CHAPTER 5

Rapid, Multiplex Optical Biodetection for Point-of-Care Applications

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1 Need for Advanced Biodetection

Despite hopes that antibiotic and vaccine therapies might one day lead to the complete eradication of infectious disease [1], new and emergent pathogens continue to pose a global threat to public health [2]. Even in the present day, infectious disease remains among the top ten causes of death in the United States for all age groups [3]. In part due to increased virulence of recent strains, but also due to a growing cohort of individuals susceptible to severe infection, the U.S. mortality rate caused by influenza has risen to roughly 36,000 per year, exceeding the current rate of HIV/AIDS related deaths in the country [4, 5]. While the victories over bubonic plague (*Yersinia pestis*), whooping cough (*Bordetella pertussis*), polio and smallpox (variola) are clearly significant, new diseases represented by human immunodeficiency virus (HIV), as well as the tragic reality of biological agents used as weapons of terrorism and mass destruction [6] – offer sobering evidence that the battle against infectious disease is far from over [7].

More than ever, the task of effectively controlling communicable diseases ultimately rests on the ability to rapidly and accurately detect infection at the earliest stages. Rapid diagnostics are crucial for identifying outbreaks of infectious disease which could potentially escalate to a full-scale epidemic or global pandemic. Various surveillance programs and strategies have been proposed or are already in place to monitor influenza at state and national levels [8, 9]. At the lower (but no less critical) local level, hospitals are concerned with highly contagious and potentially lethal nosocomial infections which can emerge or enter the clinical setting. Antimicrobial resistance in tuberculosis and methicillin-resistant *Staphylococcus aureus* (MRSA) pose particular problems since effective vaccination and/or antibiotic treatments are either limited or nonexistent [10]. Rapid identification to contain such diseases is essential for reducing the clinical workload at metropolitan hospitals [11]. In the event of a sudden or unexpected epidemic, vaccines and antibiotics may be in short supply, and so timely assessment and triage of individuals suspected of infectious disease exposure is critical for appropriate allocation of valuable resources.

Another rationale for rapid detection of infectious disease is based on the pharmacokinetics of new antimicrobial agents. For example, Tamiflu® (oseltamivir) is shown to be effective in shortening the clinical course of influen-

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za, but only when administered within 48 hours of the onset of symptoms [12]. Since standard laboratory culture and analysis requires up to 10 days for definitive results, a primary-care physician obliged to treat an acutely ill patient must make an "educated guess" based on the clinical setting, the patient's signs and reported symptoms. While most viral respiratory infections are generally self-limited, respiratory tract infections (RTI) which are bacterial in origin can be life-threatening without appropriate treatment [13, 14]. Nevertheless, in a recent survey conducted in the U.K., primary-care physicians lacking clinical serologic data were able to correctly rule-out bacterial RTIs only 60% of the time, and correctly distinguish bacterial from viral RTIs only 50% of the time [15].

Apart from speed and accuracy, we propose that multiplex capability in a biodetection platform is a practical necessity for today's diagnostic needs and applications. In the context of infectious disease detection, the list of causative pathogens which must be considered for any given clinical presentation grows with the emergence of new microbial species or strains and the introduction of potential biothreat agents into the civilian population [16]. Failure to include such "orphan" pathogens in the differential diagnosis only potentiates the risk and devastating health impact associated with these new and unfamiliar diseases [17]. However, the cost in both time and resources of performing serial diagnostic tests using conventional laboratory methods would be prohibitive and used only as an option of last-resort. Modern molecular in-vitro diagnostic techniques (which we will describe in greater detail) can be scaled-down to work with extremely small sample quantities. Moreover, these assay methods can be reduced and configured into "microarrays" that allow hundreds to thousands of individual tests to be performed on a single biological sample.

Finally, in the context of safeguarding the public health against the spread of infectious disease, we believe that a critical need exists for biodetection technology which can be scaled for portable or "point-of-care" applications. While rapid, multiplex in-vitro diagnostic instruments are readily available, the majority of these commercial systems emphasize performance over portability – incorporating delicate optics, electronics, and/or microfluidics, which ultimately relegates their use to clinical or research laboratories only [18]. A portable, albeit simpler, diagnostic instrument could conceivably play a vital role in environmental testing or field medicine, in which access to laboratory facilities is restricted or impractical for transport of contagious or contaminated materials [19]. A simpler, more economical diagnostic system offers a practical solution for widespread screening of individuals in large populations, enabling a more rapid and direct response to the emergence of infectious disease.

2
Fundamental Principles of Biodetection

The current "state of the art" in biodetection encompasses an impressive array of diagnostic instrumentation to identify and measure minute quantities of biological material on the molecular scale [20]. In the context of infectious disease, the target material can consist of the whole pathogen itself, or compo-

nents – such as protein, lipid or genetic material derived from the microorganism. Infectious disease can also be characterized by changes which occur in the affected individual, particularly when the host immune response is triggered. Changes in the serum concentration of certain host biomolecules, including cytokines and acute phase reactants, may reflect an active infectious disease process and thus represent important markers for detection [21]. Most biodetection devices currently use one of two basic molecular detection methods: nucleic acid hybridization and immunoassay.

Nucleic acid hybridization utilizes the strong binding affinity between complementary strands of DNA or RNA to capture and detect specific genetic material in complex biological solutions. This method is frequently used in conjunction with a laboratory procedure known as polymerase chain reaction (PCR, Fig. 1), to quickly replicate select genetic sequences which may be found in solution. In principle, PCR amplification of genetic material greatly improves assay sensitivity, since even a single strand of DNA could be amplified in vitro to easily detectable levels.

It should be noted, however, that PCR does not indiscriminately amplify all DNA sequences in solution. Rather, PCR amplification occurs when "primers" containing short sequences of nucleic acid hybridize to longer strands of DNA in a test solution. These primers ultimately define the terminal endpoints of the DNA chain which is replicated by PCR. Therefore, the process of PCR amplification itself plays a role in selecting specific genetic material from complex biological mixtures.

The quality of a DNA assay using PCR and hybridization depends essentially on the specificity of the primer and probe sequences used in the assay. Genetic sequences which uniquely identify a particular pathogen become known

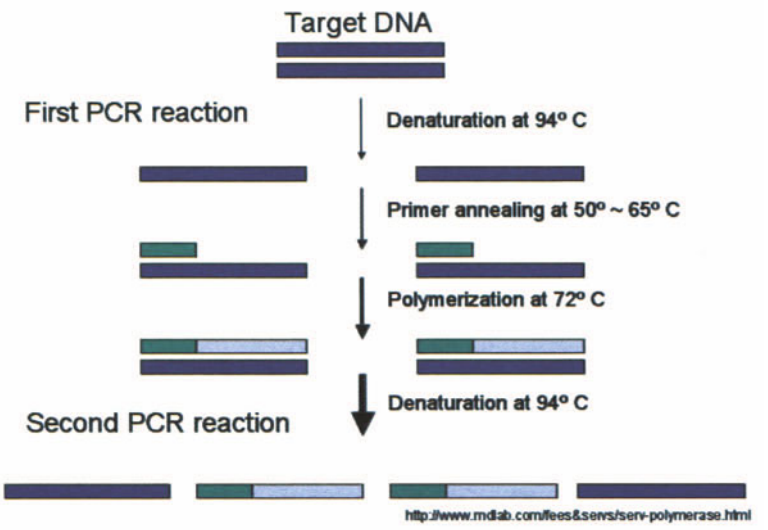


Fig. 1. Gene amplification by polymerase chain reaction (PCR, Schematic adapted from http://www.mdlab.com/fees&servs/serv-polymerase.html)

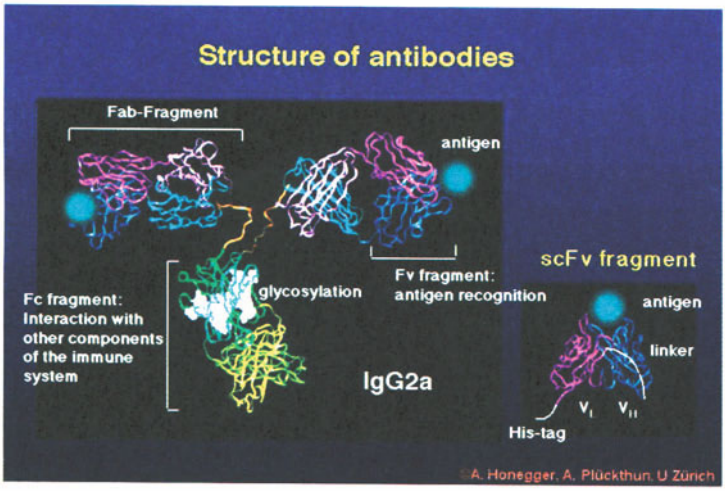


Fig. 2. Molecular/functional structure of antibodies (http://www.unizh.ch/~pluckth/slide_shows/Slides/Miniantibodies/index.htm)

as "signature" or "fingerprint" sequences for that microorganism. Signature sequences do not share homology with other genes or sequences which might also appear in the test fluid. Moreover, PCR technology has been miniaturized to the handheld scale, enabling the development of new point-of-care DNA detection instruments [18].

For the detection of non-genetic biomolecules, such as proteins, sugars and lipids, immunoassays are the method of choice. Antibodies (also known as immunoglobulins, Fig. 2) constitute a broad class of proteins whose natural function is to recognize and bind other biomolecules (antigens).

