Chapter 42
Bead-Based Suspension Arrays for the Detection and Identification of Respiratory Viruses

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Introduction

The clinical signs and symptoms associated with many infectious diseases are often too nonspecific to discriminate between causative agents, and thus, definitive diagnosis requires specific laboratory tests for all of the suspected pathogens. In particular, respiratory tract infections can be caused by numerous different viral, bacterial, and fungal pathogens that are indistinguishable by clinical diagnosis. Respiratory tract infections are also among the most common infections in humans, with approximately 6–9 episodes per year in children and 2–4 episodes per year in adults [1]. These infections cause considerable morbidity and mortality as well as high healthcare costs associated with doctor visits, hospitalizations, treatment, and absences from work and school. Early diagnosis of the etiological agent in a respiratory infection permits effective antimicrobial therapy and appropriate management of the disease.

Molecular assays designed to directly detect microbial nucleic acid sequences from patient specimens have allowed for more rapid diagnosis and treatment of infectious diseases with high accuracy and reduced turnaround time as compared to traditional immunological and culture-based methods. Further, molecular testing methodologies that permit multiplexing have the advantage that they allow for simultaneous detection of multiple nucleic acid sequences from the same sample in a single reaction vessel. Multiplexed tests reduce the time, labor, and cost of laboratory testing as compared to single reaction detection methods, and in addition to improved efficiency, also have a higher diagnostic yield by the ability to detect multiple infections. Thus, multiplexed molecular assays are an efficient method for the definitive diagnosis of respiratory infections and can also provide information on coinfections and secondary infections. Among the various multiplexing technologies

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available, microsphere- or bead-based suspension arrays have emerged as a standard molecular multiplexing technology in the clinical microbiology laboratory. As compared to planar arrays, some of the benefits of bead-based arrays include ease of use, low cost, statistical superiority, excellent sensitivity and specificity, faster hybridization kinetics, flexibility in array preparation, and rapid data acquisition [2, 3].

The Luminex® xMAP® Technology platform was the first commercial bead-based array platform to use differentially dyed microspheres of the same size to achieve multianalyte profiling for proteins and nucleic acids. Key drivers for adoption of the xMAP platform include cost savings, labor and workflow efficiencies, sample conservation, high sensitivity, and broad dynamic range. The system has the added benefit of being an open platform in that assays can be rapidly developed, optimized, and implemented by the end user. The versatility of this open architecture is evidenced by approximately 10,000 peer-reviewed publications describing a variety of applications. As a result, the platform was rapidly adopted for nucleic acid and protein analysis in various laboratory settings, and further, the Luminex® 200™ system has achieved a 510(k) clearance status for in vitro diagnostic (IVD) applications. The breadth of bioanalytical applications on the platform includes hundreds of commercially available analytes and a vast number of custom assays developed for a global install base of more than 7,000 instruments. This chapter describes the principle and uses of the xMAP Technology platform for infectious disease nucleic acid detection with an emphasis on applications for the detection and identification of respiratory viruses.

Technology Overview

The Luminex xMAP system incorporates polystyrene microspheres (beads) that are internally dyed with two or three spectrally distinct fluorochromes. Using precise amounts of each of these fluorochromes, an array is created consisting of different bead sets with specific spectral addresses. The unique spectral characteristics within individual bead sets allow each bead region to be specifically differentiated from all others in a multiplex. Each bead set can possess a different reactant on its surface and because bead sets can be distinguished by their spectral addresses and each address is associated with a specific analyte or target, they can be combined in a single reaction to measure up to 500 different analytes simultaneously. An additional fluorochrome coupled to a reporter molecule quantifies the biomolecular interaction that has occurred at the bead surface. The configuration of an xMAP nucleic assay consists of a suspension array where specific capture oligonucleotides are covalently coupled to the surfaces of the internally dyed bead sets. After completion of assay incubations with a nucleic acid sample and a detectable reporter reagent, the reactions are analyzed within a Luminex reader, classifying the beads based on the spectral address or bead identity (region) and quantifying the bound fluorophore in the reporter detection channel. The potential for simultaneous detection of 1−500 protein or nucleic acid targets provides for rapid, sensitive, and specific multiplexed molecular analyses.

xMAP Microspheres

The first generation of xMAP microspheres utilized two internal fluorescent dyes to produce a 100-membered array of spectrally distinct bead sets. Inclusion of a third internal dye has allowed the expansion from the original 100–500 bead regions (Fig. 42.1). Several varieties of fluorescent bead reagents are available from Luminex. The fundamental MicroPlex® Microspheres are 5.6 μm polystyrene beads functionalized with surface carboxyl groups for covalent attachment of capture ligands. Most of the currently available xMAP assays were developed on MicroPlex Microspheres. MagPlex® Microspheres are 6.5 μm superparamagnetic beads functionalized with surface carboxyl groups for covalent attachment of ligands. MagPlex Microspheres exhibit both high performance and low nonspecific binding, and they can be magnetically separated from solution, allowing easy automation of assay processes and simplifying assay wash steps. Microsphere reagents precoupled with unique capture oligonucleotides (oligos) are also available for nucleic acid assay development. These reagents incorporate the use of xTAG® Technology, a proprietary universal tag sorting system that allows easy optimization, development, and expansion of molecular diagnostic assays and eliminates the need to couple content-specific capture probes to the beads. xTAG oligos are optimized to be an isothermal set and have minimum cross-reactivity. Earlier assays used MicroPlex-TAG™ Microspheres which are nonmagnetic MicroPlex beads, containing two internal fluorescent dyes and precoupled with xTAG oligo capture sequences (anti-TAGs). These beads have been replaced with MagPlex-TAG™ Microspheres which are superparamagnetic MagPlex beads, containing two or three internal fluorescent dyes and precoupled with anti-TAG capture oligos.
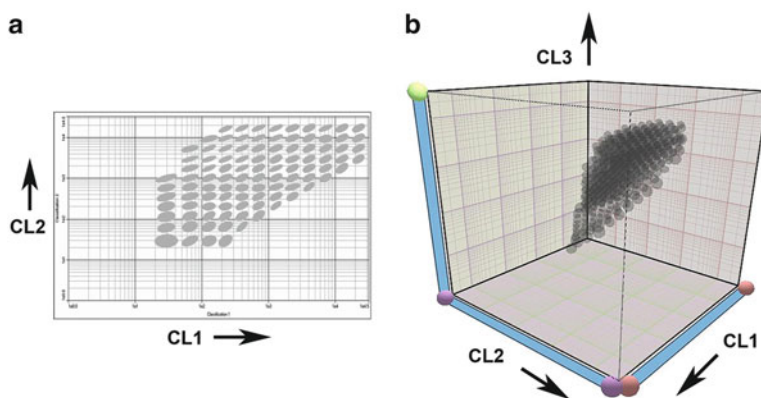

