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Chapter 5

Integrated Polymerase Chain Reaction Technologies (Sample-to-Answer Technologies)

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5.1 INTRODUCTION

5.1.1 Standard Workflow for Polymerase Chain Reaction-Based Assays

The tools available for infectious disease diagnostics and human identity testing have benefitted from major technological advances. Among the most significant of these advances was the invention of the polymerase chain reaction (PCR) in the mid-1980s (Saiki et al., 1988). This made it possible to easily and quickly amplify specific nucleic acid sequences from microscopic quantities of DNA collected from a patient specimen or recovered from an item of evidence in a criminal case.

Regardless of the type of specimen being analyzed, however, the first step in any PCR-based molecular genetic assay was the preparation of a nucleic acid extract. While this was originally performed manually (typically by boiling or chemical lysis, that is, the use of noxious organic solvents like phenol–chloroform and ethanol precipitation), a wide variety of commercially available instruments have since been developed that automate this process (Fujii et al., 2013). Automated cell lysis can be achieved through the use of detergents and chaotropic salts, heat, mechanical disruption, or simple pressure. Automated nucleic acid extraction/purification typically employs some variant of solid phase extraction whereby nucleic acids are selectively but reversibly bound to a solid substrate (typically silica), thereby allowing protein and other unwanted cellular components to be washed away, after which the now pure nucleic acids are eluted into an appropriate buffer for downstream applications. Such automated platforms provide faster and more consistent results than manual methods. The use of 96-well, 384-well, and higher density specimen processing formats has also greatly increased specimen throughput, which has led to improved economies of scale.

The second step is the PCR amplification process, which, since the 1980s, has been performed by programmable thermal cyclers. By means of heating blocks or temperature-controlled air chambers, specimens cycle through repeated rounds of DNA denaturation, target-specific primer annealing, and nascent strand extension. Here, technical advances have focused on polymerase functionalities (e.g., processivity, fidelity, stability), reduced reaction volumes, and improved thermal transitions to reduce the total amplification run times.

The third step is the detection and analysis of the amplified products of the PCR. Size or conformational fractionation by slab-gel or capillary electrophoresis (CE) has long been the default technology. Here, the availability of precast gels, capillary arrays connected to gel pumps, and higher-density formats for automated sample loading has helped to increase specimen throughput. However, the process remains somewhat laborious and time-consuming. Advances in alternative technologies for PCR amplicon detection and analysis have sought to circumvent the limitations of electrophoresis-based approaches by detecting target amplicons during the amplification process itself. These approaches employ intercalating compounds, minor-groove binding dyes, and a wide variety of hybridization probes (e.g., molecular beacons and Scorpions®) that can be detected and quantified as they bind to nascent strands of DNA created during each cycle of the PCR process.

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Another approach that avoids the need for post-amplification fractionation by gel electrophoresis is melt curve analysis. This is a postamplification assay that can be performed without the need to remove an aliquot of amplified DNA from the PCR tube. It examines the helix-dissociation profile (i.e., the melt curve) of nascent double-stranded DNA as it is subjected to increasing temperatures. The melt curve of any amplicon is a function of sequence-specific nearest-neighbor thermodynamic interactions. As a result, melt curves serve as "signatures" for the presence of an amplified target sequence. See Chapters 4 and 6 for a comprehensive discussion of the underlying principles of real-time PCR, high-resolution melt curve analysis, and their respective applications to molecular diagnostics.

The final step in PCR-based molecular assays is data interpretation, whereby the fragments detected by gel electrophoresis or CE, the real-time PCR amplification curves, and the postamplification melt curves are interpreted. For narrowly tailored and well-optimized PCR assays with clearly defined expected outcomes, interpretation can be a relatively simple and rapid process. For example, PCR-based diagnostic tests for methicillin-resistant *Staphylococcus aureus*, *Clostridium difficile*, and tuberculosis (TB) are relatively simple, low-multiplexed assays that yield well-defined clinical answers. In sharp contrast to this are PCR-based assays for human identity testing, which may be used for kinship analysis or in forensic DNA crime laboratories. The use of short tandem repeats (STRs) as target genetic markers and the highly variable nature of forensic evidence with regard to sample integrity, DNA quantity, and contributor complexity make data interpretation an especially time- and labor-intensive process, requiring manual review by a skilled laboratory analyst. Efforts to streamline this process have focused on the development of powerful expert systems (computer software that is guided by user-defined rules with the goal of being able to consistently analyze a given set of data with the same skill as a human) (Haned and Gill, 2015). Expert systems do not replace humans. Rather, they automate tedious and often time-consuming aspects of data analysis to provide analysts with quality scoring of analyzed data and explanations for the reasoning that the program uses to support a specific analytical outcome. See Chapter 21 for a comprehensive discussion of the use of DNA testing for human identity applications.

Even with the advances that have been made in laboratory automation, the traditional diagnostic assay workflow has remained largely segmented. Specimens and reagents are usually manually loaded and unloaded from different instruments and transported among different physical locations within a laboratory. At a minimum, these include dedicated pre-PCR (low-template extraction and purification) and a post-PCR (high-template amplification and postamplification analysis) areas of the laboratory. The purpose for this segregation of work areas is to minimize the chance of generating erroneous assay results (the inadvertent introduction of previously amplified nucleic acids into preamplification nucleic acid extracts or pre-amplification processes). In both a medical diagnostic context and a forensic context, undetected contamination of one specimen with PCR-amplified products from a different specimen could have catastrophic consequences.

It is widely recognized that even with stringent standard operating procedures and the utmost caution, it is impossible to completely eliminate the risk of cross-contamination when performing PCR-based assays using segmented workflows, even if the individual segments are automated. For this reason, laboratories typically employ multiple layers of controls and quality checks that are designed not only to prevent cross-contamination but also to ensure that it is detected in those rare instances when it does occur. Subsequent root cause analysis and corrective action reports are then generated in an effort to perpetually improve the overall process.

5.1.2 Fully Integrated Polymerase Chain Reaction-Based Assay Systems

There has been a remarkable shift from labor-intensive assays and segmented workflows to fully automated and integrated instruments. Where traditional DNA-based assays require extensive hands-on time to isolate nucleic acids from test specimens; amplify diagnostically informative amplicons; and convert raw electrophoretic fractionation, high-resolution melt curve, or hybridization data into diagnostically meaningful results, a new generation of easy to use, fully automated assays has greatly streamlined this entire process. Variously described as "sample-to-answer" or "sample-in, answer-out" systems in medical diagnostics or as "sample-to profile" systems in human identity testing, these instruments integrate and fully automate nucleic acid extraction, amplification, detection, data interpretation, and reporting in a single device (Park et al., 2011; Sackmann et al., 2014; Buchan and Ledeboer, 2014).

The miniaturization of instruments and reductions in reaction volumes without a loss of data output quality have been hallmarks of modern molecular biology, and these advances are at the core of the sample-to-answer assays. All necessary chemical reagents for the test specimens and some form of internal process control are prepackaged in either a liquid or dried state in disposable assay cassettes or blister packs. Typically, each specimen is processed along its own dedicated pathway, which physically isolates it from other specimens during the entire analytical process. This eliminates the opportunities for potential cross-contamination that exist in traditional segmented workflows. As a result, laboratories are able to bypass many of the burdensome and expensive procedural and infrastructural aspects of

contamination control. Most of these systems are lightweight benchtop instruments with small footprints that make them portable and thus well suited to near point-of-care diagnostics, even in lower resource environments. Similarly, sample-to-profile human identity testing systems are compact enough to be used in a police department booking station, mobile crime scene van, airport security area, or field-forward military site.

These instruments and their assay cassettes can be run with minimal hands-on operation and by staff with less technical training than is typically needed for a traditional diagnostic lab technologist or forensic analyst. By eliminating segmented workflows and assaying specimens in parallel, these systems typically have much faster turnaround times than conventional assays. In the medical diagnostic arena, the sensitivity and specificity of sample-to-answer assay systems are comparable to that of more conventional methods for the identification of the causative organism responsible for a given pathology and often times the presence in that organism of specific genes associated with antimicrobial resistance traits and/or virulence factors. Similarly, the accuracy of sample-to-profile instruments for human identity testing is comparable to that of conventional methods for the analysis of buccal swabs and other good quality reference type samples.

5.2 COMMERCIAL SAMPLE-TO-ANSWER ASSAY SYSTEMS

5.2.1 Systems for Infectious Disease Diagnostics

A number of sample-to-answer and sample-to-profile systems are commercially available (Table 5.1), including the FilmArray® (BioFire® Diagnostics/bioMérieux), the GeneXpert® (Cepheid), the Liat® PCR system (cobas®/Roche), Simplexa™ for 3M Integrated Cycler (Focus Diagnostics), the Verigene® System (Nanosphere/Luminex®), the RapidHIT™ 200 DNA profiler (IntegenX), and the DNAscan™ Rapid DNA Analysis System (NetBio/General Electric [GE] Healthcare). While certainly not an exhaustive list, these examples encompass the range of general operational principles underlying these systems. Specific details in regard to the individual assay chemistries that run on these systems will be provided in this chapter in the context of specific clinical or identity testing applications.

