Chapter 7
Multiplex Immunoassays

Sandeep Kumar Vashist

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7.1 Introduction

The simultaneous detection of multiple analytes is desired in healthcare for the diagnosis, monitoring, and management of complex diseases. Therefore, there is a dire need of cost-effective, robust, and simple multiplex IAs that can reliably detect all the desired analytes from a single sample. Multiplex IAs would enable early and accurate diagnosis of complex diseases, which would give the desired opportunity to doctors to start the treatment at the earliest possible and lead to positive health outcomes for the patient. The rapid clinical diagnosis and the immediate start of treatment are crucial in emergencies and intensive care units, which require instant clinical decisions, such as patients with stroke [1]. In contrast, in cases of complex diseases such as sepsis, the clinical diagnosis cannot be made just based on the quantitative analysis of a single biomarker as there is a need to quantify multiple

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biomarkers to determine the various types of sepsis and analyze the efficacy of the treatment regimen.

The recent years have witnessed tremendous advances in multiplex IAs, which have shown significant promise for clinical diagnostics and point-of-care testing (POCT) [2]. Many prospective multiplex IA technologies and products would make their way to the market in the coming years. Apparently, the clinical diagnostics would be the foremost priority for multiplex IAs, however, rapid POC multiplex IAs at the point-of-need would also be of greater utility in environmental testing, food safety, veterinary sciences, and other bioanalytical applications [3]. Another emerging need for multiplex IAs would be for personalized healthcare monitoring and management as many smart healthcare devices are making their way to the market [4].

Various commercial devices are available for the multiplex detection of clinical analytes and parameters, such as Abaxis Piccolo Xpress and Abbott i-STAT system. They could perform multiplex detection of a limited but appropriate number of analytes. However, they employ expensive and bulky analyzer-based benchtop systems. Similarly, mass spectrometry (MS) [5], such as matrix-assisted laser desorption/ionization (MALDI)-MS, is widely used in healthcare for the rapid detection of pathogens and clinical analytes. But MS instruments are also costly, bulky, and require skilled analysts. Therefore, there is a need to develop rapid and cost-effective POC platform-based multiplex IAs and portable readers, which could be used for multiplex analyte detection at any place and time by users having basic operational skills. There is a strong emerging trend toward smart healthcare and diagnostic devices equipped with mobile healthcare and other advanced features. It substantiates the need for smart multiplex IA formats and smart readers, which could pave the way to personalized healthcare monitoring and management. The smartphone (SP)-based diagnostic readers and IAs, which have been widely demonstrated for the detection of numerous analytes, are providing an impetus to develop prospective SP-based multiplex IAs [4, 6–8].

7.2 Overview of Multiplex Immunoassay Formats

A wide range of multiplex IA formats has been developed based on the use of various strategies. The most prominent approach for multiplex IAs is the use of multiple spots on a solid substrate, where each spot can detect a separate analyte. The strategy has been employed by many companies and researchers for the development of various IAs. However, the preparation of such spotted substrates requires complex fabrication procedure and expensive instrument with precise control of spotted volume. The realization of the IA is also complicated as most of the IA procedures employ the same conventional and prolonged multistep ELISA procedure. There is a significant risk of misinterpretation, especially when nitrocellulose or nylon membranes are used for spotting as the background signal from a non-spotted substrate can vary a lot. Additionally, the imaging readout needs to be highly precise and accurate, where the specific assay signal should come only from the spots and

should not be impacted by the background. As the morphology of the spots after the IA varies considerably, it is essential to take into account the signal from a fixed area within the spots. Some limitations of the approach are the limited number of multiplex IAs, increased complexity in imaging as the number of spots in the spotted array increases, and cross talk between the different assays as the assay components from a spot can diffuse to all adjacent spots [9–11]. The recent developments are specifically targeted at improving all these limitations. However, it remains to be seen whether such multiplex platforms could compete with clinically established predicate IAs in terms of desired bioanalytical performance.

A prospective strategy for multiplex detection is the use of different microchannels or various regions of a channel for the detection of multiple analytes. Similarly, various electrode arrays and lateral flow strips have also been employed for the multiple analytes. Of interest is the use of various beads and labels (such as dyes) for multiplex detection. Although colorimetry, fluorescence, chemiluminescence, and electrochemical signals are the most common signals that are employed for the readout of multiplex IAs [9, 12], the continuous developments in biosensors and IA formats are leading to innovative multiplex IAs.

Table 7.1 provides an overview of all major commercially-available multiplex IA technologies. The recent advances and ongoing efforts in POC and complementary technologies, such as microfluidic operations, lab-on-a-chip platforms, novel biosensor strategies, and prolonged reagent storage concepts, will pave the way to the significant improvements in multiplex IAs in the coming years [11, 41, 42].

7.3 Multiplex Immunoassays

Various commercial multiplex IAs developed to date by different companies are described here in detail together with the technical details, applications, main characteristics, and concerns.

