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A hybrid approach to device integration on a genetic analysis platform

Des Brennan1, Dorothee Jary2, Ants Kurg3,4, Evgeny Berik3, John Justice1, Margaret Aherne1, Milan Macek5 and Paul Galvin1

1 Tyndall National Institute, University College Cork, Lee Maltings, Prospect Row, Cork, Ireland
2 Department of Technology for Biology and Health, CEA-LETI, 17 Avenue Des Martyrs, 38054 Grenoble, France
3 Asper Biotech Ltd, 17a Vaksali st., Tartu 50410, Estonia
4 CEA-Institute of Molecular and Cell Biology, University of Tartu, Riia 23, 51010 Tartu, Estonia
5 Institute of Biology and Medical Genetics, Charles University, V Uvalu 84, Praha, 5CZ 150 06 Prague, Czech Republic

E-mail: des.brennan@tyndall.ie

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Abstract

Point-of-care (POC) systems require significant component integration to implement biochemical protocols associated with molecular diagnostic assays. Hybrid platforms where discrete components are combined in a single platform are a suitable approach to integration, where combining multiple device fabrication steps on a single substrate is not possible due to incompatible or costly fabrication steps. We integrate three devices each with a specific system functionality: (i) a silicon electro-wetting-on-dielectric (EWOD) device to move and mix sample and reagent droplets in an oil phase, (ii) a polymer microfluidic chip containing channels and reservoirs and (iii) an aqueous phase glass microarray for fluorescence microarray hybridization detection. The EWOD device offers the possibility of fully integrating on-chip sample preparation using nanolitre sample and reagent volumes. A key challenge is sample transfer from the oil phase EWOD device to the aqueous phase microarray for hybridization detection. The EWOD device, waveguide performance and functionality are maintained during the integration process. An on-chip biochemical protocol for arrayed primer extension (APEX) was implemented for single nucleotide polymorphism (SNiP) analysis. The prepared sample is aspirated from the EWOD oil phase to the aqueous phase microarray for hybridization. A bench-top instrumentation system was also developed around the integrated platform to drive the EWOD electrodes, implement APEX sample heating and image the microarray after hybridization.

Keywords: microfluidics, integration, EWOD, hybridization

(Some figures may appear in colour only in the online journal)