Antibodies play a critical role in the host defense, in part by monitoring the blood circulation for the presence of foreign (and possibly harmful) material that enters the body. Antibodies are sensitive and specific for their target antigen and, upon binding, can recruit other components of the immune system to respond against the foreign agent. While the pool of naturally-occurring antibodies in circulation are randomly generated and carry different specificities, certain antibody "clones" will predominate, depending on the history of recent acute exposures to selected foreign antigens. These monoclonal antibodies can thus be harvested from the blood serum and subsequently utilized for laboratory-based in vitro detection of the target antigen.

In general, DNA hybridization assays and immunoassays are complementary techniques. But since antibodies can be raised against nearly any type of biomolecule – including DNA – immunoassays have a broader range of application. (To be fair, DNA *aptamers* represent a novel application of synthetic oligonucleotides whose tertiary structure enables sensitive and specific binding to other biomolecules.) By selecting antibodies which are specific for epitopes which are exposed on the pathogen surface, immunoassays can be directly performed on biological or clinical samples, without prior sample preparation.

3
Development of Optical Methods for Biodetection

Bacteria are considered to be the smallest living cells and range in size from 0.1 to 10 μm. Traditionally these microorganisms are identified by direct visualization using light microscopy with Gram stain. The staining process reveals biochemical characteristics of the bacterial cell wall and facilitates the proper identification of bacterial species. In contrast to bacteria, viruses are much smaller (100 nm or less) and require enhanced techniques, such as electron microscopy or fluorescent labeling, for adequate visualization.

Laboratory culture plays a key role in standard detection methods. By allowing the pathogen to multiply and replicate, one effectively amplifies the signal to be detected by bioassay. Furthermore, laboratory culture itself serves as a functional assay to determine pathogen viability. Viruses, for example, are too small to be visualized by light microscopy. However, when cultured on cell monolayers, live viruses will infect and lyse cells, creating voids or "plaques" which are readily (in)visible on the the culture dish. Despite its establishment as a reference standard, laboratory culture and direct microscopic identification is a relatively costly procedure, both in time and resources. For this reason, the method is reserved for clinical cases in which more serious bacterial infections are suspected. For general or routine screening purposes a much less cumbersome diagnostic technique is necessary.

Representing a first step towards this goal, direct fluorescent assay (DFA) kits utilize specific antibodies to label various pathogens on a microscope slide. Different fluorophores covalently conjugated to different monoclonal antibodies enables several pathogens to be tested simultaneously, and the results interpreted by the color-coding determined by the fluorescent conjugation scheme. However, these kits can only be used by trained laboratory technicians and are again impractical for high-throughput applications.

3.1
Sandwich Immunoassays – ELISA

Immunoassays can be performed in various configurations; one of the most commonly used is called a *sandwich* immunoassay. The term is derived from the arrangement of stacked layers which form in the presence of the target antigen. The base layer is comprised of antibodies which are fixed onto a solid substrate. When this surface is exposed to a biological fluid specimen, the antibodies serve to capture target antigens which may be present in solution. After the treated surface is washed, the presence of bound antigen is demonstrated by incubation with detection antibodies which are labeled with either a fluorescent dye or a chromogenic enzyme. The *enzyme-linked immunosorbent assay* (ELISA, Fig. 3) has become a laboratory reference standard for measuring dilute quantities of target antigen in solution [22].

Optimized ELISA tests utilize immunoassay antibodies to the fullest extent, measuring antigen concentrations in the picomolar range. The cost of developing this level of sensitivity into an ELISA assay is a significant investment of

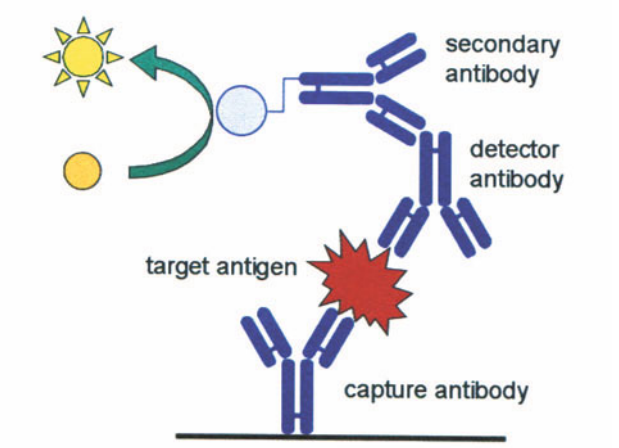


Fig. 3. Basic components of ELISA sandwich immunoassay

time and effort. ELISA assays require extensive pre-calibration with laboratory standards, and generally take half a day for results. While this is clearly an improvement over standard laboratory culture and analysis, it is still too costly to be considered for routine screening.

3.2 Lateral Flow Assays – "Strip" Tests

In order to gain speed in testing, ELISA sandwich immunoassays have been reduced to the form of lateral flow assays, commonly known as "strip" tests. Various immunoassays have been commercialized utilizing this format: for example, the i-STAT 1 handheld blood analyzer, the QuickVue influenza test by Quidel Corporation Ltd. UK, and the ZstatFlu™ test by ZymeTx, Inc. Arguably the most successful commercial implementation of the lateral flow assay concept are "home pregnancy" tests, which use monoclonal antibodies to detect the presence of human chorionic gonadotropin (hCG) in urine.

A particularly refined example of a lateral flow immunoassay is the chromatography-based handheld "smart ticket" device (Fig. 4) which is a key component of the Joint Biological Point Detection System (JBPDS) developed by the United States military (in collaboration with Canada and the United Kingdom) to detect biological warfare agents remotely.

Each JBPDS smart ticket is designed to detect one selected type of bioagent. Blue latex particles are coated with detector antibody, in order to attract and bind any targeted bioagent that may be present in a liquid sample. To determine whether any bioagent was actually found, the detection microparticles are allowed to flow across a nitrocellulose membrane strip. The strip is coated with two lines of capture antibodies: one directed also at the targeted bioagent (T) and one negative control (C) which is an antibody that directly binds to the detector antibody coated on the latex microparticle. The two lines of capture antibodies

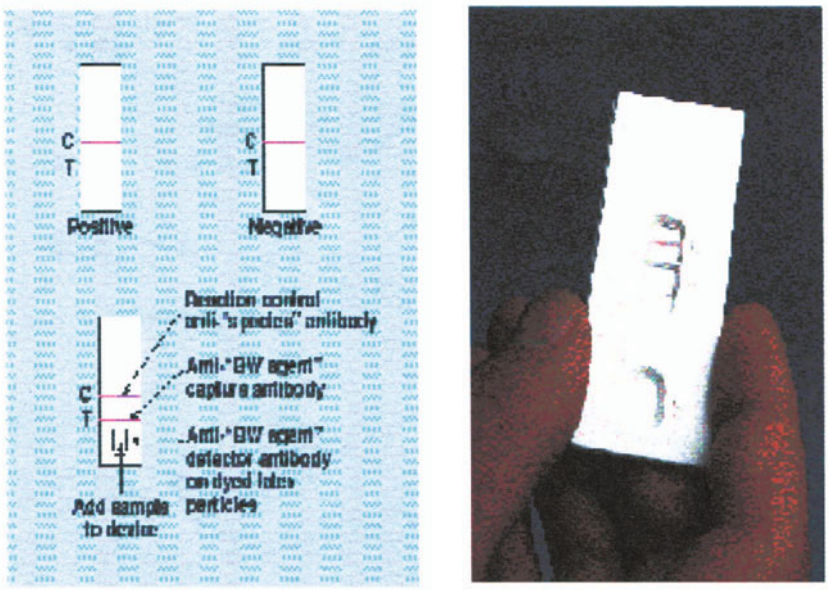


Fig. 4. JBPDS smart ticket

are initially colorless, but upon exposure to the suspended microparticles, will form either one blue line (negative result) or two (positive result), depending on the presence or absence of bioagent attached to the microparticles.

In order to expand the scope of the smart ticket device to include multiplex analysis, multiple smart tickets must be employed. The JBPDS platform uses a mechanical carousel with automated fluidics to run up to 9 smart tickets simultaneously. It should be noted that in such an arrangement, the biological sample must be divided into 9 fractions, which may adversely affect detection sensitivity.

In general, the measurements reported by lateral flow assays are less quantitative than laboratory-based ELISAs. However, for end-applications such as infectious disease screening, the device needs not so much to be precise as it should be accurate. The threshold for positive detection can be tuned to a value which is clinically relevant for proper management of the infected individual. The goal of developing a biodetector which is more rapid, more convenient and less expensive to use is to promote widespread and more frequent screening of the population to safeguard public health, and to distinguish the "worried-well" individuals from those who truly need immediate medical attention.