TABLE 5.1 Commercially Available Sample-to-Answer Assay Systems

System	Manufacturer (website)	Application	Operating principle/Detection principle	Samples/run	Approximate run time
FilmArray®	BioFire® Diagnostics/bioMérieux (www.biofiredx.com)	Infectious disease diagnostics	Nested NAAT Melt curve analysis	1	70 min
GeneXpert®	Cepheid (www.cepheid.com/us)	Infectious disease diagnostics	RT-PCR NAAT Fluorescent molecular beacon	1–80	30–150 min
Liat®	cobas®/Roche (www.usdiagnostics.roche.com/en/instrument/cobas-liat.html)	Infectious disease diagnostics	RT-PCR NAAT TaqMan® probe	1	20 min
Simplexa™ 3M Integrated Cycler	Focus Diagnostics (www.focusdx.com/product-catalog/simplexa)	Infectious disease diagnostics	RT-PCR NAAT Fluorescent probes	8	60 min
Verigene®	Nanosphere/Luminex® (www.nanosphere.us)	Infectious disease diagnostics	Microarray capture direct hybridization Light scattering by derivitized gold nanoparticles	1	150 min
DNAscan™	NetBio/GE Healthcare (www.gelifesciences.com)	Human identity testing	Multiplex PCR Microfluidic CE Electrophoresis	5	90 min
RapidHit™ 200	IntegenX (www.integenx.com)	Human identity testing	Multiplex PCR integrated conventional CE	1–7	90 min

The FilmArray® system (Fig. 5.1) provides a generic illustration of a fully integrated PCR-based sample-to-answer system. The FilmArray® system combines an automated in vitro diagnostic instrument (the base unit) with assay-specific "pouches" to detect multiple nucleic acid targets in clinical specimens (Xu et al., 2013). Each assay pouch is a disposable, self-contained, closed system that contains all the reagents required to extract, amplify, and detect specific nucleic acid targets that may be present in a clinical specimen (Poritz et al., 2011). The reservoirs in the rigid plastic component (the fitment) of the pouch contain lyophilized reagents. The flexible plastic film portion of the pouch is divided into a series of blister pack-like compartments. A series of chemical processes are executed through interactions between the pouch and actuators and sensors in the base unit. These include cell lysis and nucleic acid extraction and purification from the test specimen; first-stage multiplex PCR; and second-stage singleplex PCR and melting analysis, both of which take place in a multiwell array. To run the assay, a technician uses a syringe to load "Hydration Solution" into the pouch. This rehydrates the lyophilized reagents that are pre-packaged in the pouch fitment. A patient specimen is then mixed with "Sample Buffer," which inactivates RNases in the specimen and which will later facilitate binding of the nucleic acids to the magnetic beads for nucleic acid isolation. A sample loading syringe is then used to transfer the specimen/sample buffer mixture to the pouch. The pouch is then loaded into the base unit.

After the technician initiates a specimen run, a series of plungers, pneumatic actuators, and hard seals work together to mix the liquid reagents and move them between the blisters of the pouch (Grover et al., 2003). The FilmArray® base unit automatically performs these functions based on the run protocol selected for a specific pouch (assay chemistry) and specimen type in the instrument's operating software. Nucleic acid isolation occurs in the first three blister compartments of the pouch. The first step in processing a specimen is to lyse the outer membrane of the target microbes that may be in the patient specimen. This is done using a device called a bead-beater. A sensor in the base unit monitors the speed and operation of the bead-beater and aborts the run if the bead-beater is not working properly. Following bead-beating, the nucleic acids contained in the sample are captured, washed, and eluted by magnetic bead technology. A retractable magnet is used to capture or release the magnetic beads during washes. These steps require about 10 min to complete.

The next step in the process is reverse transcription (RT) and first-stage multiplex PCR amplification. The RT

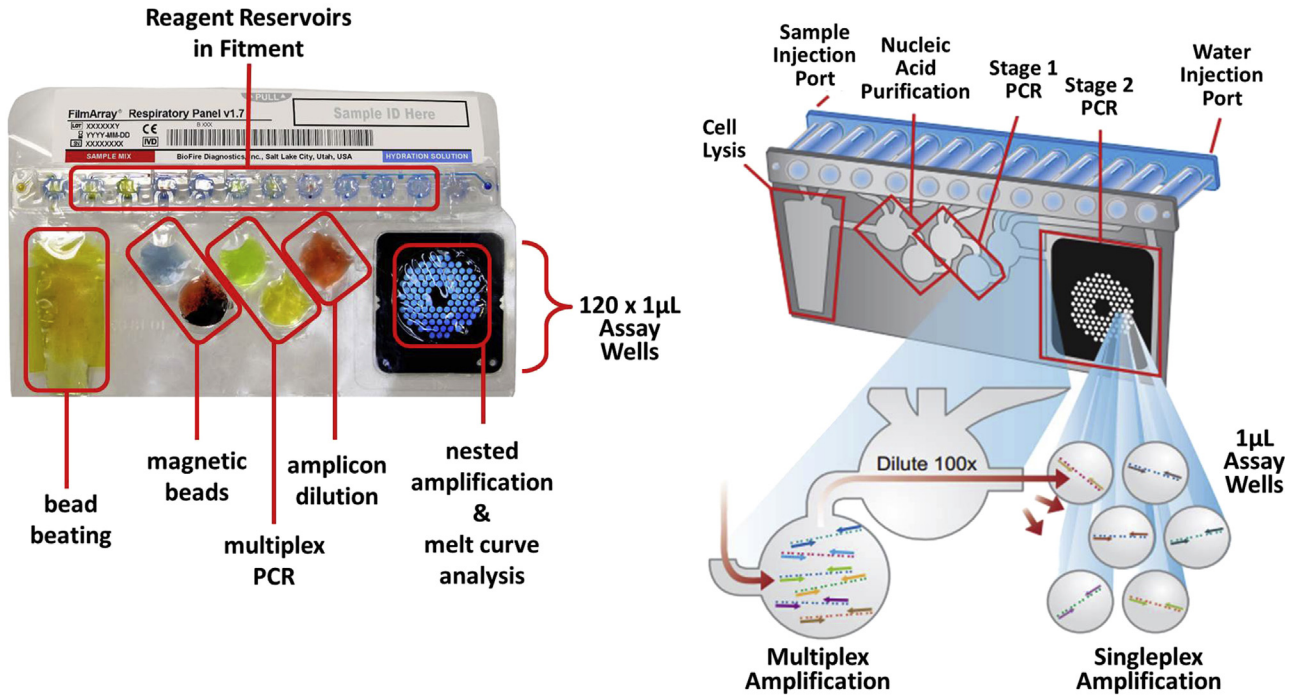

FIGURE 5.1 Photographic image (left) and schematic diagram (right) showing the salient features of a FilmArray® assay system consisting of a hard plastic fitment containing reagent reservoirs and a flexible plastic film pouch consisting of a series of reaction chambers and transfer channels. Once a sample has been introduced through the sample injection port, the pouch is inserted into a FilmArray® instrument base unit (not shown) for processing. The colored liquids shown in the photograph are to facilitate visualization. Actual FilmArray® pouches do not contain colored liquids. *PCR*, polymerase chain reaction. *Images adapted from FilmArray® 2.0 Operators Manual CE IVD.*

step is necessary to convert RNA into cDNA prior to first-stage multiplex PCR amplification in those assays where target pathogens for identification have RNA genomes (e.g., coronavirus, orthomyxovirus, picornavirus, norovirus, and sapovirus). The purified nucleic acid extract is mixed with a preheated master mix to initiate the RT step, and subsequent thermocycling for multiplex PCR amplifies all of the targets identified by the pouch as well as the process control amplicon. A Peltier device drives the thermocycling both for the RT reaction (when required) and for the first-stage PCR reactions. The purpose of the first-stage PCR is to enrich for the target nucleic acids if they are present in the patient specimen.

The amplified products of first-stage PCR are then diluted and combined with fresh PCR reagents containing a saturating fluorescent intercalating DNA dye (LCGreen® Plus). This solution is distributed to a second-stage PCR array. Each of the individual wells of the array contain primers for different assays (in triplicate) that target specific nucleic acid sequences from each of the pathogens targeted by the pouch assay as well as primers for the internal positive control(s). The annealing sites for the second-stage PCR primers are located within the sequence of the amplified products generated during the first-stage multiplex PCR (i.e., nested PCR). This serves to enhance both the sensitivity and specificity of the assay.

After completion of the last cycle of the second-stage PCR, a DNA melting curve analysis is performed to identify positive and negative PCR reactions. This is used to determine which of the targeted microbes were present in the patient specimen. During the second-stage PCR process, LCGreen® Plus dye is incorporated into the copies of DNA as they are made during each PCR cycle. When bound to double-stranded DNA, the dye fluoresces. As the temperature is increased and the copies of double-stranded DNA melt, the dye is released with a corresponding drop in fluorescence. A DNA melting curve is generated by slowly increasing the temperature of the PCR array. As this is done, the fluorescence emitted by the DNA-bound dye in each well of the array is imaged by a camera and recorded. A second Peltier device controls thermocycling for second-stage PCR and for the DNA melt analysis. As with the first-stage PCR, the thermocycling profile is controlled in accordance with the programmed run protocol for the specific reagent pouch assay being performed. Both the second-stage PCR and the melt analysis take place in the array located in the final pouch blister. Because the melting profile for the target amplicon for each microbe in the assay is known, PCR products from specific microbes can be readily identified.

Two internal positive controls are incorporated into the assay. First there is an "RNA Process Control" (RPC). The RPC employs lyophilized yeast (*Schizosaccharomyces pombe*), which is prepackaged in the assay pouch and rehydrated when the patient specimen is loaded. The yeast is carried through all stages of the test process, including lysis, nucleic acid purification, the first-stage PCR, dilution, the second-stage PCR, and the DNA melting curve analysis. An RNA transcript present in the RPC is targeted for amplification, so a positive control result indicates that all steps carried out in the assay pouch were successful. A second-stage PCR (PCR2) control is also included. The PCR2 control detects a DNA target that is dried into wells of the array along with corresponding amplification primers. A positive result indicates the success of the second-stage PCR.