7.3.1 Bead-Based Multiplex Immunoassays

A wide range of beads is used in diverse bioanalytical applications. They are available in various sizes [13], composition, and surface functionalities [2, 9] and could be bound to different enzymes, metal ions [43], quantum dots [44–46], and redox tags [47]. Moreover, they could be magnetic and nonmagnetic. Although the magnetic beads are the basis of nearly all clinical analyzer-based high-throughput immunodiagnostic assays, the non-magnetic beads are used for multiplex detection. Multiplexing can be achieved by employing beads of varying size/color, labeling them with different labels [43–47], or employing them for IAs in separate microchannels or chambers [48, 49].

Table 7.1 An overview of the major commercially available multiplex IAs

Multiplex IA type	Company	Main features	Refs.
Beads-based (xMAP® technology)	Luminex Corp., USA	• Can detect up to 100 analytes in a single 96-well microtiter plate (MTP) well using about 500 distinctly colored micron-sized polystyrene beads and a fluorescence IA • Assays could be performed using the flow cytometry-based analyzers developed by the company	[13–18]
Electrochemical ELISA	Meso Scale Diagnostics LLC, USA	• Wash-free electrochemical IA that could detect multiple analytes in complex sample matrices using carbon electrode surface-based microwell plates • Simple and easy-to-operate	[19–22]
Paper-based	Euroimmun, Germany	• Provides EUROLINE membrane test strips, i.e., line blots, based multiplex IA for the simultaneous detection of multiple antibodies in a sample • Provides EUROLineScan for the quantitative evaluation of EUROLINE membrane test strips using a flatbed scanner	[23, 24]
	Quidel Corporation, USA	• Provides Triage platform-based quantitative multiplex fluorescence LFIAs for the detection of cardiac biomarkers and drug screening in whole blood, plasma, or urine • Provides Triage® MeterPro to deliver rapid POC diagnostics results in three easy steps	[25, 26]
Array-based	Scienion, Germany	• Provides the complete technology solution for the development of multiplex IAs • Technologies include high-throughput sciFLEXARRAYER for printing arrays; sciPOLY3D polymer-based surface functionalization; sciBUFFER; assay protocols; sciREADER and software for fast readout of arrays in 96-well MTP in just 2 min; and customized solutions for multiplex IAs	[27–29]
	R-Biopharm AG, Germany	• Provides SeraSpot® microspot array for multiplex IA in 96-well MTP format • Provides SeraSight® strip • Provides SeraSpot® test kit-based ready-to-use reagents with the universal protocol for multiplex IA • Provides common ELISA processor for automated IAs • Provides SeraSight® plate mono instrument for image acquisition and interpretation of entire 96-well MTP • Provides SeraSight® strip instrument for image acquisition and interpretation of 8-well strip • Includes intelligent IA design with inbuilt reference spots for the reference curve, up to five controls for quality assurance, and a well-position marker, which is color-coded 0• Developed SpotSight® scanner and software for the image capture and interpretation-based readout of arrays in all 96 wells of MTP in 7 min 0• Provides a variety of tests for the detection of autoimmune and infectious diseases	[30]

(continued)

Table 7.1 (continued)

Multiplex IA type	Company	Main features	Refs.
	Randox, UK	• Provides the biochip array technology (BAT) for various bioanalytical applications • Developed the proprietary surface functionalization procedure for biochip and many BAT-based assays • Developed fully automated analyzers from medium to very-high throughput, i.e., Evidence Evolution, Evidence, and Evidence Investigator, and Evidence MultiSTAT	[31, 32]
	BioVendor, Germany	• Developed array-based multiplex colorimetric and fluorescent IAs • Provides Array Reader C-series and F-series reader instruments for the readout of colorimetric and fluorescent multiplex IAs, respectively, in 96-well MTP format in just 2 min	[33]
	Pictor Diagnostics Ltd., New Zealand	• Provides PictArray™ platform for semiquantitative multiplex detection using a customized ELISA-based procedure • Provides a compact PictImager™ for image capture and analysis of PictArray™	[34]
	GENSPEED Biotech, Austria	• Developed CE-certified IVD multiplex IA on a micro-ELISA chip for the detection of up to eight biomarkers for hospital-acquired infections and periodontitis in just 15 min using an array spotted using the Scienion's sciFLEXARRAYER, sciDROP, and sciPOLY 3D technologies	[35]
MF-based	Gyros Protein Technologies AB, Sweden	• Provides centrifugal MF-based Gyrolab Bioaffy CDs, i.e., a LabDisk platform with pre-integrated reagents for automated fluorescent IAs • Provides Gyrolab instrument (Gyrolab xPlore™) for running a single CD-based IA • Provides Gyrolab™ xP workstation for running up to five CDs simultaneously	[36, 37]
	Abaxis, Inc., USA	• Provides POC Piccolo Xpress™ whole blood chemistry analyzer for automated centrifugal MF-based assays • Provides several clinical laboratory improvement amendments (CLIA) waived tests for multiple analytes, biomarkers, toxins, nucleic acid, pathogens, etc. • Analyzer can perform up to 14 different tests on a LabDisk platform with pre-stored reagents	[38, 39]
	Samsung, South Korea	• Provides a handheld POC analyzer called Samsung LABGEO IB10 and centrifugal MF LabDisk platform-based fully automated IAs that detect multiple analytes in just 20 min • Detects up to three analytes in a single run	[40]