3.3 Fixed Microarrays – DNA Gene Chip

Advances in engineering technology have enabled new devices to operate on scales much smaller than previously thought possible [23]. As a result, both immunoassays and DNA-based tests can now be configured as microarrays, in

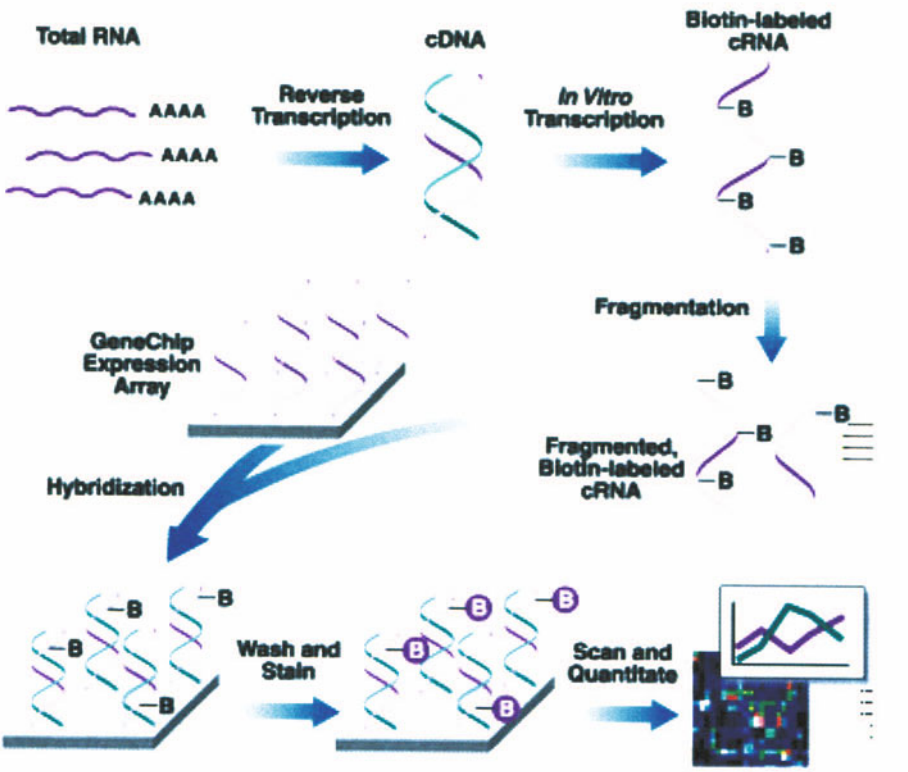


Fig. 5. "Gene chip" arrays for gene expression analysis (Affymetrix, Inc. http://www.affymetrix.com/technology/ge_analysis/index.affx)

which each array component is functionally equivalent to an ordinary strip test. The benefit of microarray technology for biomolecular detection is that detection sensitivity can be retained despite assay multiplexing.

One successful commercial application of the microarray concept is the DNA "gene chip" (Fig. 5) developed by Affymetrix Corp. [24, 25]. Fodor and colleagues first described a method of densely packing chemical probe compounds on a silicon substrate [26]. By combining solid phase chemical synthesis with photolithographic fabrication techniques, Affymetrix successfully assembled an array of 65,000 discrete nucleic acid probes into an area no larger than a few square centimeters. Each 50 μm x 50 μm microarray element was estimated to contain thousands of oligonucleotide probes. The DNA chip was designed for massively-multiplex assay applications such as gene expression profile analysis.

By incubating the gene chip with a complex biological solution such as a cell lysate, the fixed oligonucleotide probes are allowed to hybridize with segments of DNA that are present in solution. In a manner similar to the sandwich immunoassay, the surface of the gene chip is then washed, and subsequently probed for the presence of hybridized DNA. The microarray is optical-

ly analyzed by raster scanning, and finally the data is decoded by comparing the measured signals to the array pattern in which the oligonucleotide probes were deposited.

Because the individual array elements of the DNA chip are miniscule compared to the typical test strips of a lateral flow assay device, a sophisticated instrument is required to properly read the DNA microarray. The cost of purchasing such a system is more than offset by the savings in time and resources, if the same assortment of DNA tests were to be performed separately using conventional laboratory methods. But since the instrument reader is quite large and contains delicate optics, it is not rugged for portable field applications. Furthermore, a single chip design taken from concept to product can cost as much as $400,000 USD, and cannot be modified without a complete redesign. Thus in applications such as bioforensics or proteomics – in which experimental protocols are constantly evolving with the influx of new information – the cost of continually updating a DNA chip design could prove prohibitive.

3.4
Liquid Microarrays – Luminex Flow System

It is important to realize that microarrays need not be fixed in 2 dimensions [27]. The argument can be made that, for certain fixed microarray designs, individual test elements may not have equal access to the biological fluid sample [28]. In situations where the probe molecules compete for a common target ligand, the detection would be biased towards the probe element which first encounters the sample solution. One method for addressing this issue is to "un-

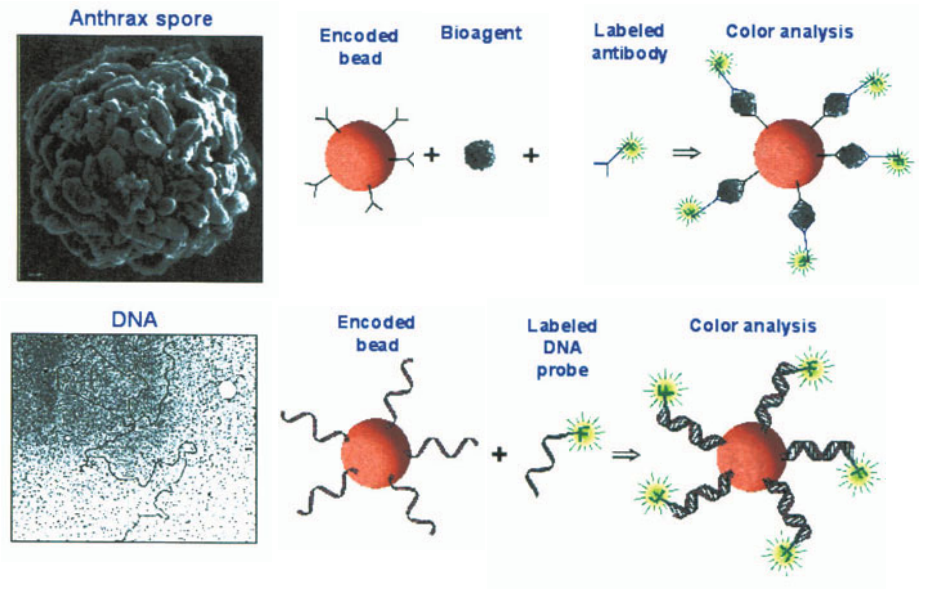


Fig. 6. Microbead-based detection of protein and DNA

fix" the microarray, allowing all probe elements to mix freely and equally with the biological fluid sample [29].

Luminex Corporation developed a multiplex bioassay system around this "liquid array" concept. Instead of designing assays to be performed on flat surfaces, as in ELISA or the Affymetrix gene chip, Luminex developed a system platform based on 5-μm diameter polystyrene microspheres that are surface-modified to facilitate chemical coupling of either capture antibodies or oligonucleotide probes (Fig. 6). The functional nature of these particles is thus similar to that of the labeled particles used by the JBPDS lateral flow assay. However, in contrast to the JBPDS method of analysis, the Luminex platform analyzes the microspheres using a customized flow cytometer (Fig. 7).

Flow cytometers are commonly found in biomedical research laboratories, and are used to analyze particles (usually living cells) suspended in solution. The core mechanism of the flow cytometer is an optical system which interrogates particles that pass through a thin glass capillary tube. Using various forms of illumination (visible or UV laser, for example), the flow analyzer is able to count and measure parameters such as particle dimension, optical absorbance and fluorescence. The microfluidic subsystem of the flow analyzer uses an inert carrier solution to draw up the biological sample at a rate and in a manner which guides the suspended particles to pass through the capillary channel in single file. In normal function, the flow cytometer can analyze thousands of particles in a matter of minutes.

In order to incorporate multiplexing capability into their diagnostic platform, Luminex introduced a method of optically encoding these latex microspheres, using discrete amounts of red and orange fluorescent dyes impregnat-

