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Multiplexing molecular diagnostics and immunoassays using emerging microarray technologies

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Enzyme-linked immunosorbent assays (ELISA) are frequently used for quantitative measurement of the presence of protein, for single-analyte testing, in a sample. The application of ELISA in a microarray format has the potential to simultaneously measure the presence and/or concentrations of numerous proteins, in multiplex testing, all contained in a small drop of test fluid. Microspot microarray technology, in combination with protein biomarkers and nucleic acid diagnostics, appears to be the future high-performance analytical platform of choice. Validation of a large number of disease markers in both molecular and protein diagnostics has paved the way for the emergence of the multiplex assay. Initially, simple low-throughput multiplex assays were tested using the immunoassay format. These were followed by low-level multiplexing and high-throughput array-based immunoassays. More recently, two types of high-level multiplexing and high-throughput diagnostic methods using microspot arrays and bead arrays have been successfully developed to complement single-analyte assays. The value in rapid diagnostic evaluation for high-throughput multiplex, diagnostic test systems based on sound assay design must take into account data screening, normalization and statistical evaluation of possible concentration measurement, data errors and automated operation. Benefits of using multiplex array platforms include improved-quality patient care, as well as cost effectiveness and time saving. These multiplex methods also set the stage for future protein/nucleic acid codetection. Currently, the one analyte at a time test scheme is still dominant; nonetheless, the multiplex microspot microarray tests evaluated in a single multiassay analyzer are expected to become a significant part of clinical diagnostic testing within the next 5–10 years. This review is focused on microspot array and bead array methods for providing high throughput and a high degree of multiplexing in diagnostic testing.

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Healthcare and codependent disease management rely heavily on diagnostics. The vision to prevent diseases, such as cancer, has motivated the research for diagnostic markers to allow for early intervention. Ideally, prognosis or very early detection of unique molecular and protein biomarkers would allow early intervention, treatment and better management of disease. Follow-up of treatment, proven or experimental, would benefit from prognostic indicators [1,2]. For example, early diagnosis and follow-up of Alzheimer's disease is a major challenge facing diagnostics developers today [3]. Since the

human genome was deciphered, pharmacogenomics related research revealed that drugs are metabolized differently as each person's genes, (e.g., those for hepatic drug metabolizing enzymes) have key differences and the corresponding enzymes have vastly different levels of activity. As a result of this research, pharmacogenomics related diagnostics are emerging [4]. In regard to disease detection, changes in DNA sequence and gene expression level have been found to be associated with a predisposition or even certain causation of diseases, including inherited metabolic diseases, cardiovascular,

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cancer and other diseases. In molecular diagnostics, these changes in DNA sequence and gene expression levels have been used in disease diagnosis. Clinical research has demonstrated that nucleic acid changes can also lead to increased drug effects when managing acute and chronic stages of disease, such as increased toxicity and/or decreased efficacy. A number of routine tests based on genetic testing have become available, enabling theranostic based on genomic informatics.

Some disease diagnostic demands have been met by *in vitro* diagnostics manufacturers. The current standard for immunoassays (IA) and for emerging molecular diagnostics is that each method of diagnosis is to analyze for one analyte. Specifically, one aliquot of a patient sample (serum, plasma or whole blood) is processed and tested for only one analyte at a time. This single-analyte approach is predominantly used, as assays tend to provide inherently variable, inaccurate, or nonspecific results. Public pressure for better tests and regulatory scrutiny for accurate diagnosis, has enticed *in vitro* diagnostics manufacturers to optimize assays to be highly specific for only a single analyte of interest. It is well recognized that assay development needs extensive research and development to optimize and achieve acceptable assay performance characteristics. As a reflection of complexity in developing even relatively simple assays, *The Immunoassay Handbook* has now become significantly longer than its previous versions [5]. In addition, assay development has been limited by underlying technologies. For IAs, lateral-flow methods have been widely applied for rapid turnaround time, to provide only information on detection versus nondetection, in reference to clinically derived threshold detection levels. For improved sensitivity, quantitation and throughput, ELISA has been a most frequently used format, based on coating an analyte onto the solid phase of a sorbent 96-well plate, and limiting the ELISA assay to that particular analyte [6]. There are many other methods that adequately determine levels of analytes, one at a time, using solid-phase detection chemistries [7].

As an example for the predominance of single-analyte methods, in a recent survey of major IA analyzers [8], only 1 out of a total of 46 analyzers was indicated for multiple-analyte detection with a limited IA option for qualitative tests for drug abuse and hormones, available in the USA since 2004.

The demands for better medical diagnostics have been addressed in part by provision of analyzers that run large options of single-analyte tests with random access capability, so that several separate tests can be carried out within the same run time. In some cases, faster turn-around times are desirable, including short turn-around time (STAT) assays, as physicians need to determine quickly if additional tests are required. Nonetheless, these approaches do not remedy the deficiencies of single-analyte assays.

In molecular diagnostics, single-analyte assays were also, until recently the accepted standard as multiple RNA or DNA-based markers need PCR [9] or similar methods to be amplified. These amplification methods, have primarily been limited to one amplicon at a time per assay. Molecular biological research has provided efficient multiplex PCR amplification methods. At the same time, microarray research has provided methods for multiplex detection of molecular diagnostic markers. The reader is referred to BOX 1 for definitions of terms used frequently in this review.