Fig. 42.1 Luminex bead maps. (**a**) Two-dimensional representation of the 100-plex bead map with Classification 1 dye (CL1) on the *x*-axis and Classification 2 dye (CL2) on the *y*-axis. *Gray ovals* indicate the positions of the 100 bead regions. (**b**) Three-dimensional representation of the 500-plex bead map with CL2 on the x- axis, Classification 3 dye (CL3) on the *y*-axis, and CL1 on the *z*-axis. *Gray ovals* show the positions of the 500 bead regions. The software allows the user to zoom in to specific areas ("slices") of the bead map for a more detailed view

Luminex Analyzers

There are currently three xMAP analyzers available through Luminex or its partners: the Luminex 200, the FLEXMAP 3D®, and the MAGPIX® (Fig. 42.2). The Luminex 200 and the FLEXMAP 3D are flow analyzers capable of multiplexing up to 100- or 500-plex, respectively, with fluidics and laser-based optics similar to traditional flow cytometry instruments. The MAGPIX utilizes a flow cell and CCD-based optics with magnetic beads for multiplexing up to 50-plex. In the flow analyzers, the beads are introduced into a rapidly flowing fluid stream and through hydrodynamic focusing, are interrogated individually as they pass by two separate lasers. A 635-nm, 10-mW red diode laser excites the fluorochromes contained within the microspheres and a 532-nm, 13-mW yttrium aluminum garnet (YAG) laser excites the reporter fluorochrome (R-phycoerythrin, Alexa 532, or Cy-3) bound to the bead surface. High-speed digital signal processing classifies the microsphere based on its spectral address and quantifies the reaction on the microsphere surface. Multiple readings are made per microsphere set, providing valid and robust statistics. Thousands of microspheres are interrogated per second resulting in a system capable of analyzing and reporting up to 500 different reactions in a single reaction vessel in just a few seconds per sample.

The Luminex 200 is designed to meet the multiplex testing needs of clinical and research laboratories. Built on proven, existing technologies including flow cytometry, microspheres, lasers, digital signal processing, and traditional chemistry, the Luminex 200 analyzer allows multiplex analysis of up to 100 analytes per reaction. The system is versatile and can be used for a variety of applications such as, immunoassays, genotyping, gene expression, and enzymatic assays. A few microliters of sample in a 20–200 μL reaction can provide up to 100 results, thus the platform is well suited for applications where sample size is limited. Robust optics and fluidics afford quantitative results over a 3–4 log dynamic range with strong concordance to enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction (PCR) and mass spectrometry. An intuitive template-based software, xPONENT®, operates the system and is designed for use with commercial kits or custom protocols and has simple assay set up, plate reading, and data analysis capabilities. The 21 CFR Part 11 compatible upgrade offers multilevel user management, full audit trail, electronic records, and electronic signatures. The system is approved for IVD use, with more than 50 xMAP-based 510(k) cleared kits available and numerous existing laboratory-developed tests (LDTs).

The FLEXMAP 3D is an enhanced flow-based multiplexing system utilizing xMAP Technology. The FLEXMAP 3D system assures rapid high-throughput analyses without compromising flexibility or performance and optimizes workflow through automation of routine tasks and integration with front-end sample preparation platforms. Enhanced optics permit multiplexing of up to 500 analytes per well and provide enhanced sensitivity with dynamic range extended to 4.5 logs. The system is compatible with both 96-well and 384-well plates and has a piercing probe which allows sealed plates to be analyzed. The dual syringe configuration processes plates 2–3 times faster than the Luminex 200. The system software, xPONENT, has
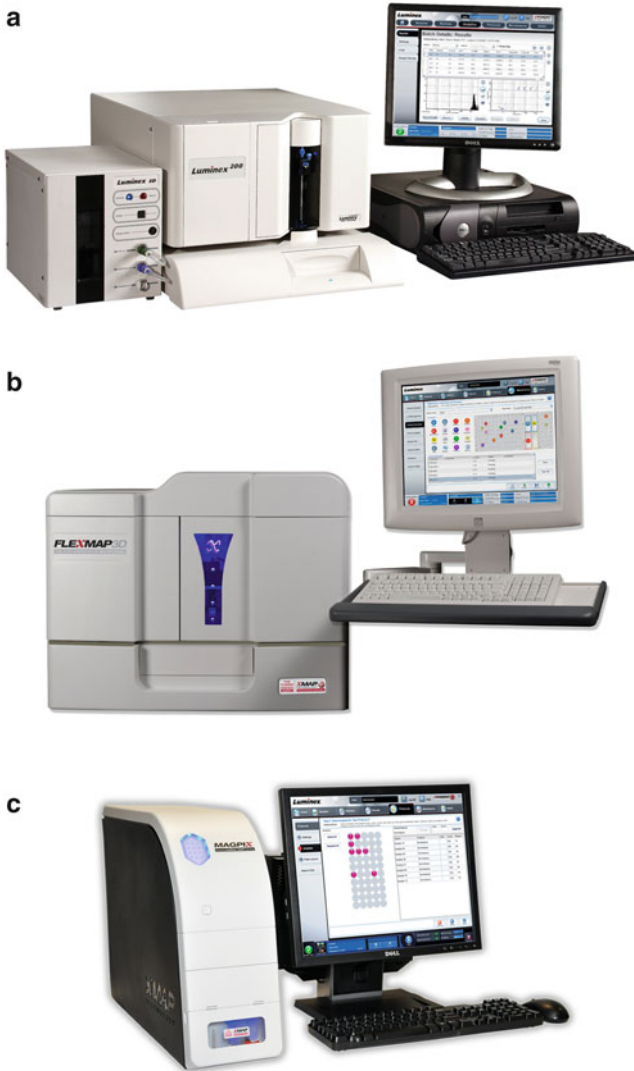
Fig. 42.2 Luminex analyzers. (**a**) The Luminex® 200™ total system includes the Luminex 200 flow analyzer, the Luminex XYP™ plate handling platform, the Luminex SD™ sheath fluid delivery system, xPONENT® software, and computer. (**b**) The FLEXMAP 3D™ system includes analyzer, plate handling, and fluid delivery systems integrated within a single unit. Also included are xPONENT® software and computer with an articulating arm to house the monitor and keyboard. (**c**) The MAGPIX® system is a compact system based on CCD imaging technology. xPONENT® software provides stream-lined start-up and shutdown protocols and minimal maintenance requirements

automation and LIS interface components and includes walk-away maintenance and calibration functions. This combination of advanced features and capabilities can help to accelerate the discovery and development process and make the FLEXMAP 3D an ideal platform for multiplexing analytes that may have broadly dissimilar levels and eliminating the need for sample reanalysis due to out-of-range results.