The FilmArray® base unit's software automatically analyzes the melt curve data from the replicate wells for each second-stage PCR reaction (both controls and test samples) and automatically generates a test report at the end of the run indicating which of the targeted microbes were detected. If either of the controls fail, all results will be reported as "Invalid." Based on independent workflow analysis studies (Butt et al., 2014), the entire process from specimen-to-answer takes just over 1 h to complete but only about 5 min of actual hands-on time.

Other commercially available platforms take a similar approach by integrating specimen processing in a single-use cartridge system, though the specifics of nucleic acid extraction, target amplification, and detection vary. The GeneXpert® system, for example, combines a base unit analyzer with small disposable cartridges (modules) that are barcoded and preloaded with all of the necessary reagents for particular assays (Lawn et al., 2013; Tortoli et al., 2012). A patient specimen is placed into the cartridge, which is then loaded into the base unit, which reads the cartridge barcode and initiates an assay-specific protocol. Nucleic acid extraction takes place in a processing chamber that contains reagents, filters, and capture technologies necessary to extract, purify, and amplify target nucleic acids. Nucleic acid extraction employs a combination of chemical and ultrasonic lysis, followed by RT-PCR and fluorescent monitoring of multiple channels for target and control signals. Integrated valves are used to facilitate fluid transfer from chamber to chamber, and thin PCR chamber walls enable rapid thermal cycling. The systems software interprets the detected signal and generates a report of the results with a total run time of about 1 h. This system's base units are available with 1-, 2-, 4-, 16-, or 80-module configurations and are equipped with a robotic loading system to minimize hands-on time even in higher volume laboratories. Each module within a base unit operates individually, and as many different assays (e.g., *Mycobacterium tuberculosis*, methicillin-resistant *S. aureus*, or *C. difficile*) as necessary can be run by a technician at the same time. Therefore this system is both flexible and scalable to the needs of the specific laboratory. This is a key advantage relative to the FilmArray® system and other systems that process one assay pouch at a time. FilmArray®, however, is

able to screen for a greater number (up to 20) of target pathogens per pouch.

The Liat™ which stands for "Lab In A Tube" system is another real-time PCR-based assay system that utilizes small disposable linear tubes (Binnicker et al., 2015; Chen et al., 2015). Each tube contains all of the reagents necessary to perform an assay. These reagents are arranged in a series of blister pack compartments. Using a transfer pipette, a patient specimen is introduced to the top of the assay tube. After the assay tube is capped, it is inserted into an analyzer base unit, which integrates all nucleic acid testing processes, including reagent preparation, target enrichment, inhibitor removal, nucleic acid extraction, amplification, and real-time detection. Specimen sampling and handling are controlled using multiple sample processing modules contained within the base unit. The specimen processing modules consist of two assemblies. The first is a moving side assembly comprised of multiple sample processing plungers and clamps. The second is a fixed-side assembly. The plungers and clamps selectively compress the Liat™ assay tube segments against the fixed-side assembly so as to selectively release reagents from individual tube blister compartments and move the sample from one compartment to another. The nucleic acid extraction process is based on lysis by chaotropic salts followed by solid phase magnetic particle-based nucleic acid purification. The detection of amplified targets employs fluorogenically labeled hydrolysis probes (e.g., *Taq*Man® probes). As with other integrated systems, an internal positive control is used to confirm the instrument performance and result determination. The internal positive control (IPC) comprises an encapsulated RNA that is prepacked in each Liat™ tube. The base analyzer unit controls reaction conditions, such as thermal cycling temperatures, in accordance with specific assay programs. Data analysis employs predefined decision algorithms to generate a report of assay results. Like the FilmArray® system, the Liat™ system processes only one specimen at a time, but results are typically generated in only 20 min.

The Simplexa™ Molecular Assay system uses disk-based centrifugal microfluidics and real-time PCR assays, which are designed to run on the 3M™ Integrated Cycler (Strohmeier et al., 2015; Miao et al., 2015). Users are given a choice of two operational modes. In the first mode, a thermocycle-only disk is used, which allows for up to 96 standard real-time PCR assays but requires that patient specimens be extracted separately so that the disk can be loaded with purified DNA. Each of 96 radially inward-oriented inlet wells is connected to one of 96 amplification wells positioned at the outer rim of the disk. Contact heating is employed for thermocycling, and real-time detection can record data in up to four fluorescence channels. In the second mode of operation, a direct amplification disk is used to provide true sample-to-answer functionality. In this second mode, space on the direct amplification disk is occupied mostly by extraction microfluidics, and therefore only eight fully integrated sample processing reactions can be run at a time. The use of the disk requires little hands-on time aside from pipetting 50 μL of reaction mix onto the disk prior to processing a 50-μL patient sample. Samples to be assayed are pipetted into wells near the center of the disk. The technician then enters the assay run parameters and initiates the run. As the disk spins, centrifugal force moves the patient specimens and reaction reagents through the reaction chambers. The direct amplification disk makes use of direct amplification chemistries that can perform nucleic acid extraction and amplification in one protocol. Infrared energy and a high-velocity fan are used to heat and cool the real-time PCR reaction chambers on the disk. Rapid temperature transitions and efficient heat transfers translate into shorter cycle times (as little as 20 s/cycle). As with the thermocycle-only disk, up to four fluorescence channels are available for target detection, and the system software collects and analyzes the results. The Simplexa™ system provides results from a patient swab in about 1 h.

A similar specimen processing approach with a quite different detection technology is employed by the Verigene® system, which uses self-contained test cartridges in conjunction with two separate instrument modules: a cartridge processor and a cartridge reader. Each disposable test cartridge is designed for the multiplex analysis of a single patient specimen. It features a microfluidic cassette that contains all of the hybridization reagents needed for the assay and captures all of the waste materials that are generated in the process. It also contains a glass slide that serves as a solid support for the microarray where any targeted nucleic acids can be captured for detection. The cartridge processor unit manages the automated nucleic acid extraction, purification, amplification (if required), and hybridization processes. In the test cartridge, genomic DNA is extracted and sheared by sonication into 300 to 500 base pair fragments. This fragment size is easily manipulated and can be readily hybridized to other molecules. The fragmented genomic DNA is allowed to hybridize to a microarray of capture probes that are attached to the glass slide in the assay cartridge. Oligonucleotide probes conjugated to gold nanoparticles are then introduced and allowed to bind to any complementary genomic DNA fragment present on the microarray. Any unbound oligonucleotide-gold nanoparticle probes are washed away while elemental silver is deposited onto the gold nanoparticle probes, which are bound and remain. This serves to amply the signal for the optical detection of light scattering from the derivitized gold nanoparticles (Giljohann et al., 2010). The detection sensitivity that is achieved through the

use of gold nanoparticles exceeds that of the fluorescent dye-based detection methods that are employed by most other sample-to-answer systems, so much so that while nucleic acid amplification is an option with the Verigene® system, it is not always necessary and can be omitted in some assays. Finally, the cartridge reader images the microarray and analyzes the results from the processed cartridges in order to generate a report. Based on independent evaluations of this system (Butt et al., 2014), results are typically generated in approximately 2.5 h, making the system competitive with other sample-to-answer systems. The total amount of hands-on time was 21 min. The longer hands-on time was attributed to the need to thaw frozen reagents and move cartridges between the analyzer and the reader.

5.2.2 Systems for Human Identity Profiling

The development of systems for human identity testing that integrate all of the required processes has in some ways been an even more challenging goal than the development of many of the sample-to-answer medical diagnostic assays. The reason for this is that medical diagnostic assays typically need to detect only the presence of an amplified DNA sequence from a targeted microorganism. Human identity testing for kinship analysis or forensic purposes, however, presents the need for identifying specific length variants for each of the STR genetic markers that are targeted for analysis. This necessitates the expansion of basic integrated PCR systems to include a size-fractionation process. In conventional forensic laboratories this is performed using CE. Incorporating this into fully integrated systems has proven to be challenging, but significant progress has been made. Successes have been achieved by interfacing nucleic acid extraction and amplification cartridges with standard CE modules or microfluidic CE. Several parallel efforts have resulted in the commercial availability of fully integrated devices that integrate all of the workflow required for sample-to-profile human identity testing.

