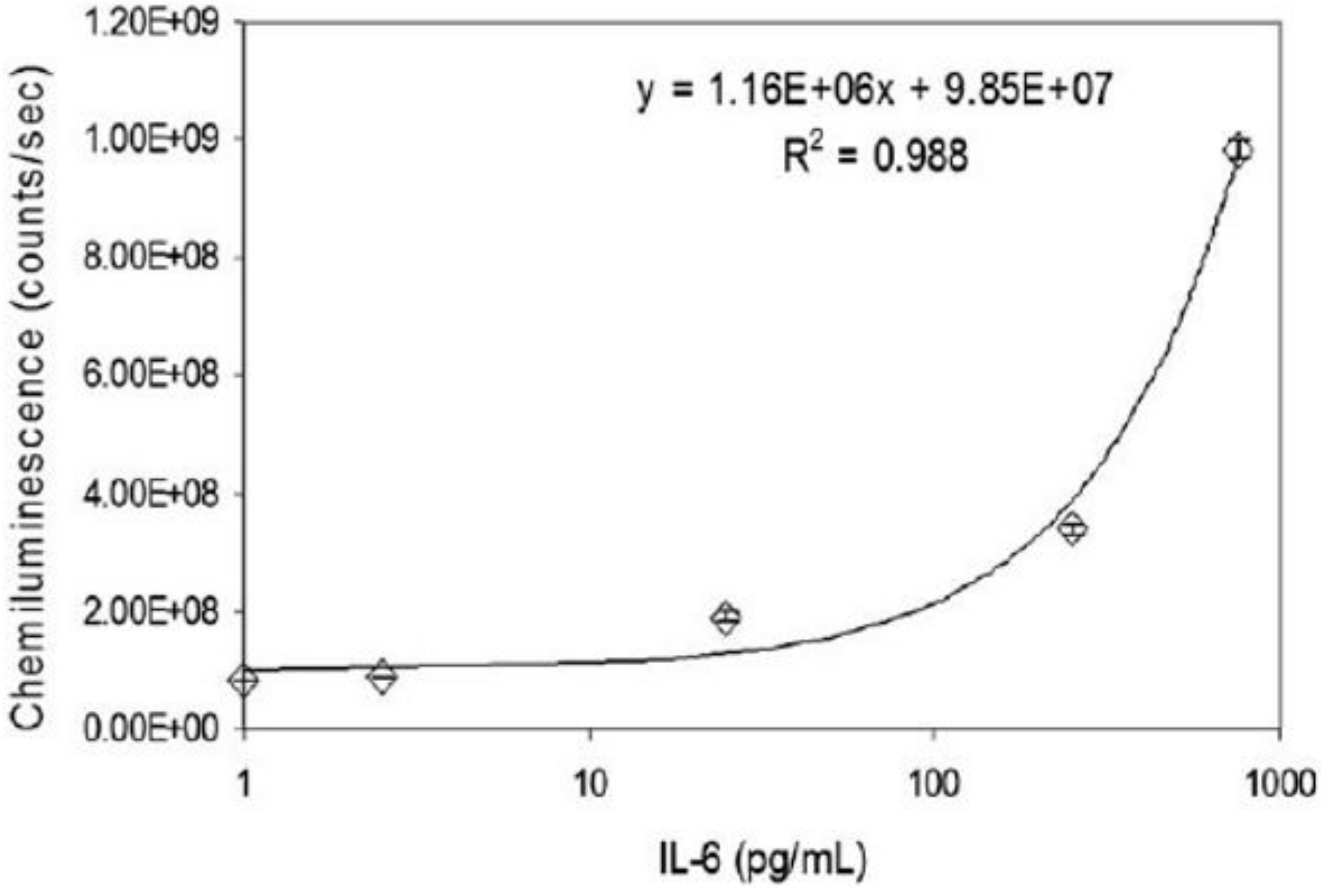

Figure 4.
Standard curve for IL-6 generated on a digital microfluidic chip. (Color version of figure is available online.)

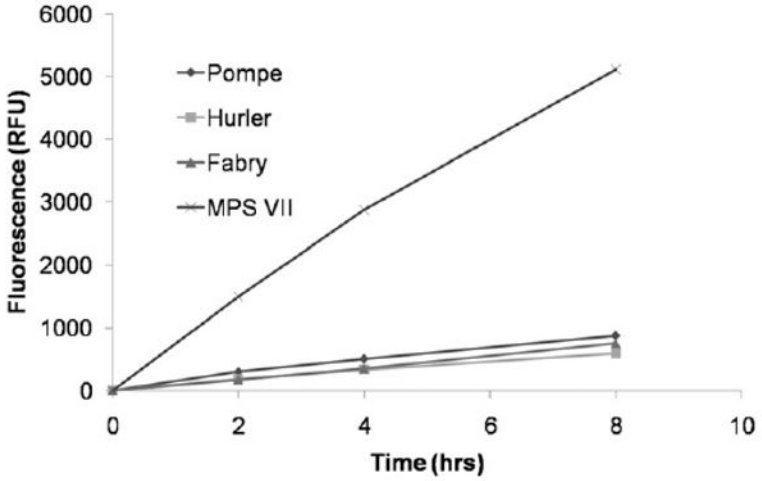

Figure 5.
Increase in fluorescence units with time for 4 lysosomal enzyme assays performed simultaneously on the same microfluidic chip.

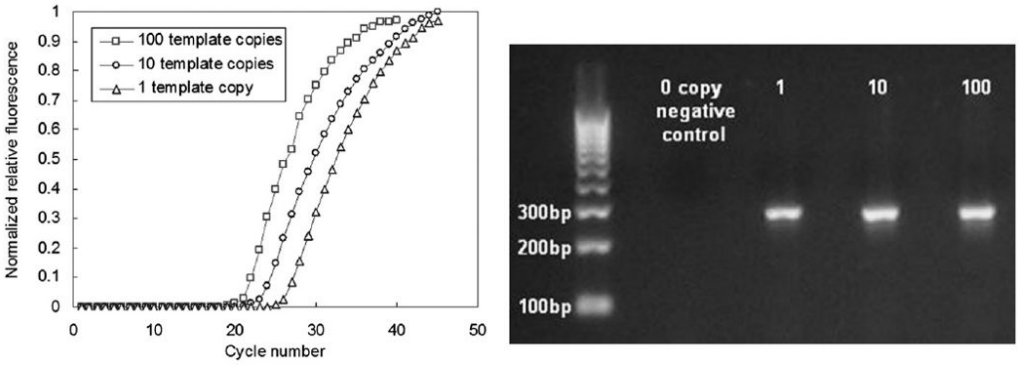

Figure 6.
Real-time on-chip PCR titration of samples (left) and corresponding gel images (right) with different number of *Candida* genome copies. Reprinted with permission.[17]