The MAGPIX, which utilizes a flow cell and robust LED/CCD-based optics, was released in July 2010. The MAGPIX system supports multiplexing of up to 50 tests in a single reaction volume using MagPlex or MagPlex-TAG beads. In MAGPIX, the reacted magnetic beads are sent through a flow cell into an imaging flow cell where a magnetic actuator pulls the beads out of suspension and holds them in place for optical analysis. Red LEDs (630-nm) excite the fluorescent dyes contained within the microspheres and green LEDs (515–521-nm) excite the reporter fluorochrome bound to the bead surface. A CCD imager identifies the bead region and quantifies the bound reporter. xPONENT for MAGPIX operates the system and can be used with commercial kits or user-developed assays. With a lower cost and a compact size (requiring only 64.8 cm bench space), MAGPIX provides an affordable multiplexing solution ideal for the low- to medium-throughput laboratory and remote laboratory testing sites.

Nucleic Acid Chemistries and Assay Development

xMAP Technology provides a flexible, open platform where users can easily develop their own custom assays. Various assay chemistries and assay development techniques have been used for nucleic acid detection on the xMAP Technology platform. Detailed sample protocols and recommendations to guide the user through the assay development process are available as downloads in the Support section of the Luminex website [4]. Assay development training is available from Luminex and custom assay development services are also available from Luminex and its partners. A general workflow for nucleic acid assay development can be described as follows: (1) Acquire materials and reagents (e.g., oligos for amplification and capture/detection, enzymes, buffers, reporter fluorophore, nucleic acid samples, and standards/controls); (2) Purify the reagent to be coupled of extraneous primary amines (e.g., Tris), if needed; (3) Conjugate the microspheres with the capture oligo; (4) Evaluate coupling efficiency using an appropriate target and detection reagent (e.g., biotinylated reverse complementary oligo); (5) Assess assay performance (background, sensitivity, specificity) using positive and negative control samples; and (6) Optimize amplification conditions and/or hybridization time, temperature, and sample input.

Microsphere Coupling

Capture oligos are modified with a spacer and terminal amine (e.g., 5′-Amino-Modifier C12) and covalently coupled to carboxylated beads using a carbodiimide

coupling procedure [5, 6]. Capture oligos should be resuspended and diluted in dH$_2$O as Tris, azide, or other amine-containing buffers can interfere in the coupling process. If oligos were previously solubilized in an amine-containing buffer, they should be desalted by column or precipitation and resuspended into dH$_2$O. Stock uncoupled carboxylated beads are washed and resuspended in 100 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 4.5. The capture oligo is added, followed by addition of *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) and incubation in the dark for 30 min. The EDC addition and incubation are repeated and then the coupled beads are washed once with 0.02 % Tween-20 and once with 0.1 % SDS. Coupled microspheres are stored in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 (TE) at 2–8 °C in the dark. When properly stored, coupled microspheres are usually stable for more than 1 year [7]. Coupling reactions can be easily scaled up or down according to user needs. Initial coupling and optimization of coupling amount (i.e., nmol oligo per million microspheres) is usually done in small coupling reactions of 2.5−5 million microspheres. Coupling reactions can then be scaled up for manufacturing and preparation of bulk reagents. For optimal coupling efficiency, it is important to perform oligo coupling reactions in as small a reaction volume as possible, typically 25−100 μL for 2.5−100 million beads [8].

Verification of coupling can be performed using a biotinylated reverse complementary positive control oligo [9]. Coupled microspheres are resuspended by vortex and sonication and combined to a final concentration of 100−150 beads of each set/μL in hybridization buffer. Target biotinylated oligos are added (5−200 fmol) and hybridized to the coupled beads for 15 min, followed by labeling with reporter (streptavidin-R-phycoerythrin) for 5 min. The reactions are then ready for analysis. Effective coupling is demonstrated by a dose response increase in median fluorescent intensity (MFI) with increasing concentration of oligo target [10].

Direct Hybridization

Several assay chemistries have been used for nucleic acid detection on the xMAP system. One approach is to use direct hybridization of a labeled PCR-amplified target DNA to bead sets bearing oligonucleotide capture probes specific for each sequence (Fig. 42.3). Direct hybridization is the simplest assay chemistry that can provide single nucleotide discrimination and takes advantage of the fact that the melting temperature for hybridization of a perfectly matched template compared to one with a single base mismatch can differ by several degrees for capture oligos approximately 15−20 nucleotides in length [11, 12]. The reaction kinetics can be adversely affected by immobilization of the reactant on a solid surface but these effects are less severe for bead-based suspension arrays; however, the diffusion rate of the immobilized capture probe can be slower and the effective concentration is reduced as compared to free DNA in solution [13]. Design of sequence-specific capture probes and PCR primers for a direct hybridization assay on the xMAP suspension array can be facilitated through the use of a tetramethylammonium chloride

Fig. 42.3 Direct
hybridization. Target DNA is
PCR-amplified with one
biotinylated and one
unlabeled primer. The PCR
products are denatured,
hybridized to probe-coupled
bead sets, and labeled for
detection with streptavidin-R-
phycoerythrin
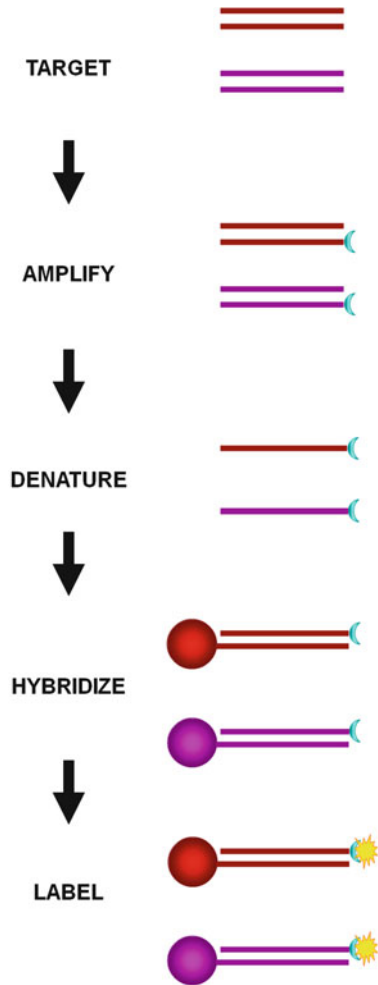
(TMAC)-containing hybridization buffer. TMAC stabilizes AT base pairs, minimiz-
ing the effect of base composition on hybridization [14, 15]. For oligos less than 200
base pairs in length, hybridization efficiency in TMAC is a function of the length of
the perfect match and less dependent on base composition. Thus, hybridization buf-
fers incorporating 3 or 4 M TMAC equalize the melting points of different probes,
allowing probes with different characteristics to be used under identical conditions
with high duplex yields [16, 17]. Typically for single nucleotide discrimination,
capture probes are designed to be matched in length at approximately 20 nucle-
otides. The probes are complementary in sequence to the labeled strand of the PCR
product and the polymorphic nucleotide is located at the center of the probe, as
mismatches in the center have a more profound effect on the equilibrium state than
mismatches near the 5′ or 3′end [18, 19].