1. Introduction

Lab-on-chip (LOC) technology has facilitated the emergence of microfluidic platforms dedicated to sample preparation, e.g., cell lysis, DNA extraction, purification, amplification and hybridization [1]. Dobson et al [2] reviewed a number of commercially available point-of-care (POC) systems highlighting the Cepheid system as a true ‘sample in, answer out’ platform. Such systems typically consist of (i) a passive disposable cartridge-type system within which biochemical processes are implemented, (ii) external pumps, valves and heaters for liquid manipulation and temperature control and (iii) detection components, e.g., fluorescence for real-time PCR. Technologies have been extensively demonstrated implementing discrete protocols for sample preparation or detection, e.g., cell lysis [3], target biomolecule amplification [4] or detection [5, 6]. A ‘sample in, answer out’ system...
must integrate biochemical processes for DNA extraction, amplification and detection in a seamless fashion. Integrating such functionality on a single platform offers the possibility for an autonomous ‘sample in, answer out’ POC system. Monolithic and hybrid systems have been reported as being able to implement biochemical processes in a seamless fashion. Woolley et al [7] developed a monolithic system to amplify DNA using PCR, delivering the sample via an ‘electrophoresis valve’ to a capillary electrophoresis (CE) detection zone identifying amplified DNA sizes. The CE channels were etched in glass and thermally bonded to a cover glass plate; fluidic reservoirs were fabricated in silicon with integrated titanium heaters. Easley et al [8] developed a fully integrated microfluidic system capable of generating a genetic profile; the system carried DNA extraction, purification, PCR amplification and laser-induced fluorescence detection on a CE channel. The fluidic channels and reservoirs were chemically etched (HF) and sealed using PDMS gaskets which also acted asvalving membranes, and resistance heaters were attached to the PCR reservoirs for thermocycling. In both approaches, sample preparation and CE-detection channels are fabricated on a single substrate facilitating integration. However, CE does not lend itself to a high-throughput analysis or mutation detection; it delivers information based on the DNA size not nucleotide sequence, and microarrays (electrical or optical) are required to identify specific mutations of interest in a high-throughput fashion. The on-chip analysis based on real-time PCR is also limited in throughput by the number of fluorescence dyes available and the complexity of optical multiplexing. Liu et al [9] developed an integrated biochip to process whole blood, adapting a modular approach integrating pumps, valves and reservoirs for sample preparation with an electrical microarray for hybridization detection. The system was composed of a plastic chip containing fluidics and reservoirs, a PCB with heaters and electrical contacts for piezoelectric pumps and an electrical eSensorTM from Motorola. All components were bonded to the PCB using double-sided pressure-sensitive adhesive (PSA). PCR was implemented on chip using a conventional thermocycling protocol (95, 55 and 72 °C) and the amplified DNA was subsequently analysed using cyclic current voltammetry. Integrated pumping and valving adds complexity to the device and the manufacturing process, while the possibility of rapidly manipulating small nanolitre volumes is limited by low flow rates and slow pump response time. The ability to move and manipulate small nanolitre droplets of reagent and sample with the electro-wetting-on-dielectric (EWOD) device is something that cannot be achieved with conventional pumps and valves; thus, EWOD is an ideal approach to low volume on-chip sample preparation. The EWOD device consists of an array of microelectrodes typically (2 mm × 2 mm); electrode combinations can be individually addressed to a high-voltage ac signal, instantaneously switching the surface property at the electrode–liquid interface from hydrophobic to hydrophilic [10–12]. Neighbouring electrodes are actuated to create a hydrodynamic pressure gradient on an aqueous droplet causing it to move in a controlled fashion. EWOD has been demonstrated in a number of biological applications; Shah et al [13] combined EWOD technology with an optical tweezers to analyse and manipulate live HeLa cells in different media. A significant challenge is presented in exchanging cells between droplets as there is significant Stokes drag on particles due to surface tension at the oil–aqueous phase interface; this also highlights the challenge in extracting aqueous droplets from the oil phase. Wang et al [14] fabricated an EWOD device with integrated heating and found no adverse effect on PCR performance although yield was reduced due to possible inhibition. They highlight localized heating causing unwanted droplet movement requiring integrated valves as a solution.

Previously, no integrated EWOD/microarray combination has demonstrated sample preparation in an integrated EWOD (oil phase) component and realizing detection on an integrated waveguide microarray (aqueous phase) component, with sample exchange between phases. The EWOD sample preparation demonstrated on the integrated system is biochemical protocols based on the SNiP analysis. SNiPs are DNA sequence variations responsible for hereditary disease when a single base in the genome sequence is altered or deleted [15]. The EWOD-prepared sample is used for integrated microarray detection based on arrayed primer extension (APEX) [16], a gene analysis technique which is fast, accurate and low cost used to screen for SNiPs. Genotype information is generated by exploiting a single-base extension mediated by a DNA polymerase to bind a dideoxynucleotide (ddNTP) labelled with a reporter dye (e.g. CY3) to its complementary target mutation. Multiplexing is typically achieved using a combination of four fluorescent reporter dyes specific to each nucleotide (adenine, guanine, cytosine, uracil); we have adopted a novel approach of spatially differentiating ddNTPs using four separate microfluidic channels. Each component is individually fabricated: (i) the polymer microfluidic is injection moulded, (ii) the EWOD device is using silicon microelectronic techniques (LETI) and (iii) the waveguide microarray is spotted using a robotic spotter (Asper Biotech). The plastic microfluidic also acts as a ground electrode to the EWOD and is processed with indium tin oxide (ITO) and silicon oxycarbide (SiOC) layers forming an electrode with a hydrophobic insulation layer. A bench-top system was assembled to implement (i) EWOD control, (ii) sample heating, (iii) fluidic exchange and (iv) optical detection.