The xMAP® technology by Luminex Corp., USA [13] is the most widely used technology to develop bead-based multiplex IAs [14–16]. It can detect a large number of analytes just in a single 96-well microtiter plate's (MTP) well using the 500 distinctly colored bead sets that have been developed by Luminex Corp. The xMAP®-based multiplex IAs are cost-effective, rapid, reproducible, accurate, high-throughput, and require less analyst's time. The IA detects the analyte in the sample by binding to capture Ab-bound color-coded micro-sized polystyrene beads known as microspheres followed by the subsequent detection by binding to biotinylated detection antibody (Ab) and streptavidin-labeled fluorescent dye (Fig. 7.1). The readout of multiplex IA in the MTP wells is performed by an analyzer having multiple lasers or LEDs and high-speed digital-signal processors. The analyzer measures the fluorescent signals from each individual microsphere particle in the well and provides the results of the multiplex IA. The excitation of the microsphere's internal dyes by the laser or LED identifies the specific microsphere set, while a second laser or LED excites the fluorescent dye on the detection Ab. After that, high-speed digital signal processors identify each individual microsphere and provide quantified results for multiple analytes after the readout of fluorescent signals from them. Recently, the company has also offered an option to employ magnetic beads, which would be very useful to diagnostic companies for the development of automated IAs as it would enable easy and rapid separation just by using a magnetic separator. The company developed two flow cytometry-based analyzers, Luminex® 100/200™, with integrated fluidics, optics, lasers, and high-speed digital signal processors, which enable the development of multiplex IAs for the detection of up to 100 analytes in a single MTP well. However, the xMAP® technology-based multiplex IAs require expensive readout and analyzer instruments [17, 18]. They also need to be rapider so that they could be employed for POCT. The ongoing research efforts are focused on the development of low-cost lab-on-c-chip (LOC)-based flow cytometers for POCT [50, 51].

7.3.2 Multiplex Electrochemiluminescent ELISA

Meso Scale Diagnostics LLC has developed an innovative wash-free and high-throughput electrochemiluminescent ELISA for multiplex detection [19]. It is a highly-sensitive IA that detects multiple analytes in complex sample matrices using carbon electrode surface-based microwell plates and a wash-free sandwich IA procedure using SULFO-TAG-labeled detection Ab, which emits light upon electrochemical stimulation (Fig. 7.2). Being wash-free, it is simple to operate and avoids numerous unnecessary and labor-intensive wash steps. It has good analytical performance that is comparable to that of Luminex *x*MAP® multiplex IA [20–22].

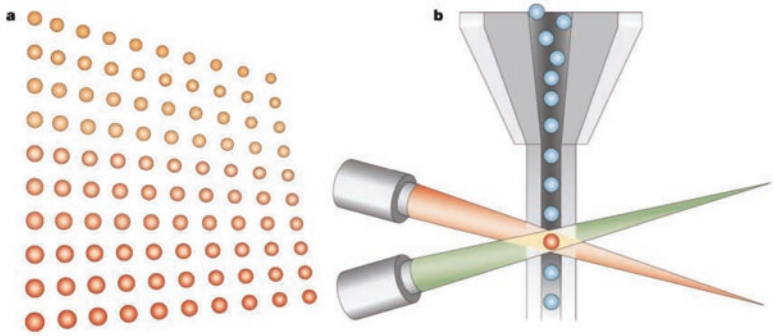

Fig. 7.1 Luminex *x*MAP® multiplex IA technology. (**a**) Polystyrene beads are internally colored with two different fluorescent dyes: red and infrared with up to 100 distinct bead regions generated by using different concentrations of red and infrared dyes. Each bead region, bound to a different capture Ab, detects its particular analyte, followed by the subsequent binding of a biotinylated detection Ab and streptavidin-conjugated phycoerythrin (reporter dye). (**b**) The beads are identified individually in a rapidly flowing fluid stream that passes by two laser beams: red classification laser (635 nm) or LED reveals the color code of the bead region, and green reporter laser (532 nm) or LED determines the analyte concentration by measuring the reporter fluorescence intensity [16]. Reproduced with permission from Elsevier B.V [16]

7.3.3 Paper-Based Multiplex Immunoassays

Several paper-based multiplex IAs have been demonstrated and are commercially-available. Lateral flow IAs (LFIAs) are the most simple, rapid, and cost-effective IA formats for POCT at homes, remote settings, decentralized laboratories, and point-of-need. LFIAs are described in depth in a separate chapter of this book. The conventional LFIA has been modified recently into multiplex formats by many companies [53]. Most multiplexed LFIAs are based on the detection of an optical signal [54–57], while a few also employ electrochemical detection [58, 59].