Optimal assay conditions are determined by evaluating the effect of hybridization temperature, probe length, and input target concentration on assay sensitivity and specificity. The probe melting temperature is influenced by length, sequence, and type and position of the mismatched base. The effect of a mismatch on hybridization is greater with increasing temperature and decreasing probe length [12, 20]. Thus, discrimination can be improved by increasing the hybridization temperature and/or decreasing the probe length. After testing known DNA samples under a set of standard hybridization conditions, nucleotides are added to the 5′ and/or 3′ ends of the probe to improve sensitivity or removed from the 5′ and/or 3′ ends of the probe to increase specificity. The position of the polymorphism within the probe sequence can be adjusted when necessary to avoid formation of secondary structures and adequate specificity can usually be achieved when the polymorphic nucleotide is between positions 8 and 14 of a 20-nucleotide probe [19].

PCR primers are typically designed to amplify 100–300 base pair regions of target sequence with one primer of each pair biotinylated at the 5′ end for labeling the target strand of the amplicon. Using a small target DNA minimizes the potential for steric hindrance to affect hybridization efficiency. In some cases, larger targets (400–1,200 base pairs) have been used successfully, suggesting that hybridization efficiency is also dependent on the sequence and overall secondary structure of target [21]. Hybridization kinetics and thermodynamic affinities of matched and mismatched sequences can be driven in a concentration-dependent manner [22]. At concentrations beyond the saturation level, the hybridization efficiency can decrease presumably due to competition of the complementary strand and renaturation of the PCR product [20, 23]. Therefore, it is also important to determine the range of target concentrations that yield efficient hybridization without sacrificing discrimination.

Solution-Based Chemistries with Bead Capture

Another approach is to use a sequence-specific enzymatic reaction in solution to determine the target sequence followed by capture onto the bead surface for detection (Fig. 42.4). This format involves the incorporation of a specific capture sequence during the enzymatic step that allows hybridization to a complementary "address" sequence on the bead surface. Commonly used enzymatic methods for sequence determination rely on the discriminating ability of DNA polymerases and DNA ligases, and include allele-specific or target-specific primer extension (ASPE or TSPE), oligonucleotide ligation assay (OLA), single base chain extension (SBCE), and target-specific PCR (TS-PCR) [24–26]. This approach takes advantage of solution-phase kinetics and permits the addressed bead sets to be used in many different assays where new sequences can be targeted by adding the appropriate capture sequence to the target-specific oligo used in the enzymatic step. Hybridization buffer and reaction conditions for detection are dependent upon the capture sequences attached to the microspheres. Commercially available Luminex MagPlex-TAG Microspheres, precoupled with xTAG capture oligos (anti-TAGs) are compatible with
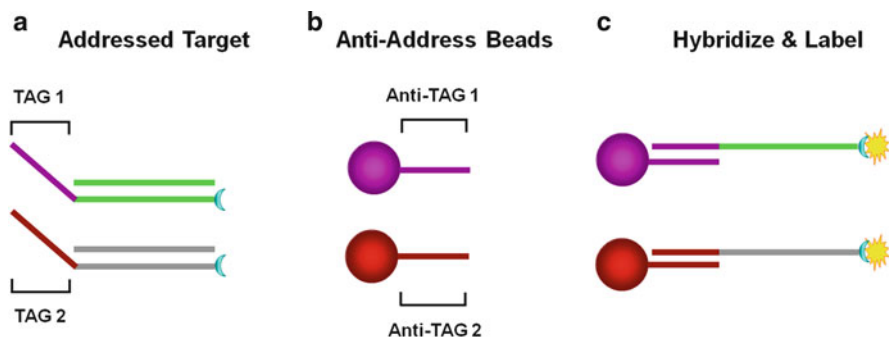
Fig. 42.4 Capture of "addressed" targets onto precoupled beads. (**a**) Solution-based enzymatic assay amplifies targets, incorporating a unique address sequence into each product. (**b**) Bead sets coupled with complementary capture sequences ("anti-address"). (**c**) Products are captured onto beads through hybridization of the anti-address capture and address sequences, and labeled with streptavidin-R-phycoerythrin

solution-based enzymatic chemistries. The assay developer appends an appropriate complementary TAG sequence to each target-specific oligo to allow capture onto the corresponding anti-TAG coupled to the bead.

Assay development parameters for enzymatic solution-based nucleic acid assays on the xMAP platform have been described and various procedures are illustrated in Fig. 42.5 [27–29]. Briefly, target-specific oligos are designed to be matched in melting temperature at 51−56 °C. For TSPE, TS-PCR, and OLA, the discriminating nucleotide is positioned at the 3′ end of the oligo, whereas for SBCE the 3′ end of the primer is positioned one nucleotide upstream. The unique capture sequence for each target is incorporated at the 5′ end of the oligo. Optimization, when needed to improve sensitivity or specificity, can be achieved by adding or removing nucleotides from the 5′ end of the target-specific sequence (immediately downstream from the capture sequence) or by targeting the opposite DNA strand.