2. Experimental details

2.1. Integration approach

A key challenge in realizing integrated diagnostic systems is maintaining biomolecule integrity. For hybridization, a DNA probe microarray is immobilized on the optical waveguide substrate prior to integration with the EWOD device and sealing with the microfluidic component. High temperature bonding strategies such as (i) anodic bonding (silicon–glass) and (ii) thermal fusion (glass–glass) can damage immobilized biomolecules. Ultrasonic welding is common for plastic bonding; however, immobilized biomolecules may be removed or denatured during the bonding process. Optically cured adhesives are extensively used to rapidly seal microfluidic
systems, eliminating channel blockage often encountered with thermally cured epoxies; however, UV radiation exposure damages DNA. The integration approach is to fix the EWOD and waveguide components in the plastic carrier substrate using a double-sided PSA (Applied Adhesives Research). The microfluidic lid is aligned to the EWOD reservoirs and glass microarray, brought into contact and sealed using the PSA as highlighted in figure 1. The PCB connects EWOD electrodes to drive electronics, and microtight fittings (Upchurch Scientific) are attached to the microfluidic lid as fluidic interconnects. The interconnects allow oil, sample and reagent loading as well as sample transfer from EWOD to microarray and post-hybridization wash. The transparent microfluidic lid facilitates fluorescent microarray imaging. The DNA probe microarray (80 spots/row, 20 spots/column) is oversized compared to microchannel dimensions, eliminating substrate alignment issues. Each spot along any given microarray row corresponds to a specific mutation of interest which is replicated along each column.

With spatial multiplexing, to screen for possible mutations associated with DNA bases A, C, G and U, the hybridization-detection step must take place in four separate microfluidic channels (1 mm wide, 0.1 mm deep, 20 mm long). Probe DNA spot size (100 μm) and pitch (200 μm) guarantee at least three spots per mutation across each channel. Following integration, the EWOD device was tested to verify performance. The EWOD microfluidic reservoirs were pre-loaded with silicone oil and the integrated system was then connected to the drive electronics. This instrumentation delivers a sinusoidal signal (120 VRMS, 3 kHz) selectively to EWOD electrodes via a Labview™ interface. As the EWOD electrodes are energized, the surface property at the solid/liquid interface changes from hydrophobic to hydrophilic, thereby moving droplets of Tris buffer in a controlled fashion.

In figure 2, a coloured dye is used to highlight the four hybridization channels, and the EWOD reservoirs are highlighted by the EWOD electrodes in the expanded view of
the EWOD device. A hydrodynamic pump and valve system implements hybridization and post-hybridization wash stages, while the EWOD component implements APEX preparation. Hydrodynamic pressure also moves the sample between the EWOD outlet reservoirs and the four hybridization channels. The EWOD electrodes define the reservoirs and fluidic paths along which the liquids move as the electrode–liquid interface becomes hydrophilic with applied voltage. The effect of liquid manipulation with changing electrode voltage is highlighted in figure 6 as a droplet is moved along the EWOD device.

2.2. Microfluidic fabrication

A Babylast 6/10P micro injection moulding machine was used to manufacture the plastic carrier substrate and the microfluidic lid from Zeonor® thermoplastic. Negative relief SU8 masters were defined on silicon substrates using photolithography. The microfluidic channel was 100 μm deep and 1 mm wide; these dimensions were required for the EWOD device and are suitable for low-cost acetate mask photolithography. The design layout was carried out on Mentor graphics CAD package and the masks printed by JD Phototools (2000 dpi). A 100 μm thick SU8 (Microchem Ltd) layer was spin coated onto a silicon wafer (single side polished, 4 inch, 750 μm thick). The substrate is exposed using an acetate mask aligner (Zeiss) defining the microfluidic design, and then the substrate is hard baked and developed using EC solvent. The master is placed in the injection mould cavity for fabrication (figure 2). Prior to injection moulding the Zeonor® pellets were dried in an oven for 40 min at 50 °C. The system temperatures were set as follows: (i) injection nozzle 195 °C, (ii) injection chamber 190 °C, (iii) plastic melt chamber 220 °C, with injection parameters of 60 bar pressure for a 3 s injection time. The manufactured microfludic lid then had thin films of ITO (180 nm) and SiOC (1000 nm) deposited. The ITO layer forms a planar ground electrode, while the SiOC forms a hydrophobic layer for the EWOD operation. To prevent plastic melting, we repeated three ITO depositions of 60 nm allowing the substrate to cool between evaporation cycles. The average surface roughness of the injection moulded parts was 70 nm, preventing liquid droplets ‘sticking’ during the EWOD droplet manipulation. To fabricate the polymer carrier substrate, a brass mould was CNC machined. The injection cavity (substrate size) was 40 mm × 45 mm containing negative relief structures forming pockets in the polymer substrate within which the devices were inserted. The EWOD device pocket was 11 mm × 12 mm × 750 μm, and the waveguide pocket was 55 mm × 25 mm × 150 μm.