A prospective multiplex IA has been developed by Euroimmun, Germany, which involves the use of EUROLINE membrane test strips for the simultaneous detection of multiple antibodies in a sample [23]. A flatbed scanner and imaging system-based EUROLineScan [24] has been developed for the quantitative evaluation of EUROLINE membrane test strips. The company has developed several multiplex IA products for the diagnosis of several diseases, such as autoimmune liver diseases, antinuclear antibody (ANA), myositis, TORCH syndrome, extractable nuclear antigens (ENA), etc.

Quidel Corporation, USA, has developed the Triage platform-based quantitative multiplex fluorescence LFIAs for the detection of cardiac biomarkers and drugs in complex sample matrices of whole blood, plasma, or urine [25, 26, 60]. The multiplex IA provides quantitative results in just 20 min using a portable fluorometer called Triage® MeterPro [60], which delivers rapid POC diagnostic results in three easy steps.

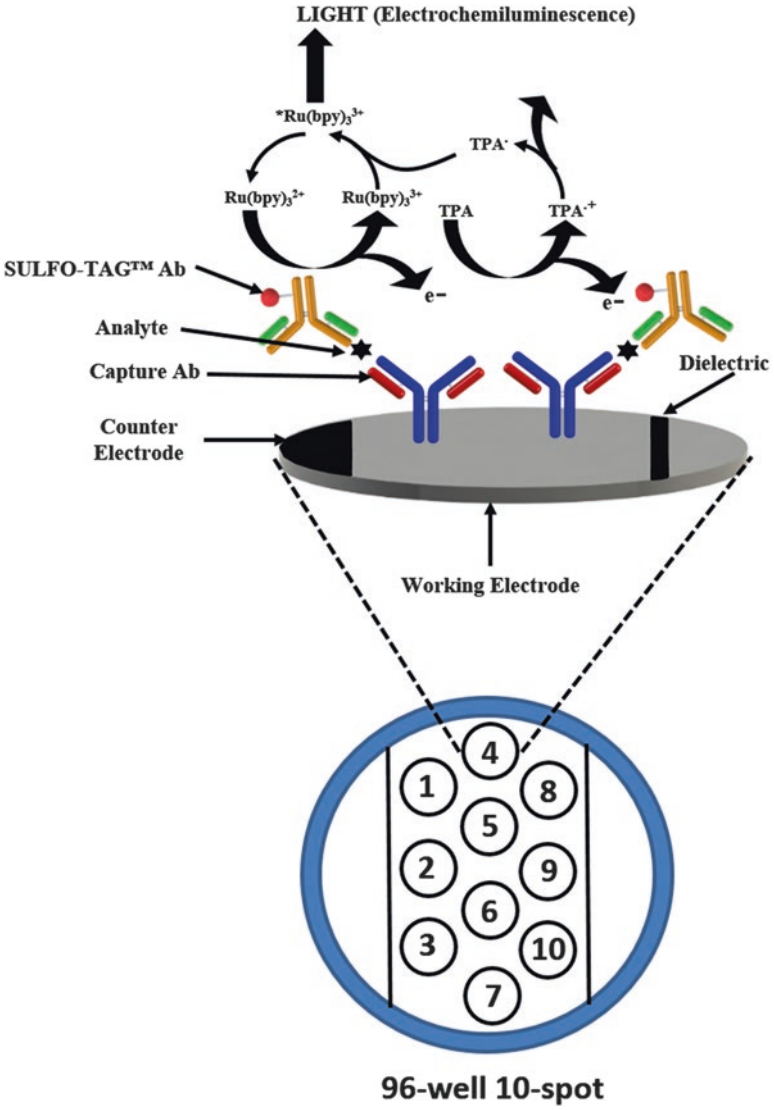

Fig. 7.2 Electrochemiluminescent ELISA-based wash-free sandwich IA for the highly sensitive detection of analytes in complex sample matrices. Upon electrochemical stimulation, light is emitted from the carbon electrode surface-based microwell plates with specific SULFO-TAG™ labels on the detection antibody. Reproduced with permission from Elsevier B.V [52]

During recent years, many innovative smart LFIA readers have been developed by several companies that have led to quantitative LFIAs. The smart readers, equipped with mobile healthcare tools and Cloud computing, have significantly increased the functionality of LFIAs. Some important examples are the smart LFIA readers developed by Cellmic, USA and BBI Solutions, UK, which are described in detail in another chapter of this book.

7.3.4 Multiplex IAs Using Microfluidic Paper-Based Analytical Devices

The use of microfluidic paper-based analytical devices (MF-PADs) has increased considerably during the last decade due to their low-cost, rapid fabrication, ability to manipulate liquids at a high level, adaptation of various microfluidic operations (such as mixing, splitting, separation, and filtration), improved assay performance, and flexibility [61–63]. They are ideal for multiplex IAs in the developing countries that have limited resources, healthcare infrastructure, and professionals. However, the MF-PAD format has not been a commercial success, which is mainly due to the increased fabrication efforts required for mass production, concerns about the reproducibility and performance of IAs, and need for the simplified operational procedure. The conventional MF-PAD-based IA, based on colorimetric detection via naked eyes, is mainly qualitative or semi-quantitative. The recent advances in the development of smart readers are paving the way to quantitative MF-PAD-based multiplex IAs.