The RapidHIT™ 200 System (Fig. 5.2) provides an illustration of this more complex workflow (Holland and Wendt, 2015; Hennessy et al., 2013). The RapidHIT™ 200 is a fully integrated sample-to-profile DNA identification system that uses four disposable cartridges in conjunction with an instrument base unit. The sample and control cartridges integrate the nucleic acid extraction and PCR amplification processes. The anode cartridge contains linear

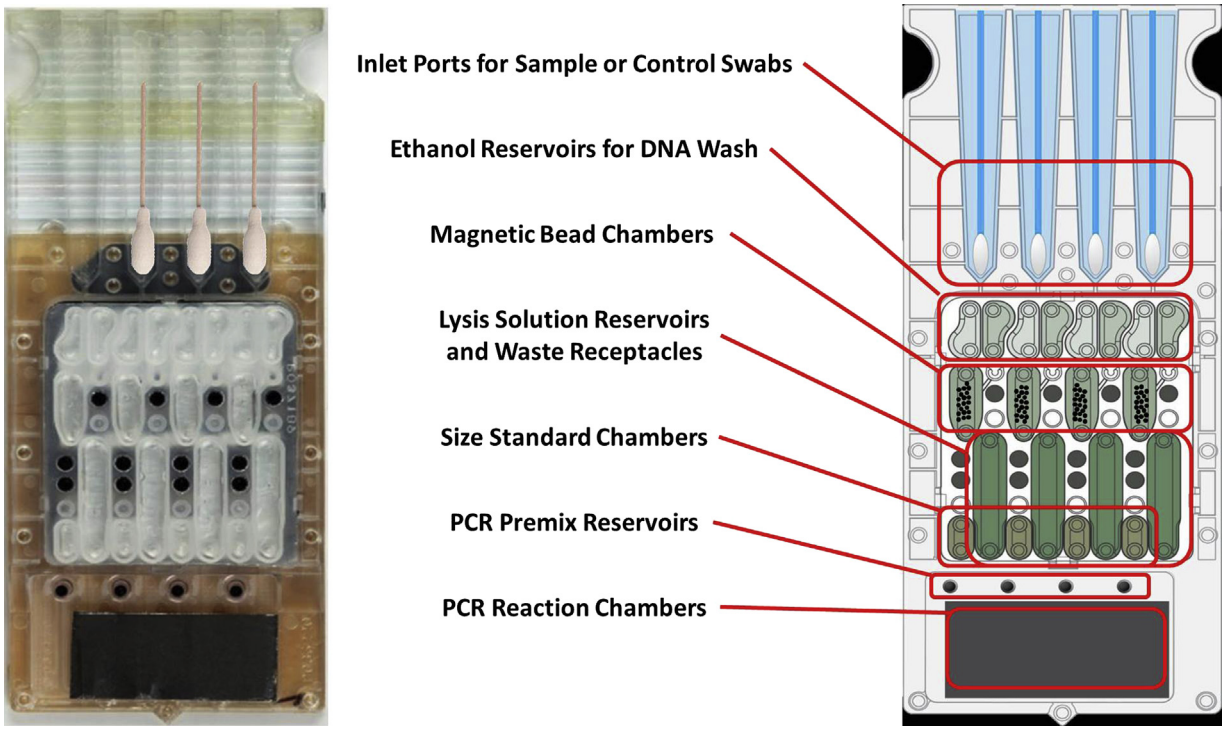

FIGURE 5.2 Photographic image (left) and schematic diagram (right) showing the salient features of a RapidHIT™ 200 assay system sample cartridge. After sample swabs are placed in the inlet ports, a lysis solution is pumped from the lysis solution reservoir to the swab. Suspended cells from the swab are then pumped to the magnetic bead chambers where the cells are lysed, and the DNA is immobilized on magnetic beads. Ethanol is pumped sequentially from each of the ethanol reservoirs to wash the immobilized DNA in the bead chamber. The used ethanol wash is deposited into the waste receptacle. Beads with purified DNA are transferred to the polymerase chain reaction (PCR) chamber and mixed with PCR premix. After PCR amplification, amplicons are fractionated off-cartridge. *Images adapted from IntegenX.*

polyacrylamide for capillary electrophoresis, an agent that dynamically coats the walls of the capillaries to reduce electroosmotic flow during electrophoresis and the anode electrode for electrophoresis. The buffer cartridge serves as the reservoir for both the CE cathode buffer and water for other processes. It is also the receptacle for waste material.

The sample and control cartridges are injection molded to produce an integrated fluidic device consisting of a series of chambers and fluid channels that interface with pneumatically driven valves and pumps that are externally actuated to transport samples and mix fluidic streams (Grover et al., 2003). The sample cartridge has four sample ports and dedicated fluidic pathways to process up to four swabs of cellular material (typically buccal swabs). The control cartridge processes one sample port and dedicated fluidic pathway. The remaining three fluidic pathways are reserved for an internal positive control (a known DNA standard); a negative control (typically no sample) to monitor for contamination of the reagents and/or the fluidic path by extraneous human DNA; and an allelic ladder, which is prepackaged in the size-standard chamber of the flow path and serves as a standard for allele determination during data analysis.

The RapidHIT™ base unit into which the cartridges are inserted for processing has four primary subsystems: one each for sample preparation, fractionation, detection, and control/analysis. The sample preparation subsystem manages the operation of the sample and control cartridges through the use of pneumatics to move liquids from one chamber to the next. Lysis employs a combination of heat and a chaotropic guanidinium salt to release DNA, which is then captured and purified using solid phase extraction on magnetic beads. The beads with bound DNA are then transferred to the reaction chamber and immobilized by fixed magnets. A multiplex PCR master mix with primers for either 16 or 24 STR loci (the PowerPlex® 16HS Fast and GlobalFiler® Express chemistries, respectively) is then added to the reaction chamber and amplified using a Peltier thermocycler. The amplified products are then moved to the size-standard chamber where they are mixed with an internal lane size standard before being pumped to the separation subsystem. After being received by the separation subsystem, which is a separate module housed in the base unit, the linear polyacrylamide from the anode cartridge is used to fill the eight separation capillaries. The amplified samples are then heat denatured, electrokinetically injected, and size fractionated by CE using buffer from the buffer cartridge. After the run, the capillaries are automatically cleaned to prepare them for the next run. The detection subsystem uses a solid-state laser to excite the fluorescently tagged STR products, which are then detected by a charge-coupled device camera as they pass by the capillary detection window. The data on fluorescence intensity and CE mobility are collected and stored by the control/analysis subsystem, which houses the embedded computer that controls all instrument operations. This subsystem automatically processes the raw data for noise filtering, baseline subtraction, spectral deconvolution, and primer peak removal. A local copy of GeneMarker® HID human identity software then identifies the specific genotypes at all STR loci in order to generate a report with the DNA profile of each sample. The total processing time from swab to DNA profile is approximately 90 min.

The DNAscan™ (Fig. 5.3) represents an alternative approach for the generation of STR-based human DNA profiles. It employs a single-use, disposable microfluidic cassette for all DNA profiling processes, including size fractionation of the PCR amplicons, together with an instrument base unit that manages the workflow (Tan et al., 2013). The functional core of the system is an injection-molded BioChipSet Cassette (BCSC), which is constructed from four major components: the smart cartridge, the gel smart cartridge, the integrated biochip, and the separation and detection biochip. The BCSC is preloaded with all of the necessary reagents for sample processing. Liquid reagents are stored in reservoirs that have aluminum foil seals bonded to both ends. Pneumatic pressure is used to burst the seals, thereby releasing the contents of the reservoir. Lyophilized reagents (e.g., PCR master mix, internal lane size standard, and allelic ladders) are also preloaded within the chambers of the BCSC and are reconstituted as the liquid phase reaction products move through the BCSC.

The smart cartridge can process up to five buccal swabs at a time. As with most other systems, the nucleic acid purification method employs chaotropic guanidinium salt lysis followed by guanidinium-mediated binding of DNA to a solid phase silica surface. The DNA extract is then PCR amplified with a PCR master mix containing primers to amplify 16 STR loci (i.e., the PowerPlex® 16 chemistry). An alternative 27-locus multiplex PCR master mix has also been developed and tested. The gel smart cartridge contains the linear polyacrylamide sieving matrix and the electrophoresis buffer, which is used for microfluidic size fractionation and detection. A significant difference from the RapidHIT™ 200 system is that rather than traditional capillaries, the separation and detection biochip performs electrophoresis in six independent microfluidic channels that are 22.5 cm long with a cross-sectional dimension of 40 μm × 100 μm. In order to minimize potential interference from plastic autofluorescence, the entire BCSC is fabricated from a cyclic olefin polymer that produces less autofluorescence than glass. The fluorescent labels on the amplified STR fragments are excited by a 488-nm solid state laser in the optical subsystem of the base unit. Laser light for excitation is transmitted to the detection window of the separation and detection biochip, and the resulting fluorescence is then detected by a series of photomultiplier