2.3. Microarray spotting

DNA probe oligonucleotides identifying 20 mutations associated with the cystic fibrosis (CF) gene were spotted as a microarray (80 × 20) onto the glass waveguide substrate. The microarray was spotted by ASPER Biotech using a conventional spotting machine VersArray ChipWriter Proarrayer (BioRad Laboratories, Hercules, CA, USA) and SMP-3 spotting pins (TeleChem International, Inc, Sunnyvale, CA, USA). A predefined volume (0.7 nl) is dispensed on the substrate achieving 100 μm diameter spots. Prior to spotting the waveguide substrate is treated with ASPER SAL surface chemistry, enabling covalent binding of the probe DNA to the surface during the immobilization step.

2.4. System instrumentation

The probe DNA microarray is immobilized on a microscope cover slide (60 mm × 25 mm × 150 μm) with the short edges polished to an optically smooth finish for efficient laser coupling. As laser radiation is edge coupled, total internal reflection (TIR) within the glass substrate confines excitation radiation within the waveguide, minimizing stray light on the detection system. Uniform radiation illuminates the substrate at an incident angle greater than the critical angle (for glass in air 63°), exciting CY3 tagged ddNTPs incorporated into the probe/target DNA structure.

The system consisted of the Starlight (SXVF-H9C 4.9 mm × 3.65 mm array) monochromatic CCD camera for detection mounted with a CY3 emission filter. The excitation source is a green laser (3 mW, 532 nm from Edinburgh Instruments) focused to a 50 μm spot incident on the polished edge of the microscope slide (figure 3). The laser is located on a goniometer for angle selection/coupling into the microscope slide. Above the critical angle, TIR occurs and the microarray of CY3 tagged DNA spots are excited and emit fluorescence radiation at 570 nm (emission peak maximum). A lens system with 1:1 magnification consisting of two high numerical aperture lenses (NA = 0.17) each with field of view f/2.8 collects light over a 20° cone from the microarray and images onto the CCD camera.

A mechanical holder fixes the integrated device in place during biosample preparation and microarray imaging. It also facilitated fluidic and electrical interconnects to the EWOD device from the drive electronics and external liquid reservoirs etc (figure 4). A high voltage (150 V peak–peak) relay (SEALEVEL REL-32.PCI) is used to multiplex the EWOD electrodes and a Labview™ software program allowed the user to selectively address electrodes for liquid manipulation. To control temperature during the APEX protocol, a thermoelectric heater was fixed on a 90° translation stage (figure 5) which engaged the device for sample heating. The translation stage disengaged the heater from the device after hybridization wash steps, leaving a clear optical path.
3. Results and discussion

3.1. Integrated EWOD performance

The EWOD microfluidic reservoirs were pre-loaded with silicone oil and the integrated system was then connected to the drive electronics. This instrumentation delivers a sinusoidal signal (120 V_{RMS}, 3 kHz) selectively to EWOD electrodes via a Labview™ interface. As the EWOD electrodes are energized, the surface property at the solid/liquid interface changes from hydrophobic to hydrophilic, thereby moving droplets of Tris buffer in a controlled fashion. Figure 6 illustrates a 64 nl liquid droplet dispensed from a 500 nl sample reservoir and moved along the integrated EWOD electrode array.

Droplet movement is instantaneous and pressure gradients are limited to activated electrodes, making discrete droplet formation, movement and mixing possible.

3.2. Integrated waveguide/microarray performance

For successful waveguide integration, surface chemistry functionality and optical excitation efficiency must be maintained; this was verified by implementing the APEX hybridization assay on the integrated waveguide component using a sample prepared on the integrated EWOD device and aspirated to the four hybridization channels. To implement the APEX protocol within a microfluidic environment, reagents are manipulated at nanolitre volumes and modified to enhance EWOD droplet movement by adding a surfactant (5% TWEEN 20). This reduces surface tension, thereby assisting reservoir loading and enhancing droplet motion. The APEX input sample is amplified human genomic DNA from a bench-top 20 μl PCR reaction. The sample protocol was initial 5 min denaturation (95 °C) followed by 35 cycles of 30 s denaturation (95 °C), 30 s annealing (55 °C) and 30 s extension (72 °C). The final PCR step was 72 °C for 4 min. During PCR some dTTPs are replaced with dUTPs. Two 20 μl tube PCRs are pooled and 23.5 μl is removed and mixed with 6.5 μl of the enzyme mix (SAP/Exo I/UNG) and incubated initially at 37 °C for 5 min. Then, 2 μl of this mix is loaded by pipette onto the activated EWOD sample reservoir a (figure 7) and further mixed for 10 min at 37 °C by moving the sample back and forth.
Figure 7. The EWOD system is used to implement elements of the APEX biochemical protocol. Reservoirs are loaded with sample, enzymes and ddNTPs which are mixed and heated. The white arrows indicate the direction of sample movement by activated EWOD electrodes as the sample is moved to each reservoir a, b, c, d, e, f and g.