Emergence of multiplex assays

Over several years, a number of multiplex assays have been developed for the research market. For example, antibody arrays have shown to be very promising tools to study autoantigen and autoantibody responses in autoimmune diseases [10]. Regulatory-agency approved diagnostics have lagged due to technical, developmental and regulatory challenges. A prime reason for this lag is the way microarray technologies have evolved. Almost from the very beginning, microarray methods have been applied to gene expression profiling. This very appropriate application for microarray technologies, which has intensified studying differential expression of a large number of genes, is matched by the ability of microarray methods to study multiple analytes at the same time. However, since microarray technologies were tightly associated with gene expression studies, the unintended outcome has been that these technologies were burdened with a great complexity of gene expression studies, mostly due to the lack of thorough characterization of gene expression markers [11,12,13].

As a result of recent technological and regulatory advances, multiplex assays, in both IAs and molecular diagnostics, have developed at a significant rate, though most are currently

Box 1. Definitions.

- Multiplex testing: testing multiple analytes in a single test using a single sample
- Degree or level of multiplexing: number of analytes tested in a single test
- Microarray: either microspot or bead array, on a solid phase so that a number of different analytes can be tested
- Coupled bead: microsphere where a specific reagent is immobilized
- Microspot: location where a specific reagent is immobilized in a planar microarray
- Microspot array: array of microspots (i.e., immobilized reagents at precise, known x, y addresses)
- Bead array: bead-based microarray, for example, immobilizing reagents on individual bead surface at precise, known addresses (color codes)
- Single-analyte testing: testing for one analyte at a time in a test sample

Table 1. Multiplex diagnostics recently cleared by the US FDA .

Assay	Approval date and commercial availability	Description and intended use
AtheNA Multi-Lyte® ANA Kit (Zeus Scientific)	FDA cleared on December 10, 2001; available; also CE marked	Luminex bead-based autoimmune disease detection; intended for the semiquantitative detection of IgG class antibody to eight separate analytes (SSA, SSB, Sm, RNP, Scl-70, Jo-1, Centromere B and Histone) in human serum, the quantitative detection of IgG class antibody to dsDNA in human serum and the qualitative detection of IgG class antibody to ANA The test system is intended to be used as an aid in the diagnosis of various systemic autoimmune disorders
Triage® Cardio Profiler (Biosite)	FDA cleared on March 31, 2003; available; also CE marked	The Triage Cardiac Panel is a fluorescence immunoassay to be used with the Triage Meter; for the measurements of four cardiovascular markers to diagnose chest pain patients, Troponin I, CK-MB, myoglobin and BNP; used as an aid in the diagnosis of myocardial infarction, in the diagnosis and assessment of severity of congestive heart failure and for the risk stratification of patients with acute coronary syndromes
Cocaine Metabolite Assay, Qualitative (Randox)	FDA cleared on December 23, 2003; available; also CE marked	Qualitative determination of the major metabolite of cocaine, benzoylecgonine, in human urine a competitive immunoassay, and for use only on the automated Evidence™ Analyser Provides only a preliminary analytical result A more specific alternative chemical method must be used to obtain a confirmed analytical result. GC/MS is the preferred confirmatory method
AmpliChip® Cytochrome P450 Genotyping test and Affymetrix GeneChip® Microarray Instrumentation System (Roche)	FDA cleared on December 23, 2004; available; also CE marked	Roche Molecular Systems, Inc. (Assays) and Affymetrix, Inc. (Instrumentation), based on five major processes: PCR amplification of purified DNA; fragmentation and labeling of the amplified products; hybridization of the amplified products to a microarray and staining of the bound products; scanning of the microarray; and determination of the CYP450 genotype and predicted phenotype; designed to identify specific nucleic acid sequences and query for the presence of known sequence polymorphisms through analysis of the pattern of hybridization to a series of probes that are specifically complementary either to wildtype or mutant sequences, with microarrays of oligonucleotide probes synthesized on a glass substrate Intended to identify a patient's CYP2C 19 genotype from genomic DNA extracted from a whole blood sample, used as an aid to clinicians in determining therapeutic strategy and treatment dose for therapeutics that are metabolized by the CYP2C19 gene product
Tag-It™ CF Kit (Tm Bioscience)	FDA cleared on May 9, 2005; available; also CE marked	Luminex xMap® color-coded bead-array method, simultaneously detect and identify a panel of mutations and variants, including those currently recommended by the ACMG/ACOG, in the <i>CFTR</i> gene in human blood specimens a qualitative genotyping test which provides information intended to be used for carrier testing in adults of reproductive age, as an aid in newborn screening, and in confirmatory diagnostic testing in newborns and children
MultiCode-PLx diagnostic test for CF (Eragen)	Licensed on May 18, 2005; under development	Bead-based CF testing (exclusively licensed to Bayer), to determine the exact mutations as recommended by ACMG and ACOG

ACMG: American College of Medical Genetics; ACOG: American College of Obstetricians and Gynecologists; CLIA: Clinical Laboratory Improvement Amendments; CF: Cystic fibrosis; CFTR: CF transmembrane conductance regulator; ds: Double stranded; GC/MS: Gas chromatography/mass spectrophotometry; Ig: Immunoglobulin.

Table 1. Multiplex diagnostics recently cleared by the US FDA (cont.).