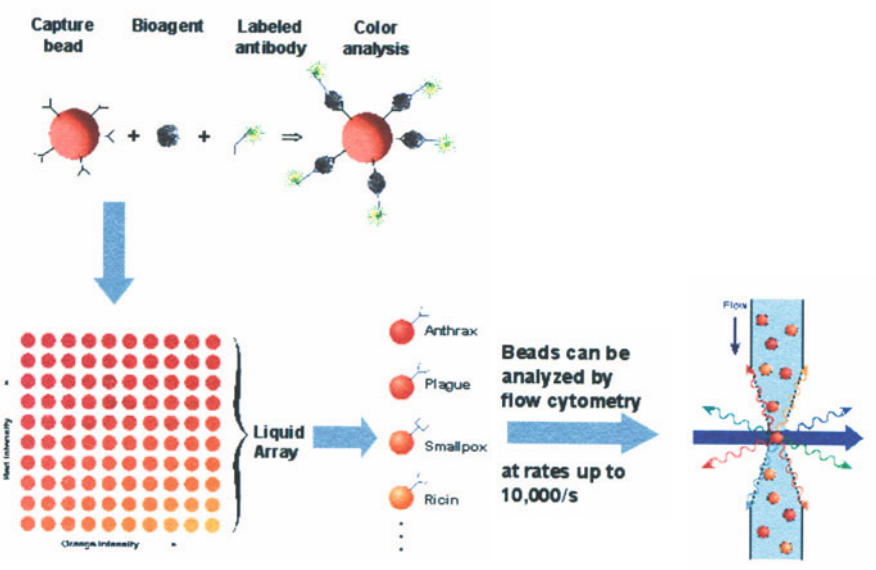


Fig. 7. Overview of microbead-based multiplex flow analysis

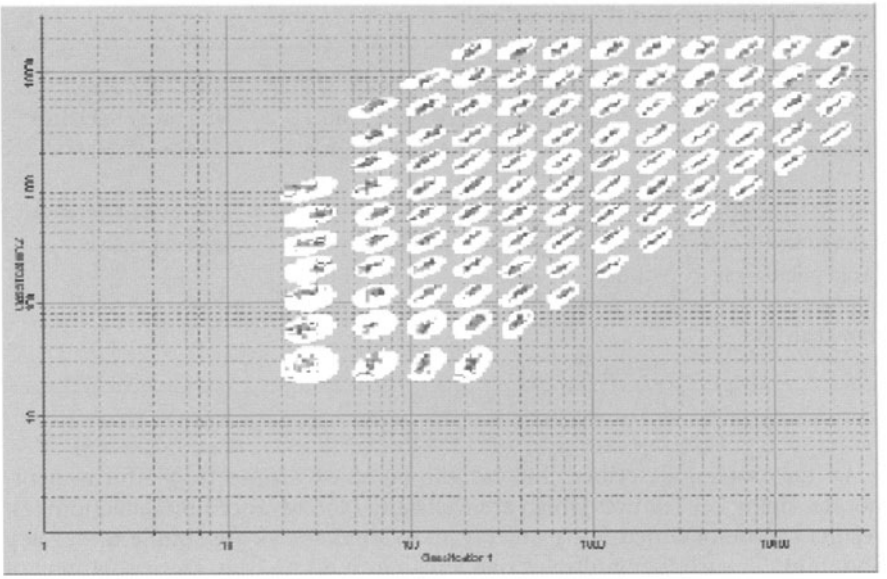


Fig. 8. LabMAP 100-plex bead map (Luminex Corporation, Austin TX)

ed into the material of each microbead. Exactly 100 distinct microbead classes were thus formed, enabling up to 100 different bioassays to be performed simultaneously on a single fluid sample (Fig. 8).

The last component of the Luminex platform is the detection element. In a typical multiplex immunoassay, an assortment of bead classes are selected for derivatization with the necessary capture antibodies. These reactive beads are then allowed to mix and diffuse freely in solution with the biological sample. After the microbeads are washed (either by centrifugation or filtration, followed by resuspension), they are subsequently treated with detector antibodies to indicate whether or not any target antigen is bound to the bead surface. The detector antibodies are linked to a common green fluorescent reporter molecule, phycoerythrin (PE).

Ultimately the assay microbeads are analyzed by the Luminex flow cytometer. Using a red laser source to measure the red and orange fluorescence intensities, the customized flow analyzer correctly determines the classification (and thus the corresponding bioassay) of each passing microsphere. Immediately following classification, a green laser source interrogates the microsphere for its corresponding reporter fluorescence – a high measured value thus indicating a positive assay result.

Because each individual microbead constitutes an independent measurement by the Luminex instrument, the data generated by a routine multiplex assay involving tens of thousands of microbeads carries much greater statistical significance, compared to other multiplex assays which may only perform measurements in duplicate or triplicate.

Table 1. Summary of Biodetection Methods

Method	Time	Multiplex	Portable	Comments
Laboratory culture	7–10 days	no	no	laboratory reference
Direct fluorescence	1 hour/ assay	serial or parallel	no	cannot automate
ELISA	<1 day	no	no	laboratory reference
Lateral flow assays	1 hour	serial or parallel	yes	least expensive
Fixed microarray	<1 day	yes	no	
Liquid array / flow analysis	1–2 hours	yes	no	
MIDS	1–2 hours	yes	yes	

Of the systems described thus far, the Luminex diagnostic platform incorporates many key features which are well suited for advanced biodetection: test results can be obtained fairly quickly, individual assays are simple to develop and modular such that multiplex panels can be built up from any assortment of derivatized microbeads, and the liquid array makes optimal use of the biological sample at hand.

Key features which the Luminex platform lacks are ruggedness and portability. Understandably, the Luminex LX100 was designed to be a sophisticated research tool of similar caliber to the Affymetrix system. However, the instrument's delicate microfluidics and optics, along with its operating power requirements, constrain its use to a laboratory benchtop environment.

4
Multiplex Immunoassay Diagnostic System (MIDS)

In response to the need for a rapid, portable, multiplex biodetection system in public health, clinical medicine and national security, we investigated the strategies developed to this point and concluded that a new system should leverage the strengths of the liquid array technology which were advanced by Luminex. Optically-encoded microbead-based immunoassays retain the advantages of speed, accuracy, cost, and multiplex-capability over other biodetection methods. However, we propose that a CCD-based optical instrument which can analyze the microbeads arranged in a 2-dimensional array is technically more rugged, scalable, and portable than a flow cytometer – and would thus be well suited for point-of-care applications. The working name for this developmental platform is Multiplex Immunoassay Diagnostic System, or MIDS (Fig. 9).

The MIDS platform consists of 3 major components which will be described in greater detail: (1) a disposable sample preprocessing unit, (2) CCD-based optical detection hardware, and (3) digital image analysis software.

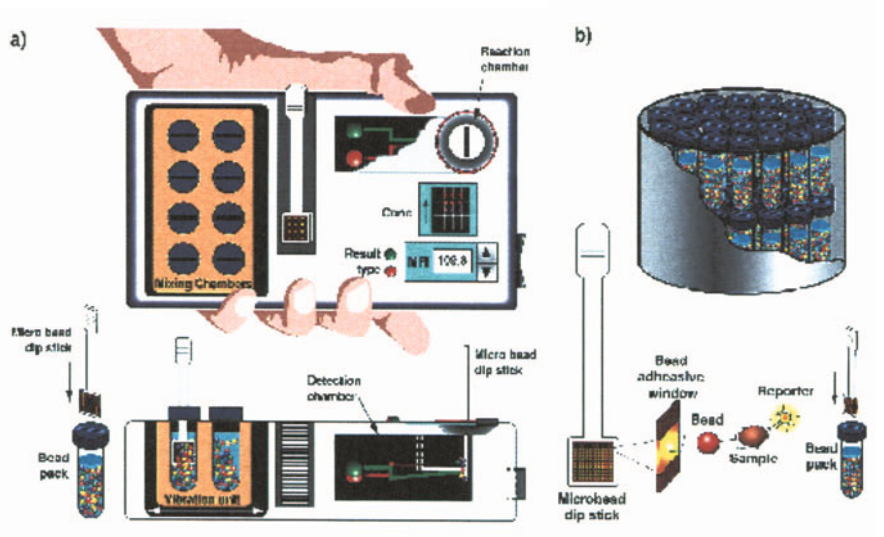


Fig. 9. Overview of MIDS concept

4.1 Disposable Sample Collection Unit

While sample collection and handling may at first seem peripheral to the discussion of optical biosensors, we put forth the argument that the ease or difficulty of detection and measurement depends entirely on the quality of sample preparation.

The disposable sample collection unit for MIDS is under active development; however, its basic roles and design have already been defined. The disposable unit performs two critical functions: (1) to receive a biological fluid specimen for immunoassay using the capture microbeads and secondary detection reagents; (2) to transfer the treated microbeads into a 2-dimensional array that will be analyzed by the optical reader.

Since the basic design of the disposable module depends primarily on the method of sample collection and on the physical characteristics of the fluid sample (i.e. blood, saliva, urine, etc.), we selected two methods most commonly used in hospitals for assessment of infectious disease: blood serum samples and nasopharyngeal (NP) swabs. Both types of fluid samples pose unique challenges for preprocessing.

Whole blood poses a unique problem for sample preparation, because the latex microspheres are similar in size to blood cells. Although filtration is a common technique to efficiently remove the microbeads from solution, whole blood contains up to 40% red blood cells by volume, and would immediately occlude the filter membrane, interfering with microsphere recovery. To address this issue, a preliminary step to prefilter or otherwise remove intact cells from the blood sample would have to be included in the sample handling procedure.

Fig. 10. Prototype disposable sample collector for MIDS

For nasopharyngeal swab samples, an extraction step is required to transfer the captured pathogens into the collection chamber. It is necessary to introduce an extraction buffer which contains a mucolytic agent to help liquefy the sample and promote release of particulates trapped by swab material. We are also considering special swab designs which will greatly improve pathogen capture and release. Because our near-term goal is to demonstrate MIDS-based detection of respiratory viruses in the hospital emergency room setting, we decided to focus development around NP swab collection, since this sampling method is more likely to detect early stages of upper respiratory tract infections, compared to blood serum samples.

The initial prototype for a NP swab-collecting disposable unit (Fig. 10) is built around a simple filter-bottom well design. The well forms a receptacle for a nasopharyngeal swab, and has a working volume of approximately 1 cm^3. Once the sample is introduced, the test microbeads are added and allowed to incubate for 20 minutes at ambient temperature. After incubation, the unbound antigen and remaining fluid sample are removed by filtration, either by applying negative pressure with a small syringe pump, or by introducing an absorbent material on the opposite surface of the filter. The microbeads are drawn down onto the

surface of the filter bottom by bulk flow across the filter membrane. The detection antibodies and reporter label are subsequently added to the resuspension medium, with intermediate wash steps, using the same filtration method.