Templates containing the target sequences for the TSPE, OLA, and SBCE reactions are generated by PCR using unlabeled primers. In TSPE, a thermostable polymerase is used to extend the primer by incorporation of dNTPs, including one which is biotin-labeled. Extension only occurs if the 3′ nucleotide of the primer is complementary and can anneal to the template DNA. OLA employs the same oligo design but a thermostable ligase is used to ligate a biotin-labeled oligo (reporter probe)

Fig. 42.5 (continued) Sample DNA is amplified and annealed to addressed target-specific primers in a reaction containing a DNA polymerase and a biotinylated ddNTP (in separate reactions for each nucleotide). Targets are extended and addressed extension products are captured onto complementary beads sets, and labeled with streptavidin-R-phycoerythrin. (**d**) TS-PCR. Sample DNA is amplified using addressed target-specific upstream primers paired with downstream biotinylated primers. Addressed PCR products are simultaneously hybridized to complementary bead sets and labeled with streptavidin-R-phycoerythrin. This example depicts a spacer between address and target-specific primer sequences to prevent amplification of the capture sequence
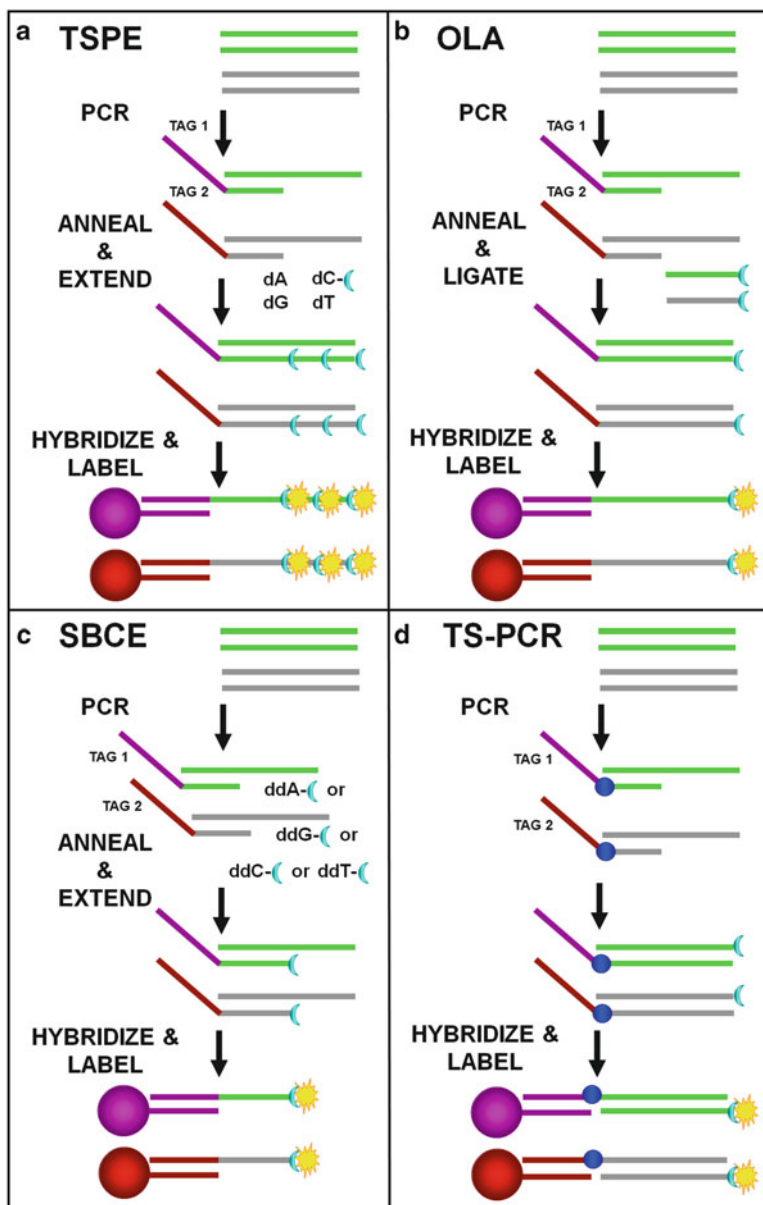
Fig. 42.5 Chemistries used for addressed microsphere capture assays. (**a**) TSPE. Sample DNA is amplified and annealed to addressed target-specific primers in a reaction containing a DNA polymerase and dNTPs (one with biotin label). Targets are extended and addressed extension products are captured onto complementary beads sets, and labeled with streptavidin-R-phycoerythrin. (**b**) OLA. Sample DNA is amplified and annealed to addressed target-specific primers in a reaction containing a DNA ligase and biotinylated reporter probe. Addressed ligation products are captured onto complementary beads sets, and labeled with streptavidin-R-phycoerythrin. (**c**) SBCE.

that is complementary to the sequence downstream from the target nucleotide. The reporter probe is designed to be matched in melting temperature at 51–56 °C, is phosphorylated at the 5′ end to provide a substrate for ligase, and is biotin-labeled at the 3′ end for fluorescent detection with streptavidin-R-phycoerythrin. For SBCE, individual reactions must be set up for each of the four possible nucleotides and a thermostable polymerase is used to incorporate a single biotin-labeled ddNTP. Extension occurs only if the nucleotide complementary to the sequence immediately downstream of the primer is present in the reaction. Targets for SBCE can be combined for each of the nucleotide reactions and if different capture sequences are used for each, the products can be multiplexed for capture onto the addressed microsphere sets. For TS-PCR, target sequences are directly amplified and labeled by PCR using one primer containing both capture and target-specific sequences paired with a biotinylated target-specific primer. This method requires a strategy which minimizes or eliminates production of the capture sequence in the nontarget strand, which would compete for hybridization of the target to the addressed bead. Some methods include asymmetric PCR, post-PCR treatment with phosphatase and exonuclease, or incorporating a spacer between the capture-specific and target-specific sequences of the primer [29]. TS-PCR chemistry eliminates the need for separate amplification and target-specific enzymatic steps, thus simplifying the assay procedure and reducing reagent costs and turnaround time.

Bead-Based Applications for Respiratory Virus Detection and Identification

Molecular analysis of infectious organisms is widely applied in healthcare and numerous applications using xMAP technology have been described for the detection of bacterial, viral, fungal and parasitic pathogens. Several commercial products are available for multiplexed detection and identification of respiratory viruses using xMAP bead-based suspension arrays, including the ResPlex™ II Panel from Qiagen, Inc. (Valencia, CA), and the xTAG RVP and xTAG RVP FAST assays from Luminex Molecular Diagnostics, Inc. (Toronto, ON). Since their development, these assays are being increasingly used for clinical diagnostics and epidemiological research, and numerous studies utilizing bead-based suspension array assays for respiratory virus detection have been reported.