Assay	Approval date and commercial availability	Description and intended use
eSensor® CF Carrier Detection System (Osmetech)	FDA cleared on March 28, 2006; available	The CFCD System uses electrochemical detection. The genotyping reaction is then loaded into cartridges that contain single-stranded capture probes bound to an array of electrodes, with each electrode containing capture probes specific for a single mutation. Cartridges are inserted into the eSensor 4800 Instrument where the single-stranded targets hybridize to the complementary sequences of the capture probes and signal probes. Detection of the target/probe complexes is achieved using alternating current voltammetry that generates specific electrical signals from the hybridized signal probes simultaneously detects mutations currently recommended by the ACMG/ACOG. The eSensor CF Carrier Detection System is not indicated for prenatal screening or for diagnostic purposes and is for Rx only professional use within the confines of a licensed laboratory, as defined by the CLIA of 1988.
Autoimmune disease assays (umedik)	Release in mid 2007; under development	Microspot array diagnostics used in simultaneous quantitative determination of autoimmune disease markers, including rheumatoid factor and anticitrullinated peptide IgM, IgG and IgA antibodies for rheumatoid arthritis, which is used as an aid in the diagnosis of rheumatoid arthritis.

ACMG: American College of Medical Genetics; ACOG: American College of Obstetricians and Gynecologists; CLIA: Clinical Laboratory Improvement Amendments; CF: Cystic fibrosis; CFTR: CF transmembrane conductance regulator; ds: Double stranded; GC/MS: Gas chromatography/mass spectrophotometry; Ig: Immunoglobulin.

marketed by small-to-mid size companies. Several companies which recently obtained US FDA clearance to market multiplex diagnostics are listed in TABLE 1. So far, more than half of these tests were molecular diagnostics based. This reflects the differences in assay development between nucleic acid-based tests and protein and small molecule-based assays. Different proteins need to be conditioned individually to maintain their conformation throughout the assay procedure due to the differences in electrical charge, hydrophobicity, post-translational modification and folding. This poses a unique challenge to the development of protein multiplex assays, as the multiple protein molecules must be immobilized under their individual optimal conditions on the microarray substrate surface and at the same time, all the multiple immobilized protein molecules must interact effectively with all analytes and reagents under a common assay condition simultaneously for all analytes in a test fluid. In contrast, nucleic acid probes and complementary targets are essentially independent of their sequences in their ability to interact with each other. Another difference is in the sourcing of reagents, with a few exceptions due to relatively rare high Guanine (G) Cytosine (C) content or secondary structure formation in probe or target sequence designs, the probes and targets can be chemically synthesized through routine oligonucleotide synthesis. While for nucleic acid diagnostic methods, probes can be designed based on the targets, due to the highly predictable complementation between the two strands of DNA molecule, the same cannot be said about protein diagnostic methods. Antibodies need to be developed based on antigens, which themselves need to be carefully prepared. Preparation and characterization of antibodies, polyclonal, monoclonal or recombinant requires extensive

efforts. Antibodies vary much in affinity and selectivity and these properties directly impact on the performance of ensuing assays. Therefore, protein assays require more effort to develop than molecular diagnostic methods.

Different ways to immobilize protein and DNA molecules and their implications to activities and long-term storage have been subject of intensive research. In general, significant research and development efforts are required for the optimal immobilization of protein reagents onto assay surfaces, both for bead and microspot arrays [14]. Since this is outside of the scope of this review, the reader is referred to the cited articles [15,16].

Advantages & limitations of multiplex assays

Time & cost of multiplex assays

Reducing cost to healthcare practitioners, patients and third party payers has been a significant driver for multiplexing assays over single-analyte assays. At the same time, to ensure quality patient care, clinicians need to have reliable diagnostic information, simultaneously on several markers, to accurately rule in and rule out disease states. Currently, aside from cost considerations, ordering test results for two or more assays to obtain test results for multiple markers, even when requested from the same testing laboratory, tends to incur additional delays. Ordering two assays at the same time could mean sending more samples and often to different laboratories. This also incurs higher costs than the standard practice of ordering one test and waiting for results to assess if further testing is required. Multiplexing is a paradigm shift which allows more economic savings using tests that produce more diagnostic information. Today's diagnostic industry is built on a mature technology base that

has driven out most if not all attainable cost reductions. Diagnostic laboratory operators clearly communicate one prominent theme – from where is the next wave of cost reduction coming. The industry believes that a next wave of laboratory diagnostic technology is needed to enable the cost reduction expectations that users have. The target cost reduction opportunities are generally viewed as originating from the following five laboratory issues.

Labor reduction:

A total of 50–80% of the labor component of running diagnostic tests can be reduced with multiplexed assays (depending on how many assays are combined into one sample testing event). Given that labor is typically 25–33% of the total test cost this reduction in labor is significant. There is currently also a structural problem – the available talent pool of trained laboratory technicians is not aligned with demand and, in fact, decreasing to a point that it is compromising growth.

Sample processing reduction:

Laboratories have two challenges with sample processing; conducting more testing with the same or smaller volumes of patient sample is critical, as increases in patient sample volumes and vials directly affect processing costs. Secondly, laboratories typically are not able to analyze all the patient tests in one analyzer resulting in multiple handling of the patient sample across multiple analyzers – directly affecting processing costs.

Reduction in outsourced diagnostic testing: for reference laboratory performance in decentralized laboratories – if a multianalyte platform is automated

Currently many esoteric and chronic tests are outsourced by a mid-sized laboratory for cost reasons. Mid-size laboratories are very aware of lost margins caused by outsourcing. Bringing a platform capable of large-laboratory performance, in terms of test type, volume, throughput and costing, into mid-sized laboratories would be extremely attractive to mid-size customers.