An exciting development is the MF-PAD for on-site liver function testing by determining the levels of aspartate aminotransferase and alanine aminotransferase in whole blood using a colorimetric IA procedure that takes just 15 min [64]. The fabrication procedure and process steps of the multiplex IA are illustrated schematically in Fig. 7.3. In another approach, the electrochemical MF-PADs were used for the multiplex detection of glucose, lactate, and uric acid in human serum using the respective oxidase enzymes [66]. The SU-8 photolithography was used to pattern the microfluidics on the device, followed by the patterning of screen-printed electrodes on the filter paper. Of interest is the electrochemiluminescent MF-PAD for the multiplex detection of four tumor markers in human plasma [67]. There have been numerous developments in electrochemical MF-PADs [68–72], which might lead to critical diagnostic applications in the near future.

7.3.5 Array-Based Multiplex Immunoassays

The most popular high-throughput multiplex IA format is based on the formation of an array of spots, where each spot detects a specific analyte. Several companies, such as Scienion, R-Biopharma, BioVendor, Pictor, Randox, etc., have developed

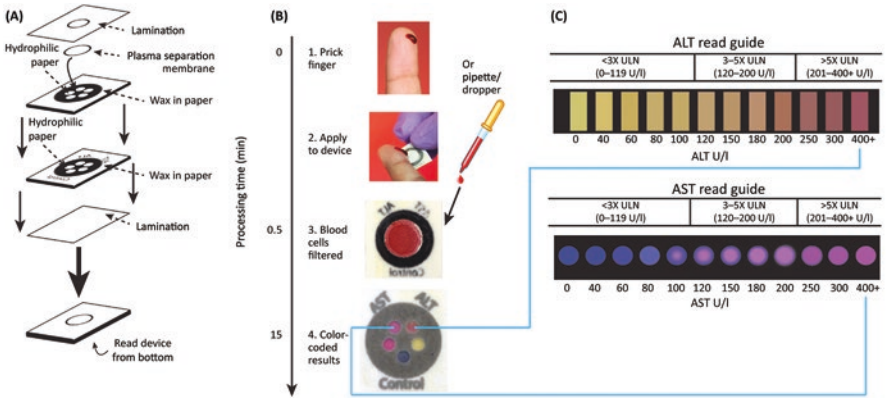

Fig. 7.3 Multiplex IA for on-site liver function testing using the microfluidic paper-based analytical device (MF-PAD). (**a**) Fabrication procedure for MF-PAD. (**b**) IA procedure. (**c**) Colorimetric readout guides for the quantitative determination of liver function enzymes. Reproduced with permission from AAAS [65]. ALT and AST stand for alanine aminotransferase and aspartate aminotransferase, respectively

the array format-based platforms for the detection of multiple analytes and the readers to determine the detection signals from each spot in the array. The main features of the products and technologies developed by various major companies are mentioned in detail in Table 7.1. The surface functionalization and immobilization of biomolecules play a prominent role in array-based multiplex IAs. One of the most widely used surface functionalization strategies is the use of silanized substrates [73] as reported by Randox and Scienion. The signal readout in case of such array platforms is mainly done by optical or electrochemical means [74]. The optical readout systems measure the fluorescence [75] or chemiluminescence [76] signals from the spots due to the binding of fluorescent- or chemiluminescent-labeled biomolecules. It is realized by image capture using a scanning charge-coupled device (CCD) or complementary metal oxide semiconductor (CMOS) camera. Some label-free microfluidic biosensor platforms have also been demonstrated employing array spots and localized surface plasmon resonance (LSPR) detection using metallic nanoparticles [77–79]. However, these are expensive as they employ complex manufacturing and process steps.

Another prospective development is the microelectrode array-based electrochemical detection of multiple analytes [80–82] by immobilizing the biorecognition elements on the microelectrodes. Various electrode materials, such as semiconductors, metals, or carbon-based materials, have been used for POC electrochemical detection [83]. Therefore, it is important to screen an optimal microelectrode material that would results in higher bioanalytical performance of an assay [84]. The ElectraSense platform from Custom Array Inc., USA is an excellent system that enables the electrochemical detection of multiple analytes on a CMOS-based Custom-Array chip with platinum microelectrodes. The signal, i.e., fluorescent

or electrochemical, is measured using a handheld reader via multimodal signal readout in less than 1 min [85, 86]. The chip can be reused for up to four times, while the biorecognition elements, i.e., DNAs and antibodies, are bound to the chip's surface via oligonucleotide hybridization. However, the chip is inappropriate for clinical diagnostics as their production is expensive and complex.