Reagent packaging, storage and delivery is another design component which we must consider for long term development of the disposable sample collector. Eventually we anticipate reagents to be supplied in dry, lyophilized form requiring rehydration prior to dispensing. In the near-term, we have developed multiple concepts for delivery of fluid reagents which require further evaluation and refinement. In general, the reagent packs must allow for isolated storage of multiple solutions and subsequent dispensing at various time intervals and rates. One method employs a syringe pre-loaded with multiple reagents in series separated by intermediate plugs. Computer control of the syringe pump would enable the various solutions to be dispensed at preset intervals between incubations. A similar strategy has been developed using a carousel mechanism which holds individually-packaged reagent "blister packs" that are ruptured and dispensed as needed. Preliminary results with the carousel design have been encouraging, but we will continue to examine details of inter-compartmental contamination, complete expulsion of the bead reagents, consistency of flow versus pressure, and accuracy of delivering small microtiter volumes. We will also consider other methods of reagent storage and dispensing, with appropriate screening of commercially available solutions – with the eventual goal of completely automating reagent dispensing, by using a simple and inexpensive disposable reagent pack that requires minimal user interface

The other primary role of the disposable sample handling unit is to transfer to assay microbeads from suspension into a planar array for imaging and analysis by the optical detector. We had previously evaluated several methods to

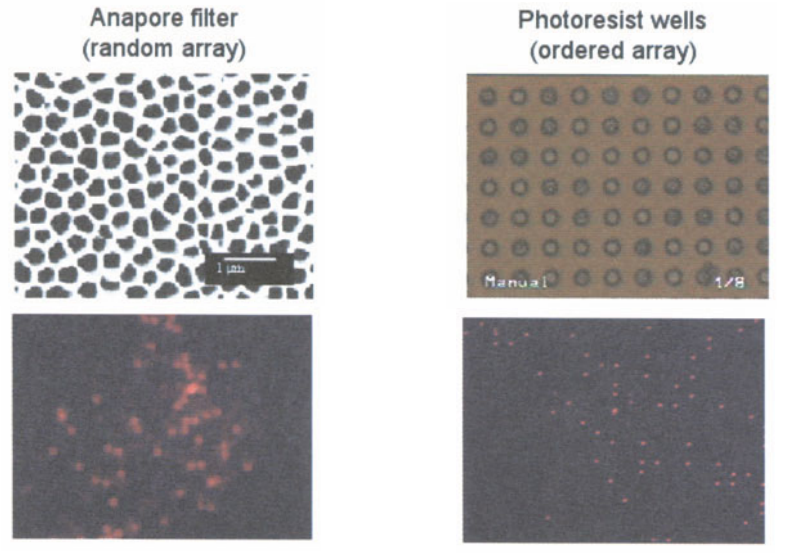


Fig. 11. Random vs. ordered 2-dimensional arrays

deposit suspended microbeads onto flat surfaces (Fig. 11). Initial investigations demonstrated partial success in immobilizing beads onto glass surfaces; however, neither surface tension nor chemical adhesion (for example, long-chain biotinylation of silanated glass slides with streptavidin-coated beads) permitted the microbeads to withstand vigorous washing. We also investigated photoresist surface technology, spinning a 5–10 μm layer of photoresist material onto glass slides and etching microscopic (10 μm diameter) wells in an ordered array across the photoresist. We found that the microbeads placed in suspension over this surface precipitated out of solution and into the wells. The resulting array was well-ordered and stable throughout moderate washing. Several disadvantages were that bead sedimentation was essentially a passive process and not well characterized. Also, the production of the microwells by photolithography was labor-intensive and would ultimately raise the cost of the disposable component of MIDS

We found a promising solution with a new filter technology by Whatman Inc., called Anopore™. Unlike conventional paper filters, Anopore is made of a semicrystalline aluminum oxide, which under electron microscopy appears almost

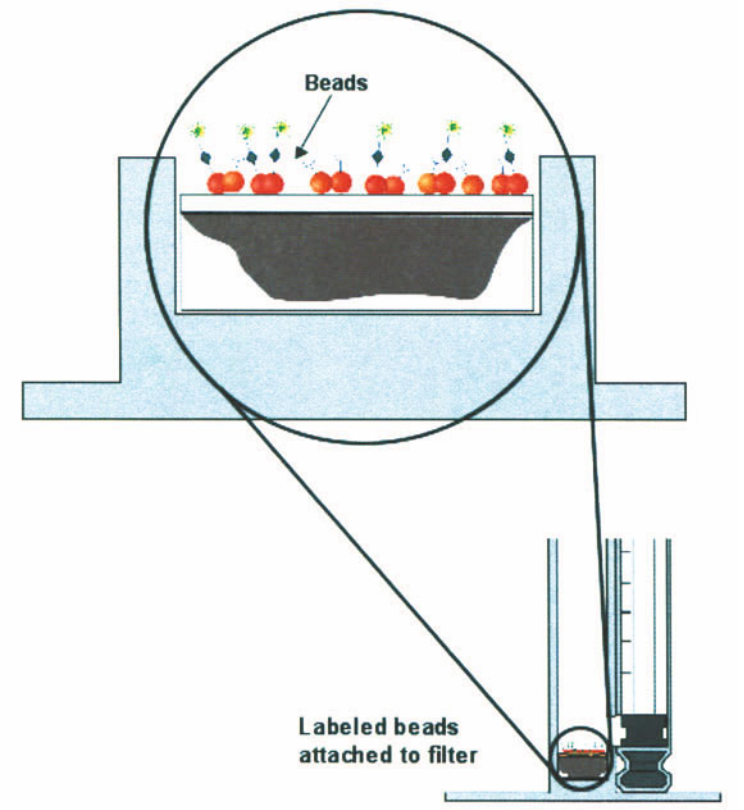


Fig. 12. Generating a 2-D microbead array by direct filtration on a flat surface

as a honeycomb structure with densely packed pores averaging 0.2 μm in diameter (compared to the 5.5 μm diameter microspheres). Furthermore, the Anopore™ material is milled flat to exacting tolerance and is optically transparent when wet. This made for an intriguing combination of features that seemed perfectly suited for the MIDS disposable device. Not only could the Anopore™ medium provide high flow rate filtration to retain the encoded microbeads during the immunoassay procedure, but it could form the substrate that would allow the derivatized beads to be imaged directly on the flat surface of the filter.

In practice, the new filter works well in the disposable sample chamber for mixing and exchanging suspension buffers during the immunoassay procedure. However, the deposition of the treated microbeads into a 2-dimensional array proved to be a more complex task. While our goal was to deposit the beads in a random but uniformly distributed monolayer on the Anopore™ surface, we found that the assay microbeads (which in the final state are coated with antibody protein, captured pathogens and reporter label) tended to form aggregates in aqueous solution. While beads which form the first layer on top of the filter surface are acceptable for image analysis, extra beads which stack on top of the first monolayer can disrupt bead classification and analysis by contributing additional fluorescence signals from their vicinity. This issue has been addressed first by adjusting the bead concentration per immunoassay, such that the total number in a given sample well is less than that required to form a complete monolayer covering the filter surface. Since the suspended latex microbeads will follow the bulk flow of solution in the reaction chamber, we have taken into consideration possible methods of controlling the quality of liquid flow across the filter membrane to promote more even distribution of the microbeads on the Anopore™ material (Fig. 12).

4.2 CCD-based Optical Hardware

We selected image-based cytometry as a basis for the MIDS detection instrument, because the hardware supporting charge coupled device camera (CCD) technology is more amenable to building small scale or portable instruments (Fig. 13). It is worth noting that the tremendous sensitivity of the photomultiplier tube used for reporter detection in flow cytometers can be matched by the relatively insensitive CCD due to the fact that CCD-based measurements can integrate fluorescence signals for many seconds, while beads in a flow system are illuminated for less than a millisecond.

For a given number of excitation sources, the degree of multiplexing for MIDS is inherently less than that of the Luminex system. There are two primary reasons for this. First, the dynamic range of the CCD is less than that of the avalanche photodiodes or photomultiplier tubes that are used in flow systems. Secondly, the distribution of fluorescence intensity for any particular (Luminex) bead class is more disperse with the imaging system because of inherent spatial variations in the excitation level over the much larger field of illumination. (A flow system concentrates the excitation in a 50-μm spot, whereas the imaging system illuminates a field measuring a millimeter or two.) In its current form,

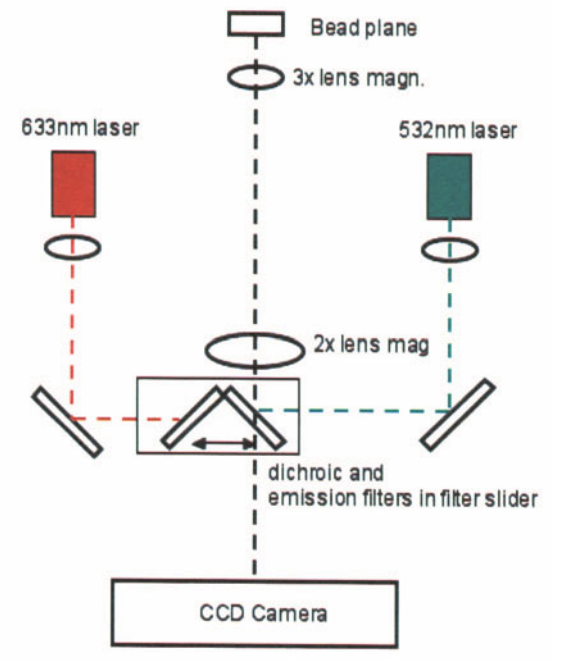


Fig. 13. Schematic of MIDS optical detection system

the benchtop prototype instrument is capable of performing the equivalent of a Luminex 6-plex assay. We are confident that the MIDS system will be able to accommodate an 8-plex assay in a basic package. By upgrading the lasers, beam conditioning, and the CCD, we estimate that up to a 32-plex will be possible. In either case, the MIDS is well suited for point of care applications which we anticipate to require 10–20 assays/panel.