Manual versus automated platforms:

A large percentage of autoimmune and infectious disease testing is still conducted on manual or semi-automated ELISA titer plates. Both technologies are extremely labor intensive and users would welcome a streamlined alternative technology.

Simplicity & breadth of testing options on one device

Any opportunity to reduce the number of platforms that exist in a mid-sized lab to satisfy the full breadth of testing options would be welcome by laboratory users, in order to reduce laboratory space limitations.

Combinatorial power of multiple markers

Multiplexing assays also improves the diagnostic power of disease markers. More often than not, single markers have clinical sensitivity and/or specificity limitations. For example, while

creatine kinase isoenzyme MB (CK-MB) and myosin light chain are good markers in screening to rule out myocardial infarction, they are not specific for cardiac diseases. Conversely, although troponin I is specific for indicating myocardial infarction, this marker lacks the sensitivity and early predictive value. The power of multiple markers is exemplified very well by the acceptance of the multiplex diagnostic methods developed by several companies using all three markers in the same assay (see TABLE 1).

Furthermore, test result information for multiple markers, each from a single analyte test, for the same sample may show variance in test outcomes among different methods. Since diagnostic tests have different clinical cut-offs and exposure to matrix effects, it is often difficult to compare and contrast information on multiple markers from multiple tests. In molecular diagnostics, many of the genes when mutated are now known to be associated with diseases. Often, any of the several mutations can affect the gene and the function of the gene product and, thus, multiple mutations are required to be detected in the diagnostic tests [17]. More than 1300 mutations have been found in the *CFTR* gene to be causative in cystic fibrosis (CF) [101]. Although $\Delta F508$ is the predominant mutation, covering well over 70% of all causative mutations, adding 20 or more mutations to this most frequent mutation in the multiplex genotyping assays is very useful in aiding the diagnosis of CF (see TABLE 1). In addition, multiple variations in a gene may interact in a complex way (e.g., some variations cancel or enhance the effects of individual variations). Therefore, all such interacting variations must be genotyped at the same time in order to provide clinically useful information. The question of clinical interpretation of multiple markers is beyond simply interpreting each marker separately. Once the information on all relevant markers is available, by way of the multiplex assays, the medical community needs to investigate the interpretation of the combinatorial value of multiple markers.

Performance advantages of microspot assays

As a result of the differences in assay platform technologies (see BOX 2), the assays developed as microspot microarray, bead microarray or traditional ELISA methods are predicted to have different assay performance. Initial published studies have already begun to validate such differences in assay performance among the three method types.

Better performance of microarraying assays over traditional ELISA assays

The traditional 96-well plate format, widely used in current single-analyte tests, is ideal for developing multiplex testing to retain high-test throughput. While ELISA assays use an entire well to immobilize a single capture reagent, this surface area is divided into many small-surface facets when immobilizing multiple reagents. In order to develop and manufacture multiplex tests, capture reagents are immobilized on small surfaces, much smaller than those in conventional assays. Employing a much smaller surface to immobilize capture reagents is also a

Box 2. Multiplexing with either bead or microspot arrays.

- Bead arrays are microspheres typically with a diameter of approximately 6 μm that can be color coded to assign individual addresses for up to 100 different populations mixed in a solution
- In a typical assay, each population can be coupled with analyte-binding capture reagents, either DNA probes or antigen/antibody capture for protein assays
- These populations of coupled beads are then mixed into a solution to complete the arraying process. Fluorescent labels have been used to detect signals
- An analyzer is used to sort out the populations based on the color code and to read signals on beads. An assay can then be developed using this bead microarray, detection reagents and an analyzer [18]
- Microspot microarrays may be made on several flat surfaces, for example, polylysine and aminopropylsilane, epoxysilane-treated glass or other material surfaces, in covalent or noncovalent linkage [19,20]
- With spot sizes between 50–250 μm, 50–300 spots can be printed in a single well of a 96-well plate. A number of companies now manufacture both the surface and well-forming accessories, and equipment to print reagent spots
- Fluorescent or chemiluminescent labels may be used for detection to achieve the high sensitivity characteristic of these microarray methods; since many spots are printed in a sample well of a 96-well plate, the spots need to be resolved through microscopic imaging
- Several microarray scanners are available which use various excitation methods, mainly with laser or white light source with filters, and PMT or CCD detection methods; assays can be developed on a microspot array platform using a microarray surface, detection reagents and an analyzer

CCD: Current charge-coupled device; PMT: Photo multiplier tube.

basis for developing better assays. More than 16 years ago, the idea of multianalyte assays using microspots was studied [21]. Microarray surfaces, at least microspot assays, were found to be better for IAs than ELISA surfaces as the microspots are much smaller than ELISA surfaces (SEE TABLE 2) and thus provide a means of developing better assays which became known as ambient assays. As a result of this difference, an analyte in a test sample for a microspot assay (an ambient assay), is less likely to be depleted or altered as a result of the assay. In contrast, for conventional assay, analyte concentration depletion is most likely the case. This difference also means that microspot assays have better accuracy and sensitivity, especially in low-analyte samples, when the spots are detected by sensitive microscopic image detection techniques [24]. Finally, microspot assays are also associated with faster kinetics as diffusion constraints are minimized.

In a comparison between ELISA and microspot methods using an identical assay antibody/antigen configuration and reagents, microspot methods were found to be several to 16-fold more sensitive and have 1–2 logs wider linear range than ELISA methods [25]. It will be interesting to see if more studies will confirm this observation that current immunological testing using ELISA and lateral flow IAs, which both suffer from relatively low sensitivity [26], become more sensitive when applying a microspot IAs format. Molecular diagnostic detection using microspot assays is expected to provide similar diagnostic improvements in results over those achieved by conventional, large surface methods including Northern blots and gel electrophoresis methods. A microspot assay was also independently verified to be an ambient format assay by Saviranta and colleagues [27].