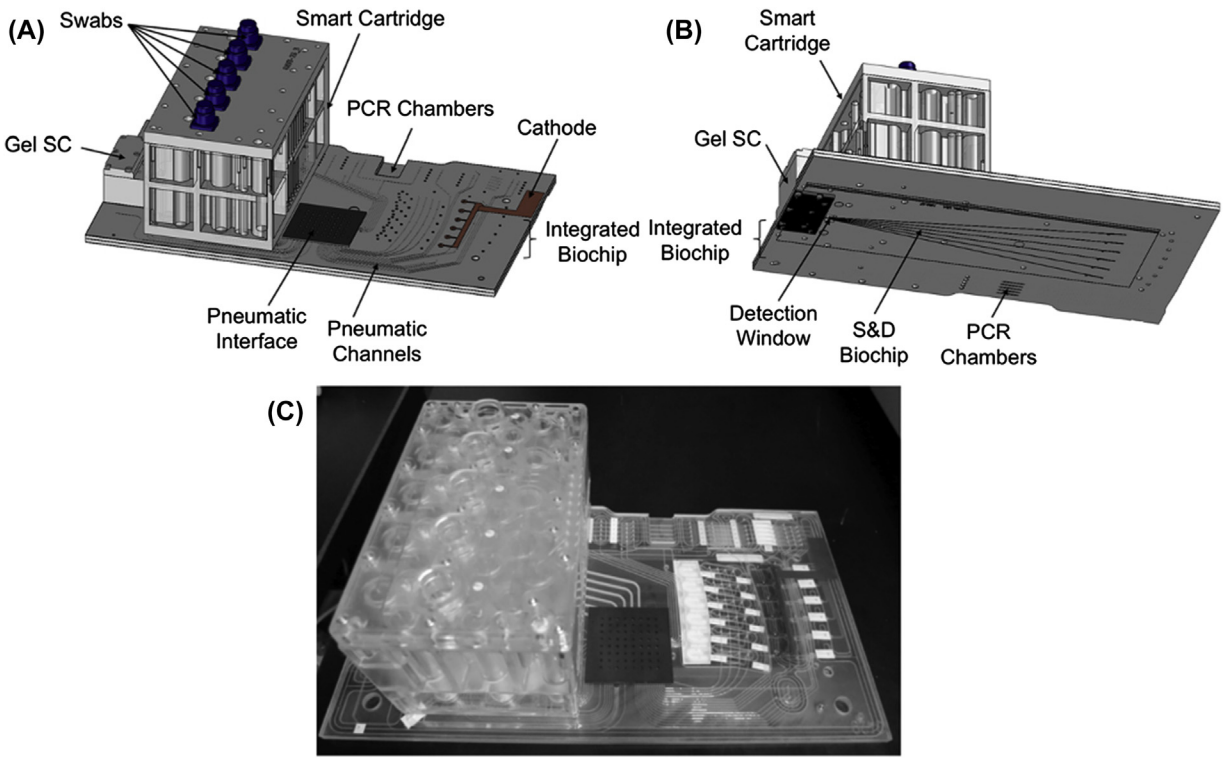

FIGURE 5.3 Structure of the DNAscan™ BioChipSet Cassette (BCSC). (A) Top view schematic showing the location of three of the four components of the BCSC (smart cartridge, gel smart cartridge, and integrated biochip). (B) Bottom view schematic showing the location of the separation and detection biochip, which contains the microfluidic CE channels. (C) Photograph of the BCSC. The primary interfaces with the instrument are via the pneumatic manifold, the polymerase chain reaction chambers, the cathode and anode (not shown, adjacent to the gel smart cartridge), and the separation and detection window. *PCR*, polymerase chain reaction; *gel SC*: gel smart cartridge; *S&D*: separation and detection. *Image from Tan, E., Turingan, R.S., Hogan, C., Vasantgadkar, S., Palombo, L., Schumm, J.W., Selden, R.F., 2013. Fully integrated, fully automated generation of short tandem repeat profiles. Invest. Genet. 4, 1. Creative Commons Attribution License.*

tube detectors. The integrated biochip component contains microfluidic channels and chambers that represent the core of the BCSC. It interfaces with the smart cartridge and facilitates nucleic acid purification by providing the means for liquid transfer from chamber to chamber. The integrated biochip also accepts purified DNA from the smart cartridge in preparation for processing to perform PCR, after which its interface with the separation and detection biochip provides for the transfer of electrophoresis-ready samples to the separation channels.

Upon completion of a sample run, the raw electropheretic data is processed to achieve baseline subtraction, spectral deconvolution, and to identify signal peaks to produce an interpretable electropherogram. Finally, expert system algorithms are used for automated allele calling. The software interprets the processed data to determine the genotypes for all amplified loci, which collectively form the DNA profile for the analyzed samples. Similar to the RapidHIT™ 200 system, the DNAscan™ system is able to generate a DNA profile through fully automated, fully integrated processing of buccal swabs in just under 90 min.

5.3 CLINICAL APPLICATIONS: PERFORMANCE FOR INFECTIOUS PATHOGEN DIAGNOSTICS

5.3.1 Respiratory Pathogens

The respiratory system is one of the most frequent sites for colonization by infectious agents. Respiratory infections are also an important driver of patient hospitalization. Several sample-to-answer assays are available for the detection of a wide variety of respiratory pathogens. PCR for influenza virus detection has many advantages over conventional diagnostic techniques. For example, detecting influenza in culture takes several days. Antigen-detection-based tests, while simple and fast, suffer from low sensitivity or require the somewhat more tedious examination of cells by fluorescence microscopy. By contrast, nucleic acid tests are fast, sensitive, specific, and well suited to sample-to-answer platforms.

The FilmArray® Respiratory Panel (RP) first gained US Food and Drug Administration (FDA) approval in 2011. The

current iteration of the FilmArray® RP targets the detection of 20 microbes, including numerous viral strains and species of bacteria (Table 5.2). Detection sensitivities vary for each organism and for different versions of the assay (Doern et al., 2013). Overall, the sensitivities reported by independent researchers vary slightly from those reported by the manufacturer in Table 5.2 but generally fall within the 90−100% range for all targeted pathogens, except for Influenza B and adenovirus which have sensitivities of 73% and 83%, respectively (Couturier et al., 2013). While the assay is intended for use with nasopharyngeal swabs, good sensitivities have also been reported for other types of specimens, including nose and throat swabs, sputum, and specimens collected from the lower respiratory tract (Branche et al., 2014; Ruggiero et al., 2014).

The Cepheid Xpert® Flu assay detects and discriminates among Influenza A, Influenza A/H1N1, and Influenza B using either nasopharyngeal swabs or nasal aspirates. It has an overall sensitivity of approximately 95%, though some researchers have seen a somewhat lower sensitivity for Influenza B (Dugas et al., 2014; Li et al., 2012). The Xpert® Flu/RSV XC is a different Xpert® assay cartridge that differentiates Influenza A, Influenza B, and Respiratory Syncytial Virus (RSV). Studies indicate that when compared with other molecular assays, the sensitivity of the Xpert® Flu/RSV XC assay for these viruses ranged from a low of 89% to more than 95% for Influenza A (Popowitch and Miller, 2015; Salez et al., 2013, 2015).

Other sample-to-answer assays approved for respiratory pathogens include the Simplexa™ Flu A/B & RSV assay, the Verigene® Respiratory Viral Plus (RV+) panel, and the Liat® Influenza A and B assay panel. Compared to in-house RT-PCR, the sensitivities of the Simplexa™ A/B & RSV assay for Influenza A, B, and RSV were 97%, 98%, and 99%, respectively (Woodberry et al., 2013). The reported sensitivity and specificity for the Verigene® RV+ was greater than 95% (Cho et al., 2015; Boku et al., 2013). Compared to the Simplexa Flu A/B & RSV assay, the Liat® Influenza A and B assay panel showed greater than 99% sensitivity for Influenza A and 100% for Influenza B (Binnicker et al., 2015).

Mycobacterium tuberculosis is the pathogen responsible for TB, which is a highly contagious and airborne disease that ranks alongside HIV/AIDS as a leading cause of mortality around the globe (WHO, 2014). Drug resistance has become a major obstacle to effective TB treatment and prevention. The spread of TB has been fueled by improper patient treatment, poor management of drug prescriptions and drug quality, and airborne transmissions of bacteria in public places. Strategies to control the spread of TB, including drug-resistant TB, have relied upon slow and labor-intensive diagnostic methods such as the acid-fast bacilli (AFB) smear test (Lawn and Nicol, 2011). Although this has been used for TB detection for more than a century, it has a low specificity and sensitivity (CDC, 2013). Accordingly, the development of faster and more accurate diagnostic tests for *M. tuberculosis* has been a top priority in the medical community (WHO, 2009). A sample-to-result molecular test for TB would have obvious advantages over conventional smear or culture tests.

The Xpert® MTB/RIF assay detects TB and resistance to rifampin in sputum specimens by targeting a TB-specific sequence. Mutations within the *rpoB* gene, which confer resistance to rifampin and are often an early marker of multidrug-resistant TB, are also detected by the assay (Boehme et al., 2010; Helb et al., 2010). Based on results from multiple evaluation studies, the sensitivity of the assay varied from 70−100% in AFB smear test-positive patients. The specificity of the assay ranged from 91−100%. The assay's ability to detect rifampin resistance exceeded 95% sensitivity and specificity (WHO, 2010). Results from controlled clinical validation studies of individuals suspected of having TB showed the sensitivity of a single direct Xpert® MTB/RIF assay to be 73% in smear-negative/culture-positive samples and 90% when three smear-negative samples were tested. The specificity of the Xpert® MTB/RIF assay was 99%. The sensitivity and specificity of rifampin resistance detection were 99% and 100%, respectively (Boehme et al., 2010; WHO, 2010).

This is not to suggest that the Xpert® MTB/RIF assay is without limitations. The need for conventional drug resistance testing is not eliminated, as it is still necessary for the detection of resistance to antibiotics other than rifampin. A negative Xpert® MTB/RIF assay result does not exclude the diagnosis of TB. The Centers for Disease Control and Prevention (CDC) reports that 15−20% of TB cases in the United States that are reported with negative culture results may also have negative nucleic acid amplification test results. This may be due to the low bacterial load or the presence of inhibitory substances in sputum specimens (CDC, 2014).