7.3.6 Microfluidics-Based Multiplex Immunoassays

Microfluidics (MF)-based multiplex IAs have been widely used for the simultaneous detection of different analytes, where each microfluidic channel is used for the quantitative analysis of a separate analyte [87]. They require an optimal MF array and design and the manipulation of fluids by a number of pneumatic valves integrated into polydimethylsiloxane (PDMS)-based devices [88–90]. An innovative MF-based multiplex IA, comprising of a disposable MF cartridge with preloaded reagents, and a handheld automated analyzer [91], detects HIV antigen, syphilis antigen, BSA, and Ab to goat IgG in each MF channel (Fig. 7.4). It can detect these four analytes in seven samples by employing four detection sites located in series in each MF channel. The multiplex IA could be performed manually or automatically by measuring the optical density signal obtained by the reduction of silver ions on the detection Ab tagged with gold nanoparticles (AuNPs). The signal is measured using a low-cost and compact reader that comprises of light-emitting diodes and photodetectors. The developed IA detects HIV and syphilis antigens in just 20 min using only 1 ml of finger-pricked whole blood. The results agreed well with those obtained by an established clinical laboratory reference test. Another prospective format is the paper-based MF device, i.e., DxBox, for the multiplex detection of malaria pfHRPII antigen and IgM antibodies to *Salmonella typhi* within 30 min in whole blood [57] (Fig. 7.5). The multiplex IA involves the delivery of sample and dried on-chip stored reagents over the multiple detection sites on the paper device via pneumatic actuation and performing the quantitative detection of analytes by imaging each spot via a flatbed scanner and determining its intensity. However, these MF-based multiplex IA formats are limited in terms of multiplexing and do not have the desired flexibility for various IA procedures.

Another emerging format is the centrifugal MF (CMF)-based multiplex IA, which employs the compact lab-on-a-disc platform, where the various IA steps are performed by MF operations by navigating the fluids through the microchannels using centrifugal forces. The most widely used CMF-based multiplex IA formats are those from Gyros Protein Technologies AB, Sweden; Abaxis, Inc., USA; and Samsung, South Korea. The automated IAs are performed in a portable analyzer, where the signal from each IA is read by optical readout. The main advantages of the CMF format are the low sample requirement, rapid sample-to-answer time, and automated operations. But there are still considerable improvements required in terms of multiplexing, robustness, and IA formats.

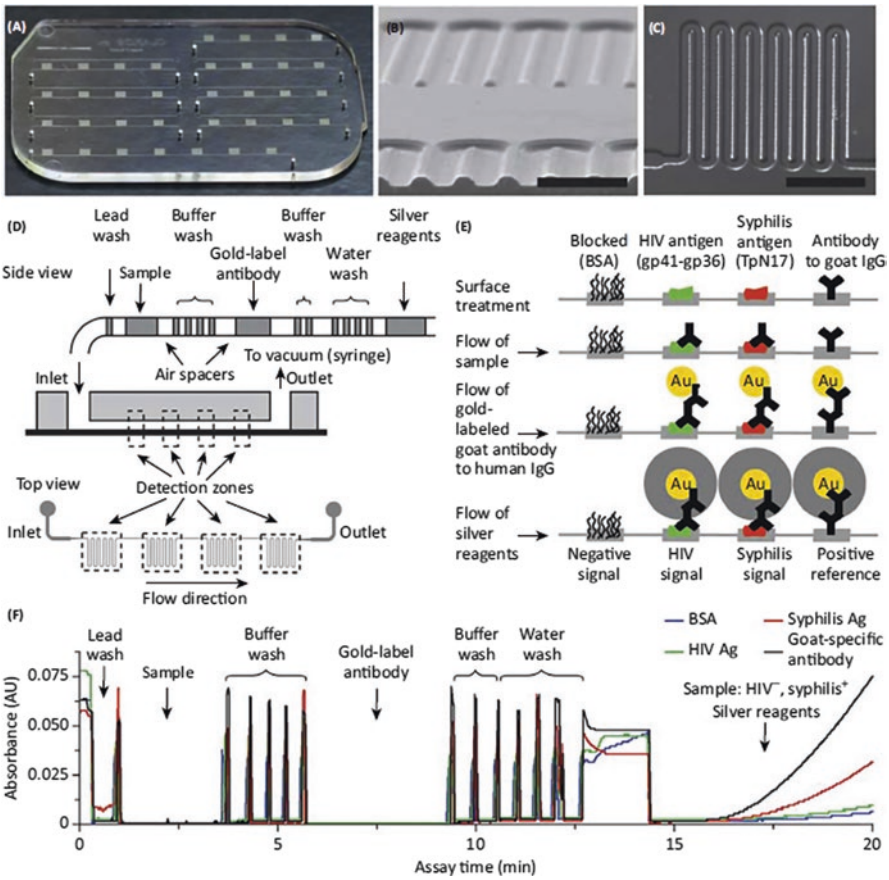

Fig. 7.4 Microfluidics (MF)-based multiplex IA platform. (**a**) MF polystyrene cassette with seven measurement units. (**b**) Scanning electron microscope image of channel cross section (scale bar: 500 mm). (**c**) Transmitted light micrograph of a single detection site (scale bar: 1 mm). (**d**) Passive delivery of a preloaded sequence of different reagents. (**e**) Schematic of assay reactions on different detection sites at various process steps. The signal detection occurs by the reduction of silver ions on detection Ab tagged with AuNPs. (**f**) Measurement of optical density signal in the developed multiplex IA for HIV and syphilis. Reproduced with permission from the Macmillan Publishers Ltd. [91]