The ResPlex II Panel is a for research use only (RUO) product that employs a novel multiplex reverse transcription-polymerase chain reaction (RT-PCR) strategy, called Tem-PCR (Target Enriched Multiplexing-PCR), combined with direct hybridization for capture onto target-specific probe-coupled bead sets. Tem-PCR uses nested gene-specific primers at low concentrations to enrich the specific targets during initial PCR cycles, followed by universal forward and reverse primers at high but unequal concentrations to achieve exponential asymmetric amplification [30]. The primer in excess is biotinylated to yield a surplus of labeled target strands that can be hybridized and detected on the complementary bead sets. The ResPlex II Panel

detects 18 respiratory viral targets: respiratory syncytial viruses (RSV) A and B, influenza A virus (Flu-A), influenza B virus (Flu-B), parainfluenza viruses (PIV) 1–4, human metapneumoviruses (hMPV) A and B, coxsackieviruses/echovirus (CVEV), rhinovirus (RhV), adenoviruses (AdV) A and E, coronaviruses (CoV) NL63, HKU1, 229E and OC43, and bocavirus (BocV) [31]. The panel also includes an internal control to monitor viral RNA isolation and PCR inhibition, and a positive control for human genomic DNA to monitor sample integrity and success of the run.

Li et al. evaluated the ResPlex II panel in a retrospective study using 360 frozen respiratory specimens and the results were compared to those obtained by a reference standard of culture and individual real-time TaqMan® RT-PCR assays [32]. Sensitivity and specificity for Flu-A, Flu-B, PIV-1, PIV-3, and RSV were 72.2–90.0 % and 99.7–100 %, respectively, and the assay could process 96 samples in 6 h. Brunstein et al. used the ResPlex II panel in combination with the ResPlex I assay, which tests for bacterial pathogens and AdV A and E, in parallel with a DFA and culture testing protocol to investigate the incidence of coinfections and the potential for complex interactions between multiple pathogens in respiratory infections [33, 34]. Coinfections were found in 27 % of the specimens tested. Using multiple statistical methods, they determined patterns of association between pathogens found in individual specimens. Potential positive correlations were found for specimens coinfected with the following: *Streptococcus pneumoniae* and *Haemophilus influenzae*, *Neisseria meningitidis* and Flu-B, and CVEV and RhV. In addition, while overall prevalence for *S. pneumoniae* was 20 %, prevalence was 26 % in RSV B positive specimens. Although the results were preliminary, the authors concluded that the data support the hypothesis that coinfection with multiple respiratory pathogens is medically relevant and that effective treatment for severe respiratory infections may require diagnosis of all pathogens involved, rather than single-pathogen reporting protocols where diagnostic testing is discontinued after identification of the first relevant pathogen.

The xTAG respiratory viral panel (RVP) assay utilizes a TSPE chemistry with xTAG Technology to identify all major respiratory viruses commonly encountered, including Flu-A subtypes H1 and H3, in a single test. The assay is approved for in vitro diagnostic use (IVD) in the United States, Canada, and Europe for 12–19 virus types and subtypes, depending on geographical location. The regulatory clearance status for each target in each region is shown in Table 42.1. The assay also includes an internal (extraction control), a positive (run) control, and xTAG Data Analysis Software (TDAS) to interpret the MFI results and establish the presence or absence of each target. RVP is compatible with the Luminex 100/200 instrument and can provide results for 96 samples in a single work day (approximately 8 h). The RVP assay was first described by Mahony et al. in a prospective evaluation using 294 nasopharyngeal swab (NP) specimens and compared to direct fluorescent antibody assay (DFA) and culture. RVP detected 112 positives as compared to 119 positives detected by DFA/culture for a sensitivity of 97 %, and further, detected an additional 61 positive specimens that were either negative by DFA/culture or positive for viruses not tested by DFA/culture [35]. Following resolution of discordants by PCR, RVP detected 180 of 183 true positives (98.5 % sensitivity) as compared to 126 of

Table 42.1 xTAG RVP targets and regulatory clearances

Viral family and subtype	RVP		
	US-IVD	Health Canada IVD	CE-IVD Europe
Respiratory syncytial virus (RSV)			
RSV A	•	•	•
RSV B	•	•	•
Influenza A (Flu-A)			
Nonspecific Flu-A	•	•	•
H1 subtype	•	•	•
H3 subtype	•	•	•
H5 subtype			•
Influenza B (Flu-B)	•	•	•
Parainfluenza 1 (PIV-1)	•	•	•
Parainfluenza 2 (PIV-2)	•	•	•
Parainfluenza 3 (PIV-3)	•	•	•
Parainfluenza 4 (PIV-4)		•	•
Metapneumovirus (hMPV)	•	•	•
Adenovirus	•	•	•
Enterovirus-Rhinovirus (EV-RhV)	•	•	•
Coronavirus (CoV) NL63		•	•
CoV HKU1		•	•
CoV 229E		•	•
CoV OC43		•	•
CoV SARS			•
MS2 bacteriophage internal control			
Lambda phage positive control			

183 true positives by DFA/culture (68.8 % sensitivity). RVP detected the presence of two viruses in 5.2 % of the specimens tested. The performance of RVP was also compared to real-time PCR in a parallel study using the same sample extracts [36]. The results were comparable for samples with Ct values less than or equal to 35 and RVP demonstrated signal to noise ratios of greater than or equal to 50.

For IVD clearance, the performance characteristics of the RVP assay were established in a multicenter clinical trial involving six sites in North America and Europe using a total of 1,464 specimens collected and tested during the 2005/2006 influenza season [37–39]. For 544 prospectively collected NP swabs, sensitivity was greater than 91 % (91.5−100 %) for all analytes except PIV-3 (84.2 %), PIV-4 (50.0 %), and AdV (78.3 %). However, only two positive specimens were available for PIV-4, and sequence analysis revealed that low overall sensitivity for AdV was mainly due to poor detection of serotypes in the AdV C species. In addition, few positive samples were available for CoV, thus sensitivity for CoV types ranged from 0.0 to 100 % or could not determined. Specificity was greater than 91 % (91.3−100 %) for all analytes, the limit of detection (LoD) was from 6×10^{-2} to 5×10^{4} median tissue culture infective dose $(TCID_{50})$/mL, and overall site-to-site reproducibility was 98.2 %. No interference was detected in 16 combinations of analyte plus potential bacterial or

viral interferent, and no cross-reactivity was observed for 26 other bacterial and viral pathogens tested. One BocV sample yielded a false positive call for RhV but was subsequently determined to be contaminated with RhV. In addition, there were no significant differences in the sensitivity of the target analytes in medicated versus unmedicated patients.