Better performance of microspot-based assays over bead-based assays

It is expected that bead array-derived assays should also be ambient assays, to some extent, as are microspot assays, based on the fact that the surface area for bead arrays are much smaller than ELISA surfaces. However, significant differences remain between microspot-array and bead-array assays. Primarily, since bead arrays have significantly larger overall surface areas than microspot arrays (SEE TABLE 2); the former may not provide truly ambient assays, in contrast to microspot assays, as experimentally proven [25–27]. The larger bead particles and the more bead particles used

Table 2. Surface areas for a microspot assay, a bead assay and an ELISA assay.

	Assay surface		
	Microarray		ELISA (96-well)
	Microspot	Bead	
Unit diameter (μm)	100	5.6	5000
Overall surface area (mm ²)	0.0078–0.078 [†]	0.098–0.98 [§]	19.6 [*]

^{*}Well of a standard 96-well plate. Note also that the actual difference in overall size may be different as the ELISA well volume (during coating stage), number of particles in a population and the number of spot replicates are variable depending on actual assays.

[†]Assuming that each capture protein has replicates of 1–10 spots.

[§]Assuming particles in a population for each sample well of 2000–20,000 [18,22,23].

per sample in a bead assay, the less likely that the resulting bead assay will be an ambient assay. Microspot-based assays have a significantly higher-fractional occupancy of binding sites by an analyte, which is relatively independent of receptor/capture concentration and sample volume even if the receptor/capture concentration is low. It is the receptor/capture concentration that determines the limit of analyte depletion in the assay. The miniaturization of array spots also has the advantage of also increasing detection sensitivity as discussed below.

The second difference lies with the density of detectable molecules, defined as the number of analyte molecules tagged with reporter. Detection in the form of fluorescence, chemiluminescence or (ultra-violet–visible) UV–vis absorbance, is quite different between microspot and bead array assays. The detection of microspot microarray assay is based on surveying the detectable molecules over the entire surface area of a spot as a unit, using microscopic image analysis. In comparison, the signal density of a bead in an assay is much lower, due to fact that beads are dispersed throughout the entire volume of a test fluid (50–200 μl for a 96-well format assay), each bead scavenging to bind analyte, effectively capturing only variations in a range of signal levels. In contrast, the higher density of signal for a microspot in a test fluid is expected to result in significantly lower detection limits and higher analytical sensitivity in microspot microarray assays.

To further restrict the sensitivity of a bead assay, in the most widely used methods [18,22,23], only the signal equivalent to that of one bead (median fluorescent intensity [18,22,23], sampled from 100 beads from a given population) is used per analyte. Therefore, the minimal detectable unit is a bead. Even assuming the best case scenario, that is, a bead, being a sphere, is sufficiently excited on half of its surface area, and that the same half sphere emits fluorescence fully into the detector, the detectable part of a bead is no more than a half sphere. This is because the bead does not always flow past the detector with the excited half sphere optimally exposed to the detector. For the microspot assay, the flat surface area of a microspot, when labeled with fluorophore, is the minimal detectable unit. When the minimal detectable units for the microspot and bead microarrays are compared, the former is 160-fold larger than the latter (TABLE 3). If less than half a sphere of a bead is consistently detected, the actual difference of area detected is expected to be even less.

Table 3. Detectable surface area for a microspot and bead-array assay.

	Assay surface		
	Microspot microarray	Bead microarray	Fold of difference
Detectable surface* (μm^2)	7850	49.2 [†]	160

*Using the diameter assumptions in TABLE 2.

[†]Surface area of half a sphere for a bead in a bead microarray assay (e.g., [18,22,23]), assuming that the detector of a reader consistently detects, at best, half-sphere surface area of a bead.

It is worth noting that other imaging detection technologies may be coupled with microspot microarray technology platforms to further increase the sensitivity of detection. For example, in a planar wave-guide microarray detection technology, microarrays are covered with highly refractive materials, forming a surface with strong evanescent fields that can be read by laser. This layer of material guides and propagates light which is read by a compatible microarray scanner. Higher sensitivity than the current charge-coupled device (CCD) and photo multiplier tube (PMT) detection method may be achieved using this imaging technique [28].

Although it is hard to predict how much more sensitive microspot assays are relative to bead assays, a recent study supports the notion that microspot assays are significantly more sensitive than bead array assays under comparable assay conditions [29]. Developed for detection of a botulinum toxin, both a microspot assay and a bead assay (a renewable surface microcolumn sensor using 36 μm beads) using the same antibody reagents and the same sandwich assay format were reported [29]. The limit of detection for the microspot assay was significantly lower than that of the bead assay. It is worth noting that the bead array assay differed from other bead-array assays in the bead diameter and detection method.

In summary, multiplex-microarray assays enable significantly improved assay performance to be developed when compared with conventional single-analyte assays. As a result of the analytical advantages of microspot-microarray assays, preliminary data indicates that microspot microarrays may be used to develop high-performance assays.

Degree of multiplexing, type of method & throughput

The current multiplex methods vary significantly in three key characteristics (i.e., the degree of multiplexing; the type of method, qualitative versus quantitative and throughput) described in TABLE 4.