Abaxis Inc., USA has developed a POC Piccolo Xpress™ whole blood chemistry analyzer [38] (Fig. 7.6a) that can perform automated IAs on a centrifugal MF-based LabDisk (CML) platform. The analyzer processes up to 14 different tests on a single barcoded LabDisk that has all the prestored reagents. The company has developed several clinical laboratory improvement amendments (CLIA) waived multi-analyte tests and other tests for biomarkers, toxins, nucleic acid, pathogens, and other analytes [39]. Similarly, Gyros Protein Technologies, Sweden has developed Gyrolab instrument (Gyrolab xPlore™ or Gyrolab™ xP workstation) [36] and

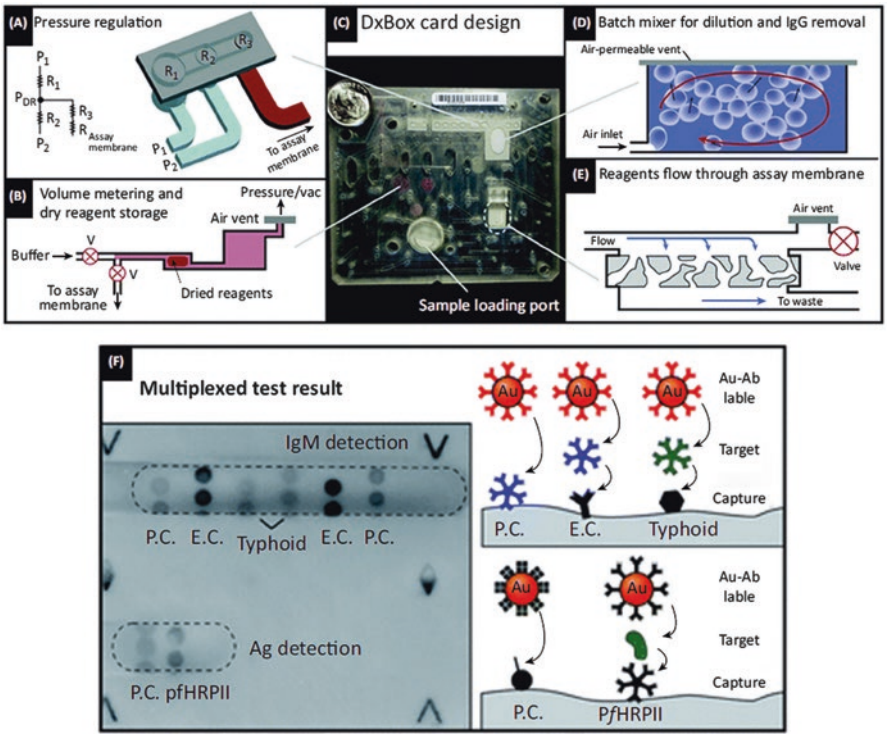

Fig. 7.5 DxBox Integrated Microfluidics (MF)-based paper card device for multiplex IAs. (**a**) Pneumatic regulation for the fluid manipulation. (**b**) On-card volume metering and freeze-dried storage of reagents. (**c**) Integrated MF cartridge. (**d**) Bath mixer for sample dilution and IgG removal. (**e**) Incubation procedure on the assay membrane. The application of an air vent and a valve removes the air between reagent deliveries and the reagents itself between different incubation steps. (**f**) Multiplex detection of IgM antibodies against typhoid infection and malaria pfHRPII antigen from human plasma. Reproduced with permission from the Royal Society of Chemistry [92]. E.C. and P.C. stand for endogenous control and process control, respectively

centrifugal MF-based Gyrolab Bioaffy CDs [37] (Fig. 7.6b). Gyrolab xPlore™ can perform a single IA on a CML platform, while Gyrolab™ xP workstation can simultaneously run up to five CML platforms. The company has also developed several IAs using a fluorescent IA procedure that involves the binding of the SA-coated bead to biotinylated capture Ab, followed by the binding of analyte and its detection via fluorescent-labeled detection Ab. Of interest is the handheld POC analyzer from Samsung, i.e., Samsung LABGEO IB10 [40], which employs CML platform-based fully automated IA for the rapid detection of multiple analytes in just 20 min. The analyzer has several advanced features, such as smart mobile healthcare tools, and can detect up to three different analytes in a single run. The CML platform contains all the prestored reagents for the IAs that are stable at room

Fig. 7.6 (**a**) (left) Centrifugal microfluidics platform, i.e., LabDisk, for multiplex detection and (right) Piccolo Xpress chemistry analyzer for fully automated IA. (**b**) (left) LabDisk platform for fully automated IA and (right) Gyrolab xPlore™ system enabling fully automated IA. Reproduced with permission from Elsevier B.V [6, 52]

temperature for a month. The company has developed IAs for troponin I, myoglobin, CK-MB, thyroid-stimulating hormone, procalcitonin, and other analytes.