5.3.2 Gastrointestinal Pathogens

Infectious diarrhea impacts millions of people around the globe each year and is associated with high rates of morbidity and mortality, especially among children. The clinical presentation of infectious gastroenteritis is not informative in terms of specifying an etiologic agent, because diarrhea is the primary symptom caused by a wide range of causative agents. The challenge of selecting an appropriate pathogen identification assay, therefore, is compounded by the fact that for many pathogens, there is no diagnostic test available at all (Buss et al., 2015). This may partially account for the fact that the etiologic agent of infectious diarrhea is only identified in about 20% of patients (Scallan et al., 2011). The rapid and accurate detection of gastrointestinal (GI) pathogens is vital to ensuring

TABLE 5.2 Characteristics of Pathogens Detected by the FilmArray Respiratory panel®

Pathogen	Classification (genome)	Season of highest incidence	Infection demographics	Sensitivity (prospective)	Sensitivity (retrospective)	Specificity (prospective)
Adenovirus	Adenovirus (DNA)	Late winter to early summer	All ages, immunocompromised	88.90%	100%	98.30%
Bocavirus	Parvovirus (DNA)	No peak season	All ages	66.70%	100%	99.80%
Coronavirus HKU1	Coronavirus (RNA)	Winter, spring	Children, adults	95.80%	n/a	99.80%
Coronavirus NL63	Coronavirus (RNA)	Winter, spring	Children, adults	95.80%	n/a	100%
Coronavirus 229E	Coronavirus (RNA)	Winter, spring	Children, adults	100%	n/a	99.80%
Coronavirus OC43	Coronavirus (RNA)	Winter, spring	Children, adults	100%	n/a	99.60%
Human Metapneumovirus	Paramyxovirus (RNA)	Winter, early spring	Children	94.60%	n/a	99.20%
Human Rhinovirus/ Enterovirus	Picornavirus (RNA)	Summer, fall, spring	All ages	92.70%	95.70%	94.60%
Influenza A	Orthomyxovirus (RNA)	Winter	All ages, 5–20% of US population	90.00%	n/a	99.80%
Influenza A/H1	Orthomyxovirus (RNA)	Winter	All ages, 5–20% of US population	n/a	100%	100%
Influenza A/H3	Orthomyxovirus (RNA)	Winter	All ages, 5–20% of US population	n/a	100%	100%
Influenza A/H1-2009	Orthomyxovirus (RNA)	Winter	All ages, 5–20% of US population	88.90%	100%	99.60%

Continued

TABLE 5.2 Characteristics of Pathogens Detected by the FilmArray Respiratory panel®—cont'd

Pathogen	Classification (genome)	Season of highest incidence	Infection demographics	Sensitivity (prospective)	Sensitivity (retrospective)	Specificity (prospective)
Influenza B	Orthomyxovirus (RNA)	Winter	All ages, 5–20% of US population	n/a	100%	100%
Parainfluenza Virus 1	Paramyxovirus (RNA)	Fall, periodicity of 1–2 years	Infants, young children, immunocompromised	100%	97.10%	99.90%
Parainfluenza Virus 2	Paramyxovirus (RNA)	Fall, periodicity of 1–2 years	Infants, young children, immunocompromised	87.40%	100%	99.80%
Parainfluenza Virus 3	Paramyxovirus (RNA)	Spring, summer	Infants, young children, immunocompromised	95.80%	100%	99.80%
Parainfluenza Virus 4	Paramyxovirus (RNA)	Unknown	All ages	100%	100%	99.90%
Respiratory Syncytial Virus	Paramyxovirus (RNA)	Winter, varies by location	Children, older adults	100%	n/a	89.10%
Bordetella pertussis	Bacterium (DNA)	No peak season	All ages	100%[a]	100%	99.90%
Chlamydophila pneumoniae	Bacterium (DNA)	No peak season	Older children, young adults, immunocompromised	100%[a]	n/a	100%
Mycoplasma pneumoniae	Bacterium (DNA)	Summer, periodicity of 4–7 years	Older children, young adults	100%[a]	90.00%	100%

CE, capillary electrophoresis; *NAAT*, nucleic acid amplification test; *RT-PCR*, reverse transcription-polymerase chain reaction.
[a]*Based on less than ten positive samples due to low clinical prevalence in the prospective study.*

appropriate therapy and infection control strategies (Liu et al., 2012; Khare et al., 2014).

Traditionally, bacterial/viral culture, microscopy to search for ova and parasites, and antigen-detection assays were the methods of choice for the identification of GI pathogens. More recently, singleplex real-time PCR assays have been developed to identify specific pathogens. These methods are often time-consuming, labor-intensive, and often offer little more than one shot in the dark after another in an effort to identify a causative pathogen. For the physician, this is not an attractive situation with respect to patient management, infection control, or public health strategies (de Boer et al., 2010). The ability to simultaneously screen for the presence of a wide variety of potential pathogens through multiplex PCR is an important feature of many sample-to-answer assays.

The FilmArray® GI panel and the Verigene® enteric pathogens (EP) panel are two such assays, both of which are commercially available and FDA approved. The FilmArray® GI panel is designed to detect 22 targets (5 viruses, 13 bacteria, and 4 parasites), and the Verigene® EP panel is designed to detect 9 targets (2 viruses, 5 bacteria, and 2 toxins) in a single assay (Khare et al., 2014).

The FilmArray® GI panel showed overall a greater than 90% level of sensitivity and specificity. In a study involving over 1500 cases, the GI panel was reported to have 100% sensitivity/positive predictive value for 12 out of 22 targets (*Plesiomonas shigelloides*, *Salmonella* spp., *Yersinia enterocolitica*, Enterotoxigenic *Escherichia coli*, Shiga toxin-producing *E. coli*, *E. coli* O157, *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Giardia lamblia*, Astrovirus, Rotavirus A, and Sapovirus). For the remaining 10 targets, the sensitivity/positive predictive value was >94.5%. Another advantage of the GI panel is the ability to detect multiple pathogens in a single assay. The use of conventional methods identified more than one pathogen in just 8.3% samples. By contrast, the GI panel indicated the presence of a mixed infection in 31.5% of the specimens (Buss et al., 2015). An assessment of 611 prospective and 839 contrived specimens conducted with the smaller Verigene® EP panel showed sensitivities of 97% for *Salmonella* spp., 93% for *Campylobacter* spp., 100% for *Shigella* spp., 100% for toxin gene *stx*1, and 97% for toxin gene *stx*2 (Novak SM et al., 2014). Taken together, these studies demonstrated that the integrated sample-to-answer diagnostic systems consistently outperform traditional culture-based methods.

Toxigenic *C. difficile* is the quintessential hospital-acquired pathogen in that it is antibiotic-driven and resistant to multiple antibiotics. The estimated prevalence of *C. difficile* infections may be as high as 50% in hospitalized patients where *C. difficile* infection is endemic, 5−7% in residents of long-term care facilities, and generally less than 2% in ambulatory adults. Carriage rates are higher in hospitalized patients who have unrelated conditions that require long-term treatment with antibiotics, which kill off other intestinal bacteria that would normally keep *C. difficile* in check. The reported incidence of *C. difficile* colitis among hospitalized inpatients ranges from 3.8 to 9.5 cases per 10,000 patient days. Rates tend to increase in proportion to the duration of a hospital stay (Dubberke et al., 2008; Kyne et al., 2002; Cohen et al., 2010). *C. difficile* causes a spectrum of diseases, ranging from antibiotic-associated diarrhea to pseudomembranous colitis (Sewell et al., 2014). The fast and accurate detection of *C. difficile* infections is important for appropriate antibiotic treatment and proper control of infection outbreaks.

There are numerous well-established methods available for the detection of *C. difficile* in stool specimens, including stool culture, toxigenic culture, antigen detection, enzyme immunoassay, and molecular testing (Karen and John, 2011). Stool culture is the most sensitive test available, but it is laborious, does not detect toxin production, and has a lengthy turnaround time of up to 4 days. Tissue culture cytotoxicity assays require more technical expertise, but they are slightly faster with a turnaround time of up to 2 days. Still, they are less specific and sensitive than PCR or toxigenic culture-based assays. Antigen-detection assays based on latex agglutination or immunochromatography are faster, producing results in under 1 h. Antigen tests, however, are nonspecific for pathogenic strains. Enzyme immunoassays, which are designed to detect *C. difficile* toxin A, toxin B, or both A and B, are fast and economical but are relatively insensitive.

Here again sample-to-answer diagnostic assays offer superior sensitivity and specificity with a fast turnaround time. The FDA-approved FilmArray® GI panel discussed previously includes a single multiplexed assay (Cdiff) for the identification of toxigenic *C. difficile*. This assay targets both the toxin A and the toxin B genes (*tcdA* and *tcdB*). Common toxigenic strains of *C. difficile* express both toxins, making the presence of either indicative of a pathogenic strain. Empirical testing and in silico sequence analyses indicate that all toxinotypes will be detected by the assay, including the epidemic North American Profile 1 NAP1/027/BI hypervirulent strain, which has been identified as a cause of hospital outbreaks worldwide (Chapin et al., 2011), Although they are detected, these strains are not specifically differentiated by the GI assay.

The Xpert® *C. difficile* and Xpert® *C. difficile*/Epi assays have both been approved by the FDA and are performed directly from a stool sample. The former targets the *C. difficile tcdB* and has been shown to have a sensitivity of 94% and a specificity of 96% (Novak-Weekley et al., 2010). The latter assay differentiates the hypervirulent NAP1/027/BI strain. Similarly, the Verigene® *C. difficile* assay has the ability to detect both toxin-encoding *tcdA* and *tcdB* genes, and it identifies the NAP1/027/BI strain

(Carroll et al., 2013). Regardless of the assay used, rapid sample-to-answer molecular testing for *C. difficile* makes it possible to facilitate a reduction in transmission during outbreaks by providing cost-effective and timely detection of the pathogen (Sewell et al., 2014).

Another GI pathogen of concern is norovirus, the most common cause of acute gastroenteritis, which is highly contagious via the fecal-oral route. Transmission of the disease is associated with crowded living environments such as cruise ships, school dormitories, daycare centers, and prisons. Accordingly, prompt identification of norovirus early in an outbreak can have a positive impact by informing appropriate actions to prevent further spread of the disease.