A high degree of multiplexing is advantageous in disease screening panels, which may contain as many as several hundred markers, (e.g., cancer screening and mutation screening). Depending on the application, the level of multiplexing may be critical such as in genotyping applications. For example, the *CFTR* gene has been reported to contain more than 1300 mutations. While real-time PCR can be used in low-level multiplex assays [30], for a test to detect CF mutation, a higher level of multiplexing is needed.

Optimal capability of multiplexing assays appears to be achievable with microspot microarrays. To date the bead array platform has been limited to 100 test bead populations and one optic detection channel. By contrast, with microspot microarrays, significantly higher microarray density, greater than 160 on x, y resolution (see below), is achievable and at the same time, multiple optical detection channels using microscopic imaging may be configured to detect signal at several fluorescence wavelengths simultaneously. On each of the spatially resolved spots, multiple analytes can be resolved using spectral responses as resolved and detected by different detection channels of a compatible reader.

Table 4. Degree of multiplexing and throughput of multiplex assays.

Assay	Degree of multiplexing	Type of method	Throughput*
Cocaine Metabolite Assay (Randox)	Protein spot array (9 plex)	Qualitative	108 samples (12 units of 9-sample cartridges)
Influenza A/H5 (Asian Lineage) virus RT PCR primer and probe set (CDC)	Real time PCR detection of DNA or RNA (4 plex)	Quantitative	96 samples
eSensor® CFCD System (Osmetech, GA, USA)	DNA array-based electronic detection system (approximately 36 plex)	Qualitative	48 samples
AmpliChip® Cytochrome P450 Genotyping test and Affymetrix GeneChip® Microarray Instrumentation System (Roche, Basel, Switzerland)	DNA array and fluorescent hybridization-based detection (30–40 plex)	Qualitative	Up to 24 samples
Triage® Cardio Profiler (Biosite, CA, USA)	Protein array and microfluidics for immunoassays (4 plex)	Quantitative	1 to a few samples
AtheNA Multi-Lyte® ANA Kit (Zeus Scientific, NJ, USA)	Protein immobilized on beads as arrays of analytes (≤ 100 plex)	Quantitative/Qualitative	96 samples
Tag-It™ CF Kit (Tm Bioscience, Ontario, Canada)	DNA immobilized on beads as arrays of analytes (≤ 100 plex)	Qualitative	24–96 samples
MultiCode-PLx diagnostic test for CF (Eragen, WI, USA)	DNA immobilized on beads as arrays of analytes	Qualitative	96 samples
OnSite MicroDiagnostics™ Rheumatoid Arthritis Assay (Umedik, Ontario Canada)	Protein spot arrays (6–168 plexes) on planar surface in 96-well format	Quantitative	96–228 samples

*Number of samples processed simultaneously in a batch.

CF: Cystic fibrosis; CFCD: CF carrier detection; RT: Reverse transcription.

For example, by combining the spatial and spectral resolution, umedik's microspot microarray analyzer is currently capable of analyzing more than 650 different microspots.

For a diagnostic test to be useful in both initial diagnosis and prognosis, it is desirable to be quantitative. In general, quantitative diagnostic methods provide more information than qualitative. For example, Alessandri and colleagues found that levels of anticitrullinated peptide antibodies and rheumatoid factor antibodies were indicative of clinical improvement following antitumor necrosis factor (TNF) therapy (infliximab) [31].

Although suitable for qualitative aspects of method development, an underlying technology may not be appropriate for quantitative methods. For example, appropriate calibrators and calibration methods need to be developed and validated. The technology has to provide for signal response to be calibrated over a reasonable analyte concentration range at an appropriate concentration standard. To this point, microspot microarrays appear to be more suitable for developing quantitative methods than bead microarrays due to their extended dynamic range and lower limit of detection. Most of the multiplex methods developed thus far have been only qualitative, partly due to molecular diagnostic genotyping methods do not demand quantitative methods and partly due to underlying

technological limitations. For example, some of the technology platforms do not allow internal calibration, and only external calibration is possible. External calibration can use up many wells, decreasing valuable sample testing space and increasing the cost of testing. In addition, external calibration methods are vulnerable to residual well-to-well variations and individual sample matrix effects that have not been considered.

Considering the highly segmented and yet centralized testing, which includes much of the diagnostic testing being carried out in reference labs, throughput of tests is a major consideration for multiplex methods. This has already proven to be a requirement of the single-test testing systems. Most of the methods which provide the high throughput volumes are based on the use of multi-well titer plates (TABLE 4).

Expert commentary

A potential limitation of multiplex assays is that the coanalytes in a multiplex assay could interfere with each other. No such interference has so far been observed or reported in practice for developed molecular diagnostic or IA methods. From a theoretical perspective, bead-based assays are more vulnerable to analyte-to-analyte interference as cross-linking can occur due to cross reactivity among various analytes, as the beads circulate in the test fluid. This interference will have little impact on microspot microarrays,

as different microspot arrays are fixed at predetermined coordinates, and normally do not interfere with each other. In our experience, when developing an autoimmune disease diagnostic platform for the quantitative/semi-quantitative simultaneous determination of six analytes including rheumatoid factors and anticitrullinated peptide antibodies for diagnosis of rheumatoid arthritis, no interference between analytes was observed. In addition, the dynamic ranges of the analytes were compatible so that all analytes could be measured when using the same dilution of a single aliquot of sample within a single-test run.