The optical system consists of the following components: an illumination source, detection electronics, analysis package, and user interface. The simplest types of light sources include light emitting diodes (LEDs), lasers, laser diodes, and filament lamps. These sources can be used in conjunction with optical filters, diffraction gratings, prisms, and other optical components to provide a specified spectral component of light. Alternative forms of radiation such as bioluminescence, phosphorescence, and others could also potentially be employed. Although typical fluorophores require excitation wavelengths in the visible portion of the spectrum (300–700 nm wavelength), other wavelengths in the infrared and ultraviolet portion of the spectrum could also prove useful for illuminating the dipstick microbead array. The transmitted, reflected, or re-emitted light from the trapped microbeads must then be propagated to an optical apparatus for detection, using photosensitive detectors such as photodiodes or photomultiplier tubes, in combination with some type of spectral and/or spatial filtering. Spatial filtering of the light is possible either by transverse scanning of the dipstick microbead array or with two-dimensional detectors such as CCD and video cameras.

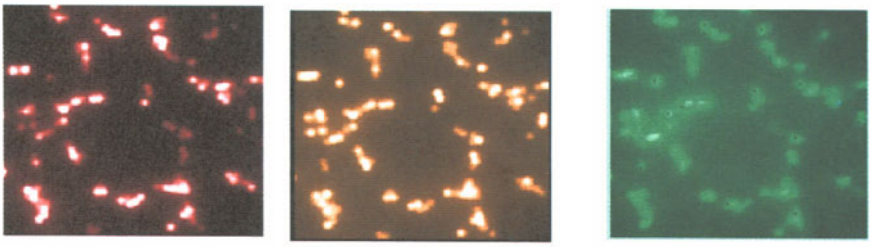


Fig. 14. Red, orange, and green component fluorescent images of random bead array (pseudocolor representation)

The criteria for evaluating system performance can be subdivided under the orthogonal functions of classification and reporting. The key variables determining system performance relate to image quality, image processing and assay analysis. System specifications are addressed concurrently by considering issues such as size, weight, cost and complexity. Lasers require power supplies and controllers, as do shutters and motorized stages. Furthermore, CCDs need need supporting mechanisms for cooling and control as well.

The optical system uses a form of Köhler illumination, by which a fluorescent sample is simultaneously illuminated from the front by a collimated laser source and imaged from the front to a CCD camera. This is done for a series of three timed exposures using a combination of dichroic beam splitters and optical bandpass filters to generate a "red-orange" image pair for bead classification and a "green" image for reporting the level of attached reporter label (Fig. 14). These images are subsequently processed and analyzed to identify bead classes and the presence of phycoerythrin reporter dye in the original sample.

A three-color component fluorescence image set must be taken for each microbead array to be analyzed. Ideally, in addition to being in focus, they would contain thousands of beads, with hundreds of CCD pixels devoted to each bead. While "megapixel" CCDs exist with necessary pixel resolution to accomplish this, such devices are both relatively large and expensive. Furthermore, the system would require a relatively high (40×) magnification, which would place greater demands on the quality of optics, beam uniformity across the image field and would reduce the depth of focus. A larger field of view also means that the available laser power is divided over a larger area, which requires longer integration times or more expensive lasers.

One of the most significant differences between Luminex's flow-based system and the MIDS' image-based method is the number of microspheres which can be analyzed per assay, which plays a large role in determining the instrument's precision. On the MIDS system the factor limiting the number of beads per image is the density of isolated beads that can be produced on a sample before it is even inserted into the optical system. The random arrays generated by bead filtration are likely to have relatively large unoccupied regions as well as those in which beads are stacked and difficult to reliably analyze. We previously described our approach to these challenges. Clearly, an ordered array is far superior for image analysis but is difficult to achieve and to fabricate when the design calls for a simple, disposable platform.

Given the constraints placed on hardware design, we must investigate other methods to compensate for the number of data points which may be collected in one assay. One option is to take several image triplets (red, orange and green) of the same sample, measuring multiple regions to collect more data points for analysis. The key drawbacks to implementing this solution are that an extra motor drive would be required as well as extra time for re-iterative image acquisition and analysis. We anticipate that the time required to collect one triplet set of images will be approximately one minute.

4.3
Digital Image Analysis Software

Currently the software for MIDS is divided into three components. LabView™ (National Instruments) controls the operation of the benchtop instrumentation, including CCD camera, shutter controls and filter wheel. As previously described, assay microbeads were deposited on Anopore™ filters and the resultant arrays viewed under the fluorescent imaging setup. Using the appropriate filter sets to extract the necessary red, orange and green fluorescence from the bead array, digital images were captured to computer with a Pixel CCD camera. Intensity measurements were made on the acquired images using subroutines written in IP-Lab™ (Scanalytics, Inc.) software. Fluorescent microbeads are identified by means of a "smart search" algorithm which sequentially raster scans each color image component. Microbead targets are identified by virtue of size, shape, and expected fluorescence intensity profile when imaged by the MIDS system. The red, orange and green images are placed into registration so that fluorescent microbeads may be sorted and their corresponding classification and reporter values saved to a data file. When displayed on a scatter plot of red vs. orange fluorescence intensity, the bead mixture can be sorted into distinct clusters of data points – resembling those of the pre-calibrated bead maps displayed using Luminex Data Collector software. The task of bead classification is performed by IgorPro (WaveMetrics, Inc.), which compares incoming data to a pre-calibrated bead map and decodes the classification of each identified bead. IgorPro also performs statistical analysis of the reporter fluorescence intensity for each bead class, ultimately reporting which classes (and hence assays) were "positive" by multiplex immunoassay.

We plan eventually to port the entire image acquisition and analysis to the LabView™ software platform, taking advantage of new functional capabilities added with the IMAQ Vision™ imaging toolset for LabView™.

4.4
Preliminary Results

We have successfully characterized the MIDS prototype using six fluorescent-encoded bead classes from Luminex. The six classes were derivatized with various capture antibodies and control reagents to simulate a general biological sensor which could detect 3 types of pathogenic material: bacterial spores (of *Bacillus globigii*), virus (MS2 bacteriophage), and protein (ovalbumin). The bead classes were derivatized according to the following scheme:

Bead class	surface conjugate	function
C200	Bovine serum albumin (BSA)	negative control
C198	r-phycoerythrin	positive detector control
C194	anti-MS2	viral probe
C170	anti-*Bacillus globigii*	bacterial probe
C168	anti-ovalbumin	protein probe
C164	anti-human IgA	positive sample control

In addition to the three experimental bead sets which probe for target pathogen, one bead class was coupled to BSA as a negative control to demonstrate nonspecific binding which might occur. C198-phycoerythrin would serve as a positive control and calibration standard for the MIDS detector. Finally, to confirm whether a biological sample was indeed placed into MIDS, a bead set was added to test for human IgA – a ubiquitous protein which is secreted by the nasal mucosa.

The simulant panel was tested in the laboratory, using calibrated solutions containing various concentrations of antigen. Briefly, aliquots of the 6-plex bead panel were incubated with variable dilutions of stock antigen (Bg or MS2, for example) in 96-well filter bottom plates for 30 minutes at room temperature on an orbital microplate shaker. After antigen was allowed to bind the capture microbeads, the wells were washed with phosphate-buffered saline, filtered, and then resuspended for 30 minutes in a solution containing the detector antibody cocktail. These detector antibodies are identical to the ones covalently bonded to the surface of the capture microspheres, but are instead biotinylated – so that they will attach to the final reporter label, which is phycoerythrin-coupled to streptavidin (Sa-PE).

The assay beads were washed and transferred to round-bottom 96-well plates for measurement on a Luminex LX100 flow cytometer. Analysis of the various bead samples showed that the reporter fluorescence intensity associated with bead classes C194 and C170 increased in proportion to the concentrations of MS2 and Bg, respectively. Positive and negative control beads showed consistent behavior – and because the samples were prepared from laboratory standards, no human IgA was present in solution, so that C164 showed little reporter fluorescence. The graph in Fig. 15 depicts the 6-plex microbead response to high concentrations ($\approx 10^7$ pfu/ml) of MS2 bacteriophage in solution.

After the Luminex flow analysis, the same bead samples were individually recovered, and then processed into 2-dimensional arrays by Anopore™ filtration, using the prototype disposable sample collectors. While remaining attached to the filter bottom, the random bead arrays were placed in the MIDS optical detector, and fluorescent red, orange, and green image triplets were acquired. The sample chamber was rotated manually several times in the holder, so that several fields could be analyzed for each microbead sample.