The Cepheid Xpert® Norovirus assay is similar in design to other GeneXpert® assays. It is performed directly from stool specimens and distinguishes between Norovirus genogroups I and II. A study of over 1400 samples found that compared to a CDC composite reference method, the Xpert® Norovirus assay demonstrated a positive percent agreement for genogroups I and II of 98% and 99%, respectively, and a negative percent agreement of 98% for both genogroups I and II (Gonzalez et al., 2016).

5.3.3 Sexually Transmitted Pathogens

Prompt diagnosis and treatment of urinary tract infections (UTIs) and sexually transmitted infections (STIs) can be critical to a positive patient outcome. Complications from delays in treatment or ineffective treatment due to misdiagnoses can result in an increased risk of acquiring other pathogens, such as HIV; dissemination of an existing infection to the circulatory and lymphatic systems; the development of pelvic inflammatory disease, which can lead to infertility; and complications with pregnancy, including damage to the fetus and/or miscarriage. The ability to rapidly diagnose such infections, particularly those that may be asymptomatic but still transmissible, enhances disease management and public health by improving the rate of treatment and the timely notification of sexual partners for follow-up testing.

Traditional diagnostic assay methods for the causative agents of UTIs and STIs include direct culture, serology, and immunochromatographic tests and nucleic acid amplification-based tests. Of these, the DNA amplification assays are generally preferred for the detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* (Papp et al., 2014). For these pathogens, the lengthy turnaround time from specimen collection to diagnosis results in a delayed administration of appropriate therapy and a decreased efficacy of infection control strategies (Liu et al., 2012; Khare et al., 2014).

As potentially useful as sample-to-answer assay systems would be in this sphere of infectious disease management, there is a narrower variety of FDA-approved or clinical laboratory improvement amendments-waived tests available. For example, there are no approved sample-to-result molecular assays for syphilis. New assays for the diagnosis and quantitation of HIV are still in the development stage. There is no FDA-approved FilmArray® panel for UTI or STI pathogens, but a first-generation STI panel was designed to detect and identify nine common STI pathogens (*C. trachomatis*, *N. gonorrhoeae*, *Treponema pallidum*, *T. vaginalis*, *Mycoplasma genitalium*, *Haemophilus ducreyi*, herpes simplex virus 1 and 2, *Ureaplasma urealyticum*). This panel was used to test 295 clinical specimens from 190 subjects. The STI panel results were compared to results from standard clinical tests performed on duplicate specimens. These included gram staining, wet mount examination, viral culture, and the serum syphilis IgG test. Concordance between the FilmArray® STI panel and standard testing was 83% for *T. vaginalis*, 98% for *C. trachomatis*, and 97% for *N. gonorrhoeae*. The assay also detected *T. pallidum* in samples from four patients who were subsequently diagnosed with syphilis by serology.

The Cepheid Xpert® CT/NG assay detects *C. trachomatis* and *N. gonorrhoeae* on vaginal swabs, endocervical swabs, and male and female urine. The sensitivity and specificity for both targets were found to exceed 95% and 99%, respectively (Causer et al., 2014). Illustrating the potential impact of rapid diagnostic sample-to-answer platforms on public health in a point-of-care context, asymptomatic men were tested on-site at a sexual health clinic using the Xpert® CT/NG assay. The rapid turnaround time of the assay enabled infected patients (14% of those tested) to receive appropriate treatment 2 days after their test. In contrast to this, the turnaround time for specimens that were tested at an off-site laboratory using conventional methods was 10 days (Gaydos, 2014). One area for potential improvement in existing assays is the lack of validation studies to support the testing of rectal and pharyngeal specimens. This would be useful, as many gonococcal and chlamydial infections of the rectal and oropharyngeal tissues are asymptomatic. Testing of 409 rectal swabs showed the sensitivity and specificity of the Xpert® CT/NG assay to be 86% and 99.2% for *C. trachomatis* and 91.1% and 100% for *N. gonorrhoeae*, respectively (Goldenberg et al., 2012).

Infection by herpes simplex virus (HSV)-1 and HSV-2 is a common cause of genital and oral STIs. After an initial acute phase infection, the viruses typically enter a latent phase. While the rate of subclinical HSV shedding tends to decrease after the first year of infection following the initial clinical episode, viral shedding may persist at high rates in some infected individuals for several years after infection (Phipps et al., 2011). In addition, HSV reactivation from latency after the primary infection can cause a clinical recurrence of the local disease accompanied by high rates of viral shedding. The continued risk that this poses both

for HSV horizontal transmission to sexual partners and vertical transmission from mother to infant makes the diagnosis of patients who may be actively shedding important to disease management. The Simplexa™ HSV 1 & 2 Direct Kit was approved for genital swab samples in 2015. According to the manufacturer, the sensitivity and specificity of the assay from genital swabs for HSV 1 and HSV 2 exceeds 97% (Focus Diagnostice, 2015).

5.3.4 Central Nervous System Pathogens

Infections of the central nervous system (CNS) are notable for their diversity and the unique challenges they present due to the potential morbidity and mortality that they cause in conjunction with inherent difficulties involved in their diagnosis and treatment. Patients with CNS infections may display depressed levels of consciousness, photophobia, altered mental states, fever, lethargy, and a wide range of other symptomology. The etiologic agents of CNS infections may range from viruses and bacteria to fungi and even parasites. Such infections often produce changes in the cerebrospinal fluid (CSF), which makes it a valuable specimen for diagnostic analyses.

Viral infection is the most common form of aseptic meningitis, and enteroviruses are the most common viral cause, particularly in pediatric cases (Hong et al., 2015). While the disease is generally self-limiting and is typically treated with supportive therapy, it can be difficult based on symptomology to differentiate it from early stage bacterial meningitis, which is a far more serious disease that can lead to death within hours or leave patients with permanent brain damage, hearing loss, and learning disabilities. Because of the potential seriousness of CNS infections, it is not unusual for a patient to be admitted and treated with broad-spectrum antibiotics until a clear diagnosis is made. Therefore it would be reasonable to postulate that the rapid and accurate diagnosis of enteroviral-associated meningitis infections would help to prevent the unnecessary use of antibiotics, shorten the duration of hospitalization, and reduce healthcare costs (Ramers et al., 2000; King et al., 2007). This represents an ideal context for accurate and fast sample-to-answer assay systems.

The FilmArray® Meningitis/Encephalitis Panel was FDA approved in 2015. The panel targets 14 pathogens for detection (*E. coli* K1, *Haemophilus influenzae*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *cytomegalovirus*, enterovirus, HSV-1, HSV-2, HHV-6, human parechovirus, varicella zoster virus, and *Cryptococcus neoformans*). A preclinical assessment of the panel using CSF specimens reported positive and negative agreements across methods of 93% and 92%, respectively. Results obtained with the Meningitis/Encephalitis Panel were compared to results with routine testing methods, and discrepancies were resolved through the use of additional nucleic acid amplification tests or by direct sequencing (Hanson et al., 2015). A second study using the panel with an HIV-infected population in Uganda detected *Cryptococcus* in the CSF of patients diagnosed with a first episode of cryptococcal meningitis by fungal culture with 100% sensitivity and specificity (Rhein et al., 2016).

The Cepheid Xpert® EV assay also tests for the presence of enterovirus RNA in CSF. The manufacturer reports that the specificity and sensitivity of the assay exceed 96% and 97%, respectively. An independent set of studies compared patients with aseptic meningitis who had been diagnosed using the Xpert® EV assay to patients diagnosed on the basis of a conventional in-house PCR assay and to patients who had not been diagnosed at all. Those patients for whom a diagnosis was confirmed on the Xpert® EV had a significantly shorter duration of broad-spectrum antibiotic administration, and fewer patients received acyclovir. The average length of stay for these patients was only 0.5 days, compared with 2 days and 4 days for patients in the group with conventional PCR or no diagnosis, respectively. The reported sensitivity for this assay was 95−100%, and the specificity was 100% (Giulieri et al., 2015; Marlowe et al., 2008; Kost et al., 2007).

In addition to their disease-causing potential in STIs, HSV-1 and HSV-2 can also cause encephalitis, with HSV-1 being more common in pediatric cases. Immediate treatment with the antiviral drug acyclovir is indicated for those patients suspected (based on symptomology) of having HSV-associated encephalitis. Left untreated, the mortality rate for these patients approaches 70% (Raschilas et al., 2002). Ironically, the administration of powerful antiviral drugs to a patient may actually interfere with traditional diagnosis by viral culture, resulting in false negative results. Such adverse effects do not impact PCR-based assays, which makes the detection of HSV using either the FilmArray® Meningitis/Encephalitis Panel or the Simplexa™ HSV 1 & 2 Direct Kit (which is approved for use with CSF specimens) faster and more reliable diagnostic options.

5.4 FORENSIC APPLICATIONS: PERFORMANCE FOR HUMAN IDENTITY TESTING

Around the world, there has been a rapid growth in demand by law enforcement agencies for Human DNA Identity testing (i.e., DNA profiling) in connection with criminal investigations. This has driven the development of more expedient techniques to handle the increasing number of samples being submitted for analysis. Multiarray capillary electrophoretic instruments that allow for the simultaneous analysis of multiple samples as well as direct PCR

amplification methodologies, which eliminate the need for extraction and quantification, have both accelerated individual steps of the DNA analysis process. Still, accurate human DNA profiling of trace and otherwise challenging samples still requires substantial hands-on time by trained forensic analysts in centralized laboratories where segmented workflows are used to extract DNA, amplify target genetic markers, fractionate amplicons, and interpret data to produce a meaningful DNA profile.