Essentially, in one microarray device designed for multiplex assays, sets of protein spots are covalently bound to the surface of a coated glass substrate within each well of a standard SBS format 96-well microtiter plate, allowing at least 90 distinct patient samples to be run. The plate includes a barcode representing the assay type and serial number of the plate. Each well has the layout of an assay microarray grid, including standard elements of grid controls, internal assay calibration series, and assay capture reagents. Each element consists of replicates of specific protein spots covalently bound to the microarray surface in predefined locations (FIGURE 1). Grid controls are placed around the grid to provide registration marks for the grid. The controls are composed of generic proteins tagged with fluorescent dye.

In our rheumatoid arthritis assay developed on the microarray platform mentioned above, the three internal assay calibration series are composed of calibrated concentrations of purified human immunoglobulin (IgG, IgM and IgA). The concentrations are precalibrated during the microarray manufacturing process using calibrators traceable to international standards thus providing an internal dynamic reference standard. Among the four rheumatoid arthritis markers, the IgM

rheumatoid factor is a more sensitive and better screening marker, whereas IgG and IgA rheumatoid factors are more specific and indicative of disease progression. Anticitrullinated peptide antibody, specifically IgG subclass [32] is, to date, the most specific marker for this disease. In addition, the assay is also capable of determining the level of two more analytes: IgA and IgM subtypes of anticitrullinated peptides.

In standard practice, since each of the markers is tested with a stand-alone test most often made by different manufacturers, only a single-analyte test is typically prescribed. Upon receiving the results, another test of an alternative marker is often ordered. Our consultation with clinicians indicated that this is mostly due to cost considerations and that physicians actually need information on multiple markers. With a multiplex assay, the results for all markers can be obtained using a multiplex single test in conjunction with a single patient sample, saving time and cost and improving patient care.

All assay operations are fully automated by the umedik fluidics workstation. The operator simply machine-loads the reagents and initiates the analyzer. Patient samples in standard bar-coded serum vials and the umedik microarray device are inserted into the fluidics station. Diluted patient serum specimens, assay controls and calibrators are incubated at room temperature in the multi-well plates. Any rheumatoid factor antibody and any anticitrullinated peptide antibodies present in the sample will bind to the immobilized human IgG and human citrullinated peptide to form antigen-antibody complexes. Each of the analytes is captured as a discrete signal either on separate spots or as a separate fluorescent wavelength onto the same spot. Unbound antibody is washed from the wells and reporter reagents are added. Excess conjugates are washed away and excess wash buffer is removed

from the well. The analyzer automatically performs the assay scan and reads the results. The results are presented as HL7-compliant data and/or as hardcopy printouts. The results are quantitative, as the signals are subtracted from background and calibrated using the calibrator titer spots which are factory-calibrated using standards traceable to applicable pertinent international standards. The multiplex microarray assays for ninety patients are currently completed in 2 h.

Five-year view

Anthony and colleagues' microarray assay review for the previous 5 years, indicated that no microspot assay had been approved using high levels of multiplexing in 2001 [33]. Later in that year, one multiplex bead microarray was approved (TABLE 1). One year later, Toder predicted in 2002 the main limiting factors to be the regulatory approval processes for DNA chips (microspot) [34]. In 2004 and

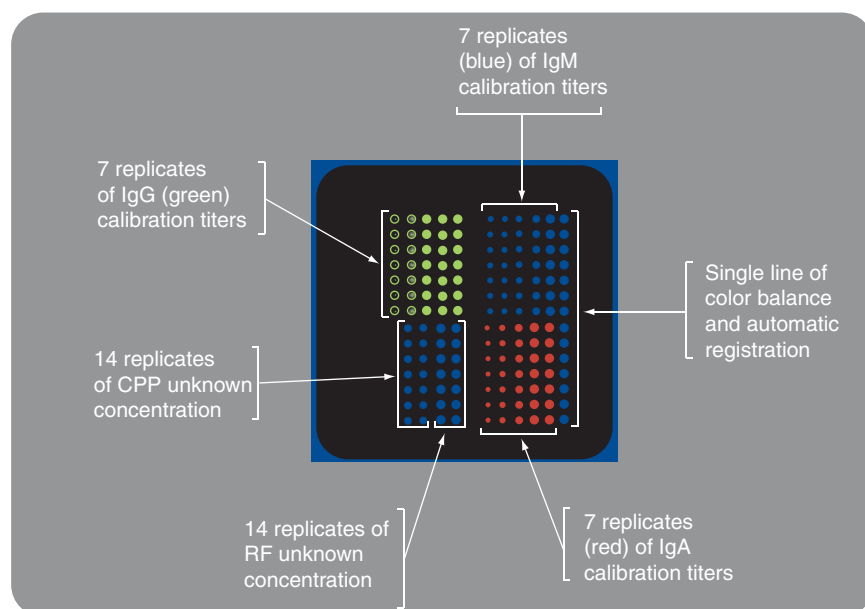


Figure 1. Microspot microarray present in each of the 96-well plate used in umedik's Rheumatoid Arthritis assay.

Ig: Immunoglobulin; RF: Rheumatoid factor.