The composite data in Fig. 16 shows the equivalent MIDS results for the same Luminex bead assay depicted in Fig. 15. The distribution of red and orange classification intensities for the microbeads detected on the MIDS platform is similar to that determined on the Luminex flow cytometer. Also similar in fashion to the

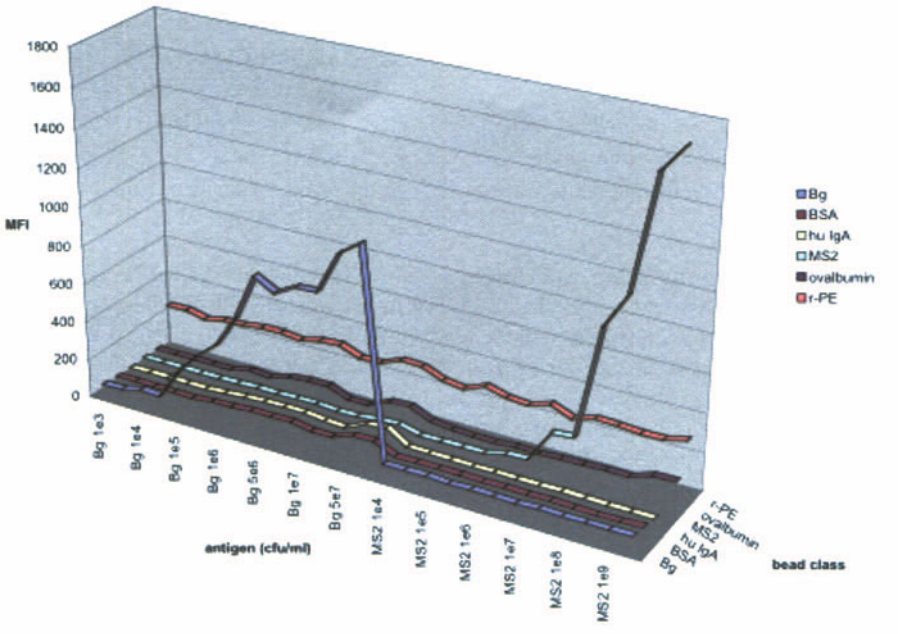
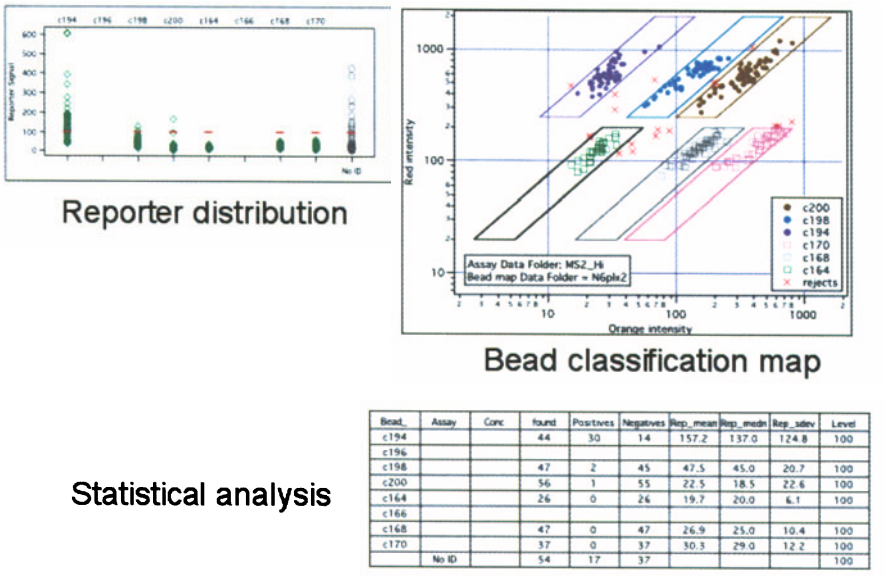


Fig. 15. Specific, concentration-dependent response of 6-plex bead assay to simulants. Luminex flow analysis

Luminex bead maps, polygonal regions are drawn to identify and distinguish the 6 bead classes. On the chart showing the distribution of reporter intensities versus bead class, C194 (anti-MS2) shows increased median reporter fluorescence as expected – while C198 (rPE) shows an intermediate level of reporter fluorescence. It should be noted that the multiplex bead set reveals inherently large variability in the reporter fluorescence intensity for any given measurement. However, the median reporter fluorescence intensity (MFI) has been shown to be a suitable population statistic that is significant for measuring antigen concentration. An arbitrary threshold level of 100 counts was set in this particular instance to demonstrate a possible method of automating MIDS detection. We expect that separate thresholds will have to be determined, based on the binding characteristics of each capture antibody used in the multiplex assay.

Since we designed MIDS to be used in point-of-care diagnostic applications, we have developed a "real" multiplex microbead panel to assess respiratory viruses that may be contained in nasopharygeal swab samples. The panel is based on the commercially available Bartels direct fluorescence assay (DFA) kit, and includes specificity against 7 common viruses: Influenza types A and B, Parainfluenza types 1, 2, and 3, adenovirus, and respiratory syncytial virus (RSV) [30]. Since the DFA kit is fully manual and requires sequential testing for all 7 targets, the development of a multiplex-capable bead panel to be used in conjunction with either MIDS or Luminex would itself be considered an advancement in diagnostic tech-



Bead_	Assay	Conc	found	Positives	Negatives	Rep_mean	Rep_medn	Rep_sdev	Level
c194			44	30	14	157.2	137.0	124.8	100
c196									
c198			47	2	45	47.5	45.0	20.7	100
c200			56	1	55	22.5	18.5	22.6	100
c164			26	0	26	19.7	20.0	6.1	100
c166									
c168			47	0	47	26.9	25.0	10.4	100
c170			37	0	37	30.3	29.0	12.2	100
	No ID		54	17	37				100

Fig. 16. Simulant 6-plex results by MIDS assay

Table 2. Respiratory virus panel.

Target Antigen	Monoclonal Antibody	Bead Class
Influenza A	A1 (Chemicon)*	151
	A3 (Chemicon)*	158
	c102 (Adv ImmunoChem)	133
Influenza B	B2 (Chemicon)*	153
	22D5 (Chemicon)*	156
	4H7 (Adv ImmunoChem	129
RSV	133/1H (Chemicon)*	150
	131/2G (Chemicon)*	155
	130/12H (Chemicon)*	147
	8C5 (Adv ImmunoChem)	142
	9C5 (Adv ImmunoChem)	139
	8B10 (Adv ImmunoChem)	140

nology. Together with positive and negative control bead sets, the respiratory panel would constitute a minimum 10-plex assay. However, in practice, respiratory viruses such as influenza have multiple strains which may have different specificities and binding affinities with different monoclonal antibodies. As a result, it may be difficult (if not altogether unreasonable) to expect just one monoclonal antibody to efficiently bind *all* strains of a chosen viral species. For this reason, 12 different monoclonal antibodies already have been selected for use in detecting only three (Flu A, Flu B, and RSV) of the seven targeted species.

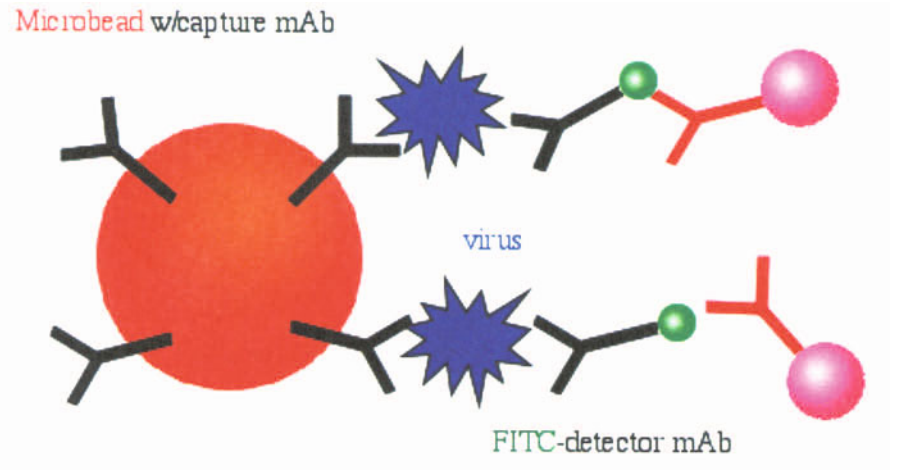


Fig. 17. Binding schematic for microbead-based respiratory viral assay

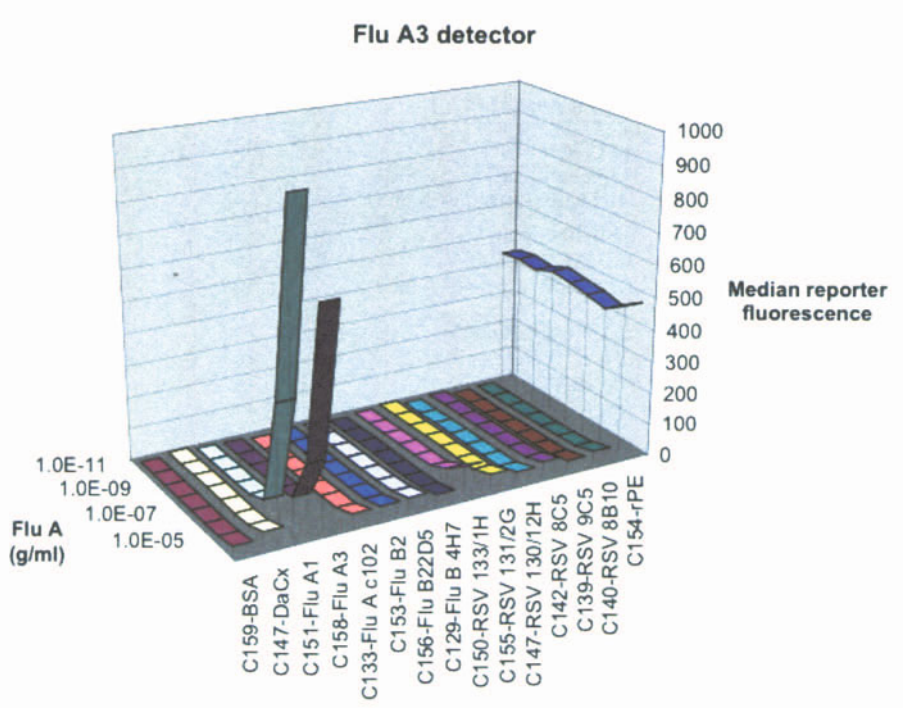


Fig. 18. Multiplex assay results by flow analysis. Representative titration curves show specific response by microbeads coated with anti-Flu A antibodies

Bead class 154 was directly coupled with R-phycoerythrin for use as a positive fluorescent control for the flow cytometer, while bead class 159 was coated with serum albumin (BSA) as a negative control bead to test non-specific reporter binding.