Figure 8. The fluidic network used to transfer the APEX sample from the EWOD device to the microarray is illustrated; it also facilitates post-hybridization wash and drying prior to fluorescence detection. (Left) Stacked view of the structure and (right) top view of the structure. Arrows indicate the flow direction generated by the external pump/valve configuration.

between reservoirs a and b, the latter containing preloaded enzyme mix. The sample was manipulated by activating the EWOD reservoir electrodes a and b (figure 7). This enabled DNA fragmentation using uracil N-glycosylase (UNG) and enzyme digestion with the shrimp alkaline phosphatase (sAP) removing any unincorporated dNTPs. The sample is then heated to 95°C for 5 min, deactivating the enzymes and cleaving the genomic DNA cleaved at uracil sites. The fragmented DNA is immediately mixed with a thermostable DNA polymerase (Thermosequenase) located in reservoir c (figure 7) and moved to reservoirs d, e, f and g, where the CY3 labelled ddNTPs are loaded and mixed. Activating EWOD electrodes along the paths indicated by the white arrows in figure 7 moves the sample to the EWOD outlet reservoirs d, e, f and g. Here, the EWOD function ceases as the external hydrodynamic pressure system takes on liquid handling for the hybridization process and transfer of the liquid from the EWOD to the optical microarray.

The four hybridization chambers are preloaded with the APEX hybridization buffer and an external programmable syringe pump (FIAlab MicroCsp 3000) is used to aspirate the sample from the EWOD outlet reservoirs (d, e, f, g) to the hybridization chambers as outlined in figure 8. After preheating the hybridization reservoirs to 57°C, the mixture in reservoirs d, e, f, g is aspirated onto the integrated microfluidic/microarray (glass waveguide) using the external pump/valve configuration. Here, the DNA hybridization and polymerase reaction (57°C) are carried out for 30 min; initially target DNA hybridizes to the immobilized probe DNA and subsequently the fluorescently labelled ddNTP is incorporated to the probe–target combination by the polymerase. For efficient aspiration, a small pump stroke (10 ml) was used.
Table 1. Outlined are the sequence of steps implemented on the system with the associated sample movement mechanism, temperature and reaction time and the reservoir/channel location with reference to figure 7.

<table>
<thead>
<tr>
<th>Analytical step</th>
<th>Flow mechanism</th>
<th>Temperature</th>
<th>Time</th>
<th>Reservoirs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Load sample (PCR product and enzyme mix)</td>
<td>EWOD electrode array</td>
<td>Room temperature</td>
<td>10 min</td>
<td>EWOD a</td>
</tr>
<tr>
<td>2. Mix sample with SAP/Exol/UNG</td>
<td>EQOD electrode array</td>
<td>37 °C</td>
<td>0 min</td>
<td>EWOD a to b</td>
</tr>
<tr>
<td>3. Denature enzymes</td>
<td>EWOD electrode array</td>
<td>95 °C</td>
<td>5 min</td>
<td>EWOD a and b</td>
</tr>
<tr>
<td>4. Move sample to Thermosequenase reservoir and mix</td>
<td>EWOD electrode array</td>
<td>57 °C</td>
<td>Immediately</td>
<td>EQOD c</td>
</tr>
<tr>
<td>5. Move/mix sample to ddNTP reservoirs</td>
<td>EWOD electrode array</td>
<td>57 °C</td>
<td>5 min</td>
<td>EWOD c to d</td>
</tr>
<tr>
<td>6. Aspirate sample to hybridization channels mix and incubate</td>
<td>Hydrodynamic pressure</td>
<td>57 °C</td>
<td>30 min</td>
<td>EWOD d, e, f, g to channels 1–4</td>
</tr>
<tr>
<td>7. Wash step (i) alconox, (ii) water, (iii) acetonitrile</td>
<td>Hydrodynamic pressure</td>
<td>(i) 45 °C, (ii) 95 °C, (iii) room temperature</td>
<td>15 min</td>
<td>Hybridization channels 1–4</td>
</tr>
</tbody>
</table>

Figure 9. The APEX reaction implemented on an integrated waveguide microarray identified 14 mutations. Faint spots identify heterozygous mutations, while bright spots identify homozygous mutations.