2005, two more assays (one DNA microspot and one DNA bead microarray, TABLE 1) were approved by the FDA. Recent development in multiplex assay technologies and the recent regulatory approvals of several multiplex assays (TABLE 1) have provided clear development and regulatory paths for developing multiplex assays. As a result of these first assay approvals, the regulatory agencies also know which aspects of multiplex assays need more stringent controls. Specifically, the FDA has issued multiplex assay guidance documents based on draft guidance first issued in 2003. In 2005, an approved guidance document was issued for multiplex instrumentation as Special Controls for multiplex microarray Class II devices [35]. These latest developments have made multiplex assays a viable, improved alternative to single-analyte assays in quality and regulatory compliance. In this context, although not specifically for multiplex assays, two recent draft guidance documents, issued a few months ago, are worth mentioning. The analyte specific reagents' (ASR) frequently asked questions document [36], which was overdue as a follow-up to clarify some of the confusions since the initial ASR guidance document [37], is relevant to both multiplex assay manufacturers and clinical laboratories, as it mentioned multiplex assays. The *In Vitro* diagnostic multivariate index assays guidance (IVDMIA) covers the use of clinical, assay and other information in an index fashion [37]. As multiplex tests provide more information, they are more likely to be used to develop IVDMIA than single-analyte tests.

With the path for regulatory compliance clearly identified, the pace of development for multiplex assays will accelerate using techniques first used for developing single-analyte tests. For example, the present liquid handling systems and automation can be applied in a similar fashion, with appropriate modification, for multiplex assays [34]. In addition, the assay format (e.g., sandwich or competitive), can be transferred from single-analyte to multiplex assay platforms.

In the next 5 years, more multiplex assays will be developed and they will begin to impact and change the way clinical testing is performed. The emergence of more multiplex methods is predicted, especially the development of extended test panel options, especially by leading multiplex assay developers. These comprehensive options will include large panels for multidisease diagnosis or even prevention, for autoimmune panels, cardiovascular panels, cancer panels, stroke panels, diabetes and infectious diseases.

As an example, infectious disease diagnosis is an extremely important area of research and development for assay manufacturers. Improved diagnostics are required to improve healthcare. Qualitative assays for the presence of specific viruses will decrease, if not eliminate, the need for viral culture to identify the presence of a specific virus, a process that can take several weeks and is often not successful [38]. Bacterial identification panels have the potential to decrease the time from clinical presentation to appropriate therapy. Increasing, it is desirable to use multiplex assays, as multiple pathogens and variants are not effectively detected by single-analyte testing methods [38,39].

Some of the multiplex panel tests may be considered large screening assays containing up to 50 different protein markers and 150 or more nucleic acid markers. Following assessment by screening assays, titer based quantitative panels for monitoring treatment outcomes may be required. The emphasis for screening panels and titer panels differs as screening panels emphasize sensitivity to rule out diseases and titer panels rely on quantitative and specific determinations to rule in and characterize diseases, monitor progression and treatment responses. Currently, single-analyte assay platform analyzers can each support as many as 100–150 assays [8]. Since each multiplex assay is used to test multiple analytes, in order to screen a similar number of analytes, the size of options for multiplex assays will be smaller than these numbers. For example, to cover a current menu of 100–150

Key issues

- Multiplex assays for protein and DNA diagnostics have numerous quality of patient care and cost advantages.
- Several multiplex assays have been approved by the US FDA in the last 3 years. Based on these predicate devices, the regulatory path has been paved to approve more multiplexing assays.
- To streamline development, multiplex assays should be developed based on well-established, current single-analyte assays.
- Multiplex assays need to be developed with optimal performance, including compatible limits of detection, lack of interference among analytes and optimal sensitivity, specificity and reproducibility for the determination of all analytes.
- Multiplexing assays will need to feature all key advantages of standard single-analyte assays developed to date, including automation, laboratory information system and high throughput.
- Multiplex assays using microspot arrays are destined to improve single-analyte assays in sensitivity, dynamic range and optimal kinetics.
- Microspot microarray assays are expected to continue to outperform bead assays in supporting high levels of multiplexing, better detection and minimizing interference among analytes within the same assay.
- Nucleic acid and protein hybrid assays will emerge as a new standard for patient testing to provide wide-ranging diagnostic information and improve the quality of patient care.

tests, only a menu of 20–30-multiplex tests (assuming 5-analyte multiplex assays) is required. Although no companies have, as yet, implemented big test options for multiplex assays, and clearly the first multiplex microarray assays are only a recent development, it is a matter of time for large numbers of assays to be offered within the next few years. Microarray methods will have reached not only parity with the conventional methods, but will begin to surpass conventional single tests for improving healthcare outcomes, in a somewhat longer time period.

To date, protein and DNA/RNA markers have been used as separate tests to provide assay results. For this reason, this review addresses both molecular diagnostics and protein IAs. There is a clinical need for using and testing with both types of tests in the same multiplex assay as complementary tests. For example, in an infectious disease assay, the molecular diagnostic markers may be indicating infection with great sensitivity, while the phenotypic protein markers may be used in staging the diseases and monitoring effects of treatment. Thus, once the initial diagnosis is clearly indicated as based on a

multiplex assay, treatment can commence without delay, responding to the disease-staging information already provided by the same multiplex assay. Technical feasibility of such a test in which both protein and DNA analytes are co-tested has been established [40]. Molecular diagnostics and protein IA are soon predicated to be codependant.

There is a trend to merge microfluidics with microarray-based bioassays. Microfluidics is an active area of research and development with the potential to reduce the assay run time and cost of reagents, and reduce sample volume requirement, which could further strengthen the power of microspot arrays for multiplexing assays [41]. This combination may ultimately enable rapid point of care diagnostics that are also multiplexed.

Disclosure

Michael Ling, PhD, is Director of Assay Development, Claude Ricks is President and Chief Operating Officer and Peter Lea, PhD, is Chief Scientific Officer of umedik Inc, Toronto, Ontario, Canada.

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