The charts shown below (Fig. 19) demonstrate bead capture efficiency as a function of antibody density on the microbead surface. The density is inferred by the concentration of capture antibody used when first derivatizing carboxylated Luminex microbeads. For each monoclonal antibody, 3 bead sets were derivatized with one of 3 antibody concentrations: high (1 mg/mL), medium (0.5 mg/mL) and low (0.1 mg/mL). Although the data suggests that antigen capture and binding efficiency are nearly saturated at even the lower antibody concentrations, we take advantage of the opportunity to demonstrate specificity and uniformity of response of each bead class for its respective target antigen.

4.5 Discussion

In summary, we have developed a benchtop optical system using conventional hardware currently available in our lab to demonstrate fluorescent imaging of two-dimensional microbead arrays. This setup is adequate to demonstrate fea-

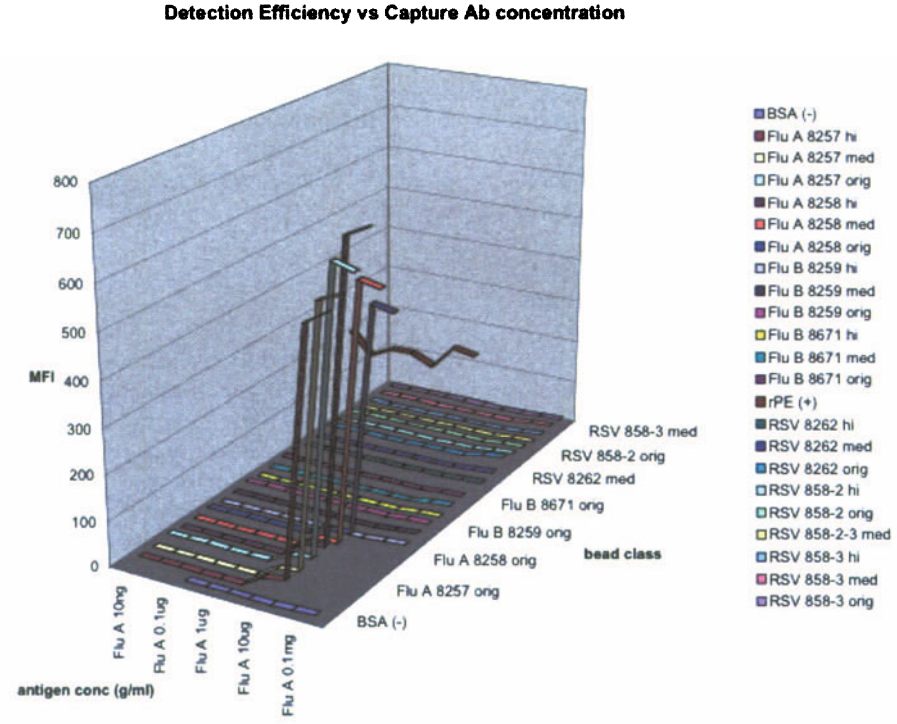


Fig. 19. Comparison of viral detection efficiency vs. capture antibody density

sibility, but components chosen specifically for this application will yield significantly better results and enable miniaturization of the entire system.

We have focused development efforts in three primary areas: sample preparation and bead arrays, optical imaging, and application development. We have successfully demonstrated bead deposition in an ordered array onto photoresist-coated glass substrates with binding efficiencies of >80%. We have also successfully captured beads in random planar arrays onto specialized filter substrates. Imaging bead arrays with a CCD camera, we have demonstrated fluorescence intensity based discrimination of up to 6 different bead classes. We are currently extending this bead decoding algorithm to allow simultaneous identification of up to 20 pathogens within a single test. Finally, we have been exploring suitable applications for MIDS such as infectious disease detection, cancer screening, and other biomarkers, with the intention of fully leveraging this unique technology.

Our preliminary studies strongly suggest that a rapid portable MIDS platform is a viable approach for point-of-care diagnostic applications. For a given microbead assay, the results achieved thus far with MIDS successfully approximate, if not match, the results obtained by conventional benchtop analysis with the Luminex flow cytometer. As the Luminex diagnostic system gains broader acceptance in clinical laboratories and is validated against other reference standards, we believe that the MIDS device concept will also gain acceptance. It should be emphasized that the objective of developing the MIDS platform is *not* to supplant the Luminex flow cytometric system – rather, MIDS will perform a critical role in point-of-care diagnostics and field medicine, where the Luminex flow analyzer cannot readily function. As an initial screening tool, MIDS must be able to detect the presence or absence of microbial pathogens in a clinical sample. More importantly, it must be able to correctly rule-out cases that do not require further work-up. For such an application, we are confident that the technology in MIDS will provide satisfactory results.

5 Conclusions and Future Directions

Not only for infectious disease applications, but for broader clinical diagnostic and biomedical research applications – multiplex biodetection using microarray technology will continue to evolve and advance. Currently the Luminex microsphere-based diagnostic system is the most mature technology applicable to the liquid array format. However, the method of optically-encoding beads by discretizing fluorescence intensity is better suited for analysis by flow-based (rather than image-based) cytometry, because of the greater inherent variability in accurately measuring fluorescence intensity across a 2-dimensional field. Flow cytometers bypass this issue by analyzing arrays which may be considered zero-dimensional, since the relative orientation of microbead and detector is fixed in space.

We will investigate alternative methods of designing multiplex-capable optical probes. Some alternative bead-based technologies include varying bead size or bead material (i.e., polystyrene, silica, etc.), and incorporating ferromagnetic

particles into the bead material to enable new methods of manipulating beads in a microarray. We will also follow developments of Quantum Dot technology (spectral coding) and NanoBarCodes (pattern coding) and other multiplex methods for appropriate insertion into the MIDS platform should they prove to have significant advantages. We are, however, encouraged that the rationale for the basic components of the MIDS concept remains essentially valid for all types of multiplex optical probes. That is, regardless of the particle-labeling technology, samples need to be introduced to the instrument, mixed with particles and other reagents, and optically measured. We believe that this new paradigm will play a critical role in guiding the future development of optical biosensors .

References

1. WHO (2002) Communicable Disease Surveillance and Response. World Health Organization Regional Office for Europe, Copenhagen
2. Noah D, Fidas G (2000) The Global Infectious Disease Threat and Its Implications for the United States. Gordon DF, Ed. National Intelligence Council, Washington, DC
3. Anderson RA (2001) Deaths: Leading Causes for 1999. National Vital Statistics Reports, Vol. 49, No. 11. CDC
4. Simonsen L, Fukuda K, Schonberger LB, Cox NJ (2000) J Infect Dis 181: 831
5. Ghendon Y (1992) World Health Stat Q 45: 306
6. Eldad A (2002) Harefuah 141: 21
7. Binder S, Levitt AM (2002) Emerging Infectious Diseases: A Strategy for the 21st Century. Centers for Disease Control
8. Meltzer MI, Cox NJ, Fukuda K (1999) Emerg Infect Dis 5: 659
9. Snacken R, Kendal AP, Haaheim LR, Wood JM (1999) Emerg Infect Dis 5: 195
10. Fong WK, Modrusan Z, McNevin JP, Marostenmaki J, Zin B, Bekkaoui F (2000) J Clin Microbiol 38: 2525
11. Brundtland GH (2000) World Health Organization Report on Infectious Diseases 2000
12. Uphoff H, Metzger C (2002) Dtsch Med Wochenschr 127: 1096
13. Goldmann DA (2001) Emerg Infect Dis 7: 249
14. Greenberg SB (2002) Curr Opin Pulm Med 8: 201
15. Lieberman D, Shvartzman P, Korsonsky I, Lieberman D (2001) Br J Gen Pract 51: 998
16. Franz DR, Zajtchuk R (2002) Dis Mon 48: 493
17. Gensheimer KF, Fukuda K, Brammer L, Cox N, Patriarca PA, Strikes RA (2002) Vaccine 20: S63
18. Nadder TS, Langley MR (2001) Clin Lab Sci 14: 252
19. Petersen K, McMillan W (2002) IVD Technology
20. Tang YW, Procop GW, Persing DH (1997) Clin Chem 43: 2021
21. McElhaney JE, Gravenstein S, Krause P, Hooton JW, Upshaw CM, Drinka P (1998) Clin Diagn Lab Immunol 5: 840
22. al-Nakib W, Dearden CJ, Tyrrell DA (1989) J Med Virol 29: 268
23. Mazzola LT, Fodor SP (1995) Biophys J 68: 1653
24. Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ (1999) Nat Genet 21: 20
25. Gabig M, Wegrzyn G (2001) Acta Biochim Pol 48: 615
26. Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991) Science 251: 767
27. Vignali DA (2000) J Immunol Methods 243: 243
28. Schaertl S, Meyer-Almes FJ (2001) Expert Rev Mol Diagn 1: 456
29. Pickering JW, Martins TB, Schroder MC, Hill HR (2002) Clin Diagn Lab Immunol 9: 872
30. Irmen KE, Kelleher JJ (2000) Clin Diagn Lab Immunol 7: 396