Further, the electrochemical detection-based microfluidic multiplex IAs could lead to rapid analyte detection and compact devices. An interesting development was the "MultiLab" platform-based MF electrochemical biosensor for multiplex detection of up to eight analytes [93], which employs a microfluidic channel network with eight separate immobilization sections in parallel that are combined with a single electrochemical measurement cell comprising multiple working electrodes. It was employed for the rapid quantitative detection of two antibiotics in human plasma in 15 min. The IA format was cost-effective and simple and required less sample and reagents, but it lacked an automated operational procedure via a portable analyzer.

7.4 Critiques and Outlook

The simultaneous detection of multiple analytes is an essential requirement for the clinical diagnosis of many complex diseases and differentiating among the similar ones. Moreover, the existing trend toward the use of clinical score, determined by assigning specific weightage to each biomarker in the multiplex panel, as a real-time and easy-to-use visual indicator of patient health for a particular disease emphasizes the need for multiplex IAs. The last decade has witnessed many multiplex IAs-based IVD products in the market from various companies, such as Luminex Corp., Meso Scale Diagnostics LLC, Gyrolab, Abaxis, Randox, R-Biopharm, BioVendor, TestLine, Scienion, Pictor, etc. Although the multiplex IAs from Gyrolab, Abaxis, Randox, and Luminex Corp. are among the most widely used, the other companies are also looking into increasing their multiplex IAs' portfolio. Further, a wide range of researchers is continuously developing innovative multiplex IAs employing the latest advances in lab-on-a-chip, microfluidic technologies, POC platforms, novel biosensors, new IA formats (such as wash-free IAs), system integration, smart system technologies, smart applications, and mobile healthcare. The cost-effectiveness, simplicity, robustness, analytical performance, ease of manufacture, and the clinical utility will be the critical factors for any multiplex IA format to be commercially- and clinically-viable. Doubtlessly, the use of multiplex IAs will be increasingly growing in clinical diagnostics and healthcare.

However, it remains to be seen whether the multiplex IAs could fulfill all the rigorous bioanalytical requirements as required by the regulatory and healthcare authorities for the clinical diagnostics [94]. The bioanalytical performances of all the IAs for the various biomarkers in the multiple IA should align well with the established predicate IAs for each biomarker. Therefore, there is a need for rigorous clinical validation of multiplex IAs. If a single biomarker in the multiplex IA format does not meet the desired bioanalytical performance, the whole multiplex IA will fail due to nonalignment with the predicate. In addition, there are several limitations in selected multiplex IA formats, which will impact the clinical analysis. As an example, most array-based multiplex IAs employ the controls and calibration spots, which is different from that of clinical analyzer-based assays that use calibrators, reference standards, and controls as samples. The spotted calibrators and controls doesn't simulate the actual IA procedure. Therefore, there is a need for critically investigating the IA format for its analytical performance.

The multiplex IAs would be very useful for physician office labs (POLs), remote settings, developing nations, and personalized healthcare monitoring. The multiplex IAs should have the desired precision, accuracy, sensitivity, specificity, reproducibility, robustness, and stability. Moreover, they should be rapid, simple to operate, low-cost, and easy to mass-manufacture. The multiplex IA kits should have prolonged storage stability for realistic applications. The array- and bead-based multiplex IAs have limitations as they require complex and expensive manufacturing and readout systems. On the other hand, the MF- and paper-based multiplex IAs are

limited in terms of multiplexing. The realization and implementation of MF operations in multiplex IAs have simplified the IA format and led to the development of fully integrated MF cartridges for IAs, which obviate any manual fluid handling by the users. The advances in the system integration, engineering, and software have further led to advanced readers for multiplex IAs.

The current international trend is firmly focused on the use of smartphones (SP) as POC diagnostic readers [95]. Several companies have developed the rapid diagnostic test readers for LFIAs, which can be used at the point-of-need without any need for continuous electricity as they are equipped with rechargeable batteries. These inexpensive smart readers have tremendously increased the outreach of clinical diagnostics as they can be used at any place at any time. Further, they have turned the qualitative LFIAs into semi- or fully quantitative LFIAs. Apart from LFIAs, SP-based readers have been developed for a wide range of IA formats and bioanalytical applications [96]. However, there is a stringent requirement to ensure the safety of patient's data in accordance with the regulatory guidelines.

7.5 Conclusions

A wide range of multiplex IAs has been developed based on various IA formats. Although the most used formats are based on the use of various beads, an array of spots, and LFIA, there is an emerging trend toward POC, PADs, and MF-based multiplex IAs. However, despite several companies that have developed and are commercializing the multiplex IA-based IVD kits, the multiplex IA is still in the nascent stages in terms of technology development. The bioanalytical performance, costs, manufacturability, automation, and data analysis will play a key role in the market penetration and acceptance of multiplex IAs. There is no doubt that multiplex detection would be highly useful for the clinical diagnosis of complex diseases and would enable differentiation between closely related diseases. However, there is a need for stringent clinical validation of multiplex IAs and their alignment with established clinically accredited IAs. The coming years will witness numerous multiplex IAs making their way into the market. They would be based on novel IA formats and advances in complementary assisting technologies. The improvements in the technology would pave the way to regulatory-compliant and robust multiplex IAs, which would play a key role in healthcare monitoring and management.

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