The sequence of protocol steps, sample manipulation mechanism, reaction temperatures and times are outlined in table 1; the reagent mixing and incubation steps are implemented on the EWOD component, while the hybridization step is implemented by the pump/valve pressure-driven system. The EWOD reservoir locations and hybridization channels outlined in table 1 are highlighted in figure 7. The EWOD system is an oil-phase mechanism, while hybridization takes place in an aqueous phase with the exchange of sample between the two achieved by pressure-driven aspiration.

4. Conclusion

We have demonstrated the feasibility of integrating an EWOD device to implement APEX sample preparation steps with a microarray for hybridization detection using CY3-labelled ddNTPs. Conventionally, a multi-dye assay requiring up to four excitation sources is used for colour multiplexing in the standard APEX hybridization assays. We have adapted a single-label approach with the potential to spatially multiplex across four microfluidic hybridization channels, eliminating the need for additional optical components. The integration approach for the hybrid system combines EWOD liquid handling with waveguide fluorescence detection achieving fluidic interconnect capable of sample exchange between the EWOD oil phase and the microarray aqueous phase. The fluorescence intensity from the microarray is attenuated due to imaging through the plastic microfluidic lid; however, mutations were clearly identified with the optical array scanning system. A stringent post-hybridization wash is required in microfluidic-based assays and this is achieved with an external pumping system removing any non-specifically bound biomolecules from the array surface; such stringency could be difficult to achieve with on-EWOD hybridization.
due to the low flow-generated surface forces. The integration strategy maintained the EWOD performance with liquids loaded onto reservoirs, dispensed and moved with EWOD electrodes in a similar fashion to a conventional EWOD device. However, reagents were modified by the addition of surfactant to enhance mobility. The viability of immobilized microarray probe DNA oligonucleotides is maintained in the integrated system with similar spot intensity profiles for homozygous and heterozygous mutations observed, compared to the standard microarray APEX results. The on-chip implementation of the APEX protocol was achieved by moving and mixing sample with reagents on the EWOD device, and hybridization was achieved on the integrated microarray. The APEX sample was aspirated from the EWOD chambers to the microarray and the hybridization assay identified 14 mutations from the DNA sample associated with CF. Bench-top instrumentation was assembled to drive the EWOD device, thermally control reactions and image the fluorescence microarray. Ideally with a POC system, whole biological samples such as blood or saliva are processed through a number of biochemical protocols from ‘sample in’ to ‘answer out’. DNA extraction, target biomolecule amplification and detection are the three core biochemical protocols to be implemented on a POC system. The DNA-extraction protocol would require a number of wash steps to be implemented on an integrated EWOD device. Many commercially available DNA extraction kits use paramagnetic beads as capture sites for double-stranded DNA as cells are lysed and debris is washed away. Biomolecule amplification is required to enrich target concentration to suitable detection levels; for DNA, this is typically achieved using the PCR amplification. To realize a ‘sample in, answer out’ system, these biochemical protocols have to be implemented on the EWOD system but it is feasible to redesign the EWOD system, increasing chamber numbers to implement additional protocols in an integrated fashion. In future work, the possibility of implementing the hybridization detection assay on the EWOD device within the oil phase will be evaluated; however, this is challenging due to wash stringency and the oil fluorescence. Many commercially available POC systems, e.g., Cepheid’s GeneXpert® system or Idaho Technologies FilmArray technology use external pumps and valves to deliver sample and reagents to a cartridge-type system where biochemical reactions take place. While many instruments are based on a real-time PCR approach, the microarray approach offers high throughput mutation detection on a single assay. The possibility of implementing all POC protocols on an EWOD-type device would offer the possibility for reduced size, portability and simplified instrumentation in emerging next-generation systems.

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