The full integration of all steps in the DNA processing workflow into a compact system has been termed "rapid DNA." Automated rapid DNA systems allow for the generation of full STR DNA profiles in a fraction of the time required by conventional laboratory methods with minimal user intervention. This provides forensic investigators and law enforcement with the ability to identify possible perpetrators more quickly, which represents an advantage when trying to obtain a warrant or to apprehend a suspect. Aside from assisting law enforcement and military intelligence with the generation of investigative leads, these rapid DNA systems also provide forensic laboratories with faster sample processing times for the analysis of reference samples.

The full potential value of these sample-to-profile systems became evident after the US Supreme Court decision in Maryland v. King, 133S.Ct. 1958 (2013). The court ruled that the collection and analysis of a buccal swab from an arrestee for DNA profiling purposes was a legitimate police booking procedure. Moreover, the court ruled that the arrestee's DNA profile could be used by law enforcement to search a criminal DNA database as part of determining whether or not to release the individual who was arrested. This decision made instantly obvious the need for and value of being able to generate DNA profiles of arrestees at police booking stations rather than at off-site forensic laboratories.

The first fully integrated rapid DNA system for human identification was the RapidHIT™ 200 Human DNA Identification System from IntegenX. When used with the GlobalFiler® Express assay, the system produces a DNA profile consisting of 21 autosomal and 3 sex-determining markers from buccal swabs and other sample types in less than 2 h. Regardless of its speed, this integrated system would not be of use for forensic purposes if it did not meet acceptable sensitivity, precision, and accuracy standards. When used with reference quality buccal swabs, the platform has over an 88% success rate in producing complete profiles (Jovanovich et al., 2015). When a dilution series of DNA is placed onto swabs and introduced into the platform, full profiles are obtained with 200 ng of DNA, and partial profiles are obtained with as little as 10 ng of input DNA. Buccal swabs analyzed on the RapidHIT™ 200 system have been found to be 100% concordant with profiles generated by traditional laboratory methods, and resultant alleles size within 0.5 bp of corresponding alleles in the allelic ladder, demonstrating acceptable precision (Hennessy et al., 2014). These data support the use of the RapidHIT™ 200 system for the analysis of single-source buccal samples for the expedient profiling of reference-quality samples.

Aside from aiding forensic laboratories in processing reference samples more quickly, there are multiple applications for rapid DNA testing of reference samples. Federal immigration officials are investigating the use of this technology for the analysis of reference type samples to verify that children entering the United States are related to their accompanying adults. The Department of Homeland Security would like to employ rapid DNA testing to support or reject claims of familial relatedness that are used to justify permission to immigrate. At borders and ports, this technology could help to ensure that individuals entering the county are not in terrorist DNA databases. Another rapid DNA system, the DNAscan™ from Healthcare and NetBio, was the first rapid DNA system approved for the upload of generated reference DNA profiles into the National DNA Index System by the FBI. This clearly demonstrates that these systems have the ability to generate actionable intelligence for law enforcement (Tan et al., 2013).

In the United States, police agencies in Arizona, Florida, and South Carolina, to name a few, have also begun using this platform for the generation of investigative leads for casework samples. The rate of DNA profiling success for casework samples appears to be sample-dependent, but full profiles have been generated from cigarette butts (range of success 0−100%, $n = 29$ samples from 6 donors); drinking items (range of success 6−100%, $n = 13$ samples from 10 donors); and chewing gum (range of success 0−100%, $n = 23$ samples from 16 donors) (Verheij et al., 2013). Given this high variability in success rates for profile detection with forensic type samples, additional improvements in the sensitivity of these systems will be needed to allow for their application to the analysis of more challenging sample types, such as touch or contact DNA samples, which typically have only trace quantities of DNA.

When DNA quantities are not as limiting as with touch type samples, it has been demonstrated that it is possible to produce full DNA profiles using traditional laboratory methodologies following a reextraction of samples previously analyzed by the RapidHIT™ system. This indicated that the RapidHIT™ system does not consume all available biological material (Thong et al., 2015; Verheij et al., 2013). This is important considering the limited nature of many forensic type samples, since it demonstrates that if further efficiencies in DNA extraction can be achieved, the success rate with casework samples might be improved.

Police are interested in the analysis of casework samples such sample-to-profile integrated systems in order to

determine, prior to suspect release, whether an individual is potentially connected to an item of evidence from a crime scene. Military applications for casework samples include analyses of munitions and weapons to determine attribution. Currently, however, FBI policy requires all casework samples to undergo a human-specific quantification step. As this step is not part of the integrated workflow on any sample-to-profile system, casework samples analyzed using rapid DNA systems must still be reanalyzed following a traditional laboratory workflow. This exemplifies how advances in technology can push ahead of existing policy. The more widespread use of sample-to-profile systems for casework samples, therefore, will necessitate a change in official policy, taking into account the technical capabilities of these new platforms. Alternatively, a quantification module would need to be added to rapid DNA systems, even though it is not necessary for the accurate and reliable operation of the instrument.

5.5 CONTINUING EVOLUTION OF SAMPLE-TO-ANSWER TECHNOLOGIES

The development of PCR technology initiated a transformational change in the field of molecular biology. The rapid growth in the "big data analytics" of the human genome has been a major driving force behind a similar transformational change in medical diagnostics. Where health care once meant diagnostic and treatment options tailored to the physiology of the average patient, physicians and other healthcare professionals now speak of the promise of "Precision Medicine" personalized to a patient's unique physiology and genetic background. An important part of making personalized care a reality is the ability to obtain patient test data in a manner that is timelier than that possible using traditional labor-intensive assay methods. One of the important goals of molecular diagnostics, therefore, has always been the development of faster and more cost-effective approaches to performing diagnostic assays. Initially, this took the form of an emphasis on large-scale automation and high-throughput instrumentation in centralized laboratories using one-size-fits-all segmented workflows.

By bringing analyses closer to the patient in the form of "Point-of-Care Diagnostics" it was hoped that several process steps could be eliminated, thereby facilitating a shorter time to result, a faster health management response, better therapeutic turnaround times, and ultimately a greater opportunity for improved patient outcomes. Making this goal a reality, however, was not to be achieved by relying on large-scale automation but rather on process integration and miniaturization driven largely by advances in engineering guided by the diagnostic needs of physicians and patients. Specifically, advances in microfluidics and microscale automation made it possible to fully integrate, on a handheld scale, the previously segmented processes of nucleic acid extraction and purification, PCR amplification, amplicon detection, and even such complex tasks as electrophoretic size fractionation. With miniaturization also came the ability to reduce processing times so as produce answers more quickly. So simple, reliable, and user friendly are these fully integrated diagnostic systems that the terms "sample-to-answer" and "sample-to-profile" testing have entered the common lexicon of both the molecular diagnostic and the forensic/human identity testing communities.

The economic forces of the diagnostics industry have motivated both well-established players like GE and a host of new market entrants to develop and make commercially available a rapidly expanding selection of diagnostic and DNA profiling tools. A major area of focus of these commercial systems has been assays for infectious disease diagnostics. Given that this industry is still in its infancy, however, there is still enormous room for growth and technological improvement. The menu of available assays can be expanded to new pathogen panels, drug resistance panels, and assays for genetic markers of human disease predisposition. Cepheid has already moved in this direction with their Xpert® FII & FV assay for human gene variants associated with thrombophilia. Of course as infectious pathogens mutate, there will be a need for modified assays, and existing assays can be improved for the use of more patient sample types. On the engineering side, continued advances in material science and fabrication technologies will drive the development of true point-of-care diagnostics instruments that are smaller, faster, and portable enough to be used at a patient's bedside.

While this industry is well positioned for a bright future, one potential obstacle that will need to be addressed in regard to sample-to-answer devices is cost, not so much for the instrument base units but rather the costs per assay, which, in most cases, are appreciably higher than the price of more traditional assay methods. Of course it can be argued that, as with all new technologies, costs will invariably drop as the technology matures. Moreover, since many of these assays target multiple pathogens in a single assay run, the cost per pathogen targeted provides users with a better overall value than if the same series of tests had all been performed on an à la carte basis. It also may be worth asking if the ability to easy test for a wide range of pathogens in a simple 1-h test will produce a shift in how physicians think about patient diagnostics. Will multiplex panels reduce the amount of time that physicians would normally spend trying to narrow a preliminary diagnosis to a "most likely causative agent" that can be tested for? Will routine testing for pathogens that might not rise to the top of a physician's list of "most likely suspects" provide new insights on the complexity of disease processes and ensure more responsive care for those patients whose conditions do not fall within the most probable etiology?

The importance of validation studies demonstrating the reliability, precision, and accuracy of sample-to-answer assays cannot be overstated, but these will need to go hand-in-hand with rigorous assessments of the impact of these systems on patient care. It is necessary but not sufficient that an assay be "fit for purpose". Ultimately, the long-term success of these amazing systems will rest on the ability to show a clear value. For sample-to-profile systems used for human identity testing, that value might be quantified in terms of crimes prevented or solved. For medical diagnostic assays, it will be necessary to demonstrate that their use results in shorter hospital stays, the elimination of unnecessary or ineffectual treatments, improved patient outcomes, and an overall quantifiable reduction in healthcare costs.

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