In a comparison study, ResPlex II and RVP suspension array assays detected more viruses than culture for 202 adult respiratory specimens, including NP and other swabs, bronchoalveolar lavages (BAL), lung biopsies, pleural fluids and sputa [40]. RVP showed 100 % sensitivity and negative predictive value (NPV), 93 % specificity, and near perfect agreement with culture. ResPlex II had slightly higher specificity (94 %), 80 % positive predictive value (PPV), and 89 % sensitivity. Coinfections were detected in 1–4.5 % of samples, most of which included a PIV, furthermore PIV-1 and PIV-2 were only detected in multiple infections. The ResPlex II was easiest to use with one reagent preparation/amplification step and one hybridization/detection step, generating results in 5.5 h, whereas RVP was more labor intensive with five reagent preparation steps and two thermal cycler programs, resulting in a 7–8 h turnaround time. The ResPlex II (RUO) offered a broader coverage of virus types than the RVP (IVD) assay, but RVP had the ability to differentiate human from nonhuman Flu-A subtypes. The authors concluded that as compared to culture, multiplexed molecular assays could provide enhanced sensitivity, faster turnaround time, and broader coverage, and should prove to be a useful tool for respiratory virus identification as an aid in patient management and outbreak investigations.

Multiplexed molecular assays can be useful as a screening tool for the detection of novel or emerging viral strains and for outbreak investigations. Wong et al. used the RVP assay in a retrospective study of 1,108 specimens from 244 suspected respiratory virus outbreaks to assess utility for enhanced respiratory outbreak investigation [41]. When used in combination with DFA and various in-house nucleic acid amplification tests (NATs), RVP testing improved detection of a viral etiological agent from 72.5 to 90.8 % of outbreaks. The RVP assay also proved to be an effective aid for the detection of 2009 Flu-A/H1N1 strains [42–44]. While the assay can detect the matrix gene of 2009 Flu-A/H1N1, it cannot identify the hemagglutinin (HA) gene of 2009 Flu-A/H1N1 in clinical specimens. In New York during the 2009 Flu-A/H1N1 outbreak, the RVP assay was used to test 375 samples that initially tested positive for Flu-A by rapid antigen tests, DFA, and culture, or were from patients with suspected infection. RVP identified 201 samples as Flu-A, with 60 identified as seasonal (H1 and H3) strains but the remaining 141 were unsubtypeable (negative for both H1 and H3). The CDC real-time RT-PCR assay specific for 2009 Flu-A/H1N1 was used to further test 101 of these specimens and 99 were identified as positive for 2009 Flu-A/H1N1. These data indicated that the predictability of unsubtypeable Flu-A identified by the RVP assay to be pandemic Flu-A/H1N1 was high. Implementation of RVP screening in the testing algorithm enabled the laboratory to rapidly subtype Flu-A, rule out seasonal H1 and H3 subtypes, detect additional respiratory viruses, and identify probable pandemic Flu-A/H1N1 cases.

The xTAG RVP FAST assay is a newer version of the xTAG RVP assay, modified to have a simpler protocol with a faster turnaround time. The assay has been

Table 42.2 xTAG RVP FAST targets and regulatory clearances

Viral family and subtype	RVP		
	US-IVD	Health Canada IVD	CE-IVD Europe
Respiratory syncytial virus (RSV)		•	•
Influenza A (Flu-A)			
Nonspecific Flu-A	•	•	•
H1 subtype	•	•	•
H3 subtype	•	•	•
Influenza B (Flu-B)	•	•	•
Parainfluenza 1 (PIV-1)		•	•
Parainfluenza 2 (PIV-2)		•	•
Parainfluenza 3 (PIV-3)		•	•
Parainfluenza 4 (PIV-4)		•	•
Metapneumovirus (hMPV)	•	•	•
Adenovirus	•	•	•
Enterovirus-Rhinovirus (EV-RhV)	•	•	•
Coronavirus (CoV) NL63		•	•
CoV HKU1		•	•
CoV 229E		•	•
CoV OC43		•	•
Bocavirus (BocV)		•	•
MS2 bacteriophage internal control			
Lambda phage positive control			

approved for IVD use in the United States, Canada, and Europe for 8−17 virus types and subtypes (Table 42.2). The targets detected have been modified slightly from the RVP assay and, depending on geographical location, can additionally detect BocV. RVP FAST uses TS-PCR chemistry where a multiplexed RT-PCR reaction is performed using target-specific upstream primers containing a TAG capture sequence paired with biotinylated downstream primers to amplify the target sequences in a single reaction. The amplified product is added to a combined hybridization/detection reaction containing the corresponding anti-TAG bead sets and streptavidin-R-phycoerythrin reporter, and then the completed reactions are read on the Luminex analyzer to determine the MFI signal. The raw fluorescence values are then analyzed using TDAS for RVP FAST software and each target interpreted as present or absent. The RVP FAST assay combines amplification with target-specific extension, and uses a combined hybridization and detection step, resulting in a turn-around time of less than 5 h (including nucleic acid extraction). Further, the risk of contamination from post-PCR manipulation is minimized by the reduction of operator handling steps to a single post-PCR transfer.

The performance characteristics of RVP FAST for IVD use were determined from two datasets resulting from multicenter clinical studies of 1,518 total specimens (EU/Canada study) and 1,191 NP swab specimens (US study) submitted for viral testing at three independent laboratories [45, 46]. For the combined dataset ($n=1518$), sensitivity was greater than 91 % (91.2−100 %) for all analytes with the exception of PIV where sensitivity was 76 % for PIV-2, 76.29 % for PIV-3, and 80 % for PIV-1, respectively. Specificity was 89.2−99.33 % and LoD ranged from

0.1 to 1,000 $TCID_{50}$/mL, depending on analyte. An additional 285 specimens were collected and tested for a subset of targets and showed sensitivity of 96.49–100 % and specificity of 89.47–98.57 % when compared to real-time RT-PCR [45]. For the NP swab dataset ($n = 1,191$), sensitivity and specificity was 90.9–97.2 and 92.5–99.3 %, respectively [46]. A coinfection was identified in 3 % of these specimens and was most commonly due to RSV/RhV at 1.1 % prevalence and Flu-A/RhV at 0.4 % prevalence. The LoDs for the viral analyte targets were determined by serial dilution of high titer stocks in Universal Transport Medium (UTM) and ranged from 1.4×10^{-2} to 3.9×10 [2] $TCID_{50}$/mL. No cross-reactivity was observed for 30 (20 bacterial and 10 viral) pathogens tested and no interference was detected in 14 combinations of analyte plus potential bacterial or viral interferent. In addition, there were no significant differences in the sensitivity of the target analytes in medicated versus unmedicated patients.

RVP FAST was compared to culture, DFA, and a combination of single and multiplex real-time RT-PCR assays in a retrospective study of 286 respiratory specimens [47]. At least one respiratory virus was detected in 13.6 % of specimens by culture and DFA combined, in 49.7 % by real-time RT-PCR, and in 46.2 % by RVP FAST. Using real-time RT-PCR results as the gold standard, RVP FAST had 78.8 % sensitivity and 99.6 % specificity. Specimens not detected by RVP FAST generally had low viral loads or contained AdV. For some viral targets, few positive samples were available in the set tested, which suggests that further study using larger numbers of positive samples is needed to fully assess sensitivity of RVP FAST for all targets. Overall, the investigators found RVP FAST to be rapid and easy to perform in comparison to the multiple real-time RT-PCR assays that would be required for equal target coverage. Pabbaraju et al. compared RVP FAST with the original RVP assay on 334 respiratory specimens that had been previously characterized for a variety of respiratory virus targets [48]. Samples were tested in parallel by both assays and the RVP assay was found to be more sensitive than RVP FAST for the combined targets, with sensitivities of 88.6 and 77.5 %, respectively. However, RVP FAST was tenfold more sensitive for the detection of RSV A. Targets not detected by RVP FAST were primarily Flu-B, PIV-2, and CoV 229E. In addition, a small number of samples positive for Flu-A, RSV B, hMNV, and PIV-1 were not detected by the RVP assay and generally had low viral loads. Reproducibility was similar between the two assays but RVP FAST exhibited better reproducibility for detection of AdV at the LoD. The authors concluded that RVP FAST met the diagnostic needs for sensitivity and specificity in their laboratory with the exception of Flu-B, which had decreased sensitivity for Flu-B in specimens collected in 2010. Reduced sensitivity for PIV-2 and CoV 229E was of lesser concern because PIV-2 has low prevalence and detection of CoV 229E does not affect established patient management protocols. Their preliminary studies also indicated that use of RVP FAST will lead to cost savings and improved turnaround time as a result of the shorter protocol and a reduction in hands-on time to 1.5 h.

The openness of the xMAP platform lends itself to the rapid development of new assays as analytes of interest are identified and described. As an example, the emergence of antiviral resistance in influenza has raised concerns about use of antiviral drugs and treatment in response to future pandemic outbreaks. Amantadine resistance

has increased dramatically for Flu-A/H3N2 and Flu-A/H1N1, and increasing oseltamivir resistance for seasonal Flu-A/H1N1 has been reported worldwide since 2007 [49–51]. Currently, resistance testing is done by sequencing or phenotypic assays which can be costly and time consuming; however, a multiplexed bead-based array could be applied for this purpose in combination with or in addition to a comprehensive respiratory viral panel. This is illustrated in a report of a multiplexed bead-based LDT that was developed for simultaneous identification of Flu-A subtypes and the oseltamivir resistance genotype [52]. The assay employed the use of degenerate primers for amplification of the HA and neuraminidase (NA) genes and eight target-specific primers with TAG modifications for TSPE and subsequent detection on MicroPlex-xTAG beads. The assay was evaluated using 54 NP specimens that were Flu-A positive by DFA or real-time RT-PCR for the matrix gene, and was capable of correctly identifying the Flu-A subtype and the oseltamivir sensitive (H275) and resistance (H275Y) alleles in all samples. Of the 17 seasonal Flu-A/H1N1 isolates, H275Y was identified in 15, and of the 24 pandemic 2009 Flu-A/H1N1 isolates, 12 were H275 (sensitive), 3 were H275Y (resistant), and 9 revealed a combination of both alleles. The assay was also inexpensive to run with a combined reagent cost of $6.95 Canadian dollars (CAD) per test.

Cost analysis studies indicate that use of a multiplexed bead-based array for diagnosis of respiratory viral infections can be less expensive than routine strategies using DFA and/or culture. A 2009 cost analysis revealed that the average diagnostic cost per pediatric inpatient was lowest for a strategy using xTAG RVP testing alone as compared to DFA alone, DFA plus culture, and DFA plus RVP assay [53]. When all four diagnostic strategies were compared, RVP alone was the least costly strategy when the prevalence of infection was 11 % or higher but DFA alone was less costly when the prevalence was less than 11 %. They estimated a savings of $291 CAD per case if the xTAG RVP test alone was used to replace DFA plus culture, and a savings of $529,620 CAD per year in direct costs for the four hospitals included in the study. An operational workflow analysis using lean methodology principles was employed in another study to evaluate potential advantages of a multiplexed bead-based array for laboratory workflow and associated cost [54]. Implementation of the RVP assay resulted in a standardized workflow that decreased laboratory costs and improved efficiency as compared to DFA and culture for diagnosis of respiratory viruses. Workflow was evaluated in terms of total hands-on time and number of operator steps, and all hospital and laboratory costs associated with testing were calculated for each test for both positive and negative result scenarios. Combining the scores for all samples included in the analysis revealed that the hands-on time to completion for 1,015 samples was 80 h for RVP as compared to 503 h for DFA and culture. Total cost per sample was $99.75 United States dollar (USD) for DFA-positive samples (no culture or additional testing performed) and ranged from $329.68 to $429.07 USD for DFA-negative samples which are reflexed to culture with variable time to completion. The total cost for RVP was $135.03 USD, regardless of result. Further, although RVP required more time, steps, and was slightly more expensive per test than DFA screening alone, only 23 % of the sample population studied were DFA-positive. The additional time and costs associated

with follow-up culture for the large percentage of DFA-negative specimens were far greater. Thus, the bead-based array assays not only provide increased diagnostic capability for respiratory viral infections but can also maximize efficiency and reduce the costs associated with diagnosis.

Conclusions

Recent advances in molecular diagnostics have provided the clinical laboratory with new methods that allow rapid diagnosis and treatment of infectious diseases. Among these, multiplexed bead-based suspension arrays have emerged as a rapid, high-throughput, and cost-effective means to provide physicians with reliable and actionable results for timely and improved patient care. In particular, bead-based suspension array assays have been demonstrated as a method of choice for the comprehensive detection and identification of respiratory viruses and have improved diagnosis, allowed identification of coinfections, provided valuable epidemiological data, and aided in the response to outbreaks of respiratory infections. With the availability of assays cleared for IVD use, this technology should become easy to implement, even in diagnostic laboratories with limited experience in molecular techniques. It can be anticipated that as new assays are developed and existing assays are continually enhanced and improved with simpler protocols, streamlined workflows, and automation, these assays will continue to prove valuable for diagnosis of respiratory viruses and other infectious diseases.

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