

## Workshop Report

# Report From a Workshop on Multianalyte Microsphere Assays

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Multiplexed assays using fluorescent microspheres is an exciting technique that has been gaining popularity among researchers, particularly those in the public health field. Part of its popularity is due to its flexibility, as both immunoassays and oligonucleotide hybridization assays can be developed on this platform. This report summarizes a workshop held by the Centers for Disease Control and Prevention that discussed issues surrounding these assays and the Luminex 100 xMAP instrument. Topics included instrumentation, assay design, sample matrix and volume, quality control, and development of commercial applications. *Cytometry (Clin. Cytometry)* 50:239–242, 2002. Published 2002 Wiley-Liss, Inc.<sup>†</sup>

**Key terms:** Luminex; microbeads; suspension array assays

The versatility and potential for multiplexing provided by microsphere suspension arrays have attracted the attention of public health laboratories. A number of public health scientists have been exploring the newest multiplexing platform available for assays, the Luminex 100 xMAP (formerly called LabMAP, i.e., laboratory multiple analyte profiling). On April 2, 2001, the Centers for Disease Control and Prevention (CDC) held the “Multi-Analyte Fluorescent Bead Assay Workshop,” a forum in which scientists from public health, academics, and industry could share their knowledge and experiences with the Luminex 100 system.

The Luminex 100 is a flow cytometer that employs two lasers, a 635-nm diode laser to excite the red and infrared classifier fluorochromes and a 523-nm frequency doubling Nd-YAG laser to excite the orange reporter fluorochrome, phycoerythrin (PE). One hundred bead sets can be detected because each set is impregnated with a unique ratio of red and infrared dyes. Each bead set has a unique distribution of fluorescent intensities that can be resolved by the instrument. Antibodies, antigens, or oligonucleotide primers are coupled to the beads to create an immunoassay or an oligonucleotide hybridization assay, respec-

tively. The secondary antibody or polymerase chain reaction (PCR) product is biotinylated and streptavidin-PE is used as the reporter. Theoretically, with 100 bead sets, 100 analytes can be detected in one sample.

During the recent period of rapid development for Luminex 100, users have not always been aware of each other's activities. Some investigators have depended ex-

Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the U.S. Department of Health and Human Services.

The authors regret to report the death of Dr. Rob Colinas (December 2000), a major force behind the development of the hemoglobin mutation analysis work using the Luminex technology reported here. We will miss Rob's intellectual contributions almost as much as his warm and outgoing personality.

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Received 1 December 2001; Accepted 18 June 2002

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/cyto.10140

clusively on home-brew methodologies, whereas others are using the commercial kits even though they have not been approved as in vitro diagnostic (IVD) devices by the U.S. Food and Drug Administration. This workshop was the first international meeting allowing laboratories to discuss their experiences with the Luminex 100.

During the five workshop sessions, participants discussed instrumentation, various aspects of assay design, sample matrix, sample volume, problems encountered, quality control issues, and development of commercial applications for both immunoassays and oligonucleotide hybridization assays. In this report, we highlight the issues and discussion points addressed during the workshop.

The first discussion centered on the advantages and limitations of the Luminex 100 instrument and how it differs from the traditional flow cytometer and its applications. The Luminex 100 is a small, compact, inexpensive flow cytometer that measures the fluorescence from microspheres or beads. It is smaller than a traditional bench-top cytometer, the result of the newly designed electronics module. The instrument uses a low-powered red diode laser and avalanche photodiodes for the two red light detectors, a compact optical detection system, a robotic sample injection system, and 14-bit digital pulse processing electronics. Some participants suggested that with the current digital pulse processor, the low end of the signal is no better than that provided by traditional electronics and any data in that region should be considered uncertain. However, when a 16-bit processor is incorporated into the system, reading at the lower end should improve. Also, the filter configuration for PE is not optimal for ultra low-level fluorescence detection. The optical filters can be changed but optional filters are not currently available from Luminex Corporation (Austin, TX).

Minimal training is required to operate the Luminex 100 because the software almost completely controls the instrument and maintenance is not complex. However, although the instrument is small and compact, it is not designed for portability. Consequently, in its current configuration, it is not practical for field applications.

Workshop participants discussed issues related to assay design. Although all the problems associated with immunoassays in general exist, some are unique to the microsphere format. Variables for any immunoassay include determining which monoclonal or polyclonal antibodies are optimal, the affinity of the antibody for the analyte, the length of the incubation times, and the necessity of washes for the assay. Aspects of the assays that are unique to the bead format include the morphologic features of the bead, the fluorescent spectral characteristics of the chosen bead sets, the optimal antibody concentration for coating beads, and the surface chemistry of the bead. These variables must be determined to optimize the sensitivity and specificity of the assay.

Assay development has occurred independently in a number of laboratories, so there are no uniform procedures for assay development and verification. Comparisons have been made with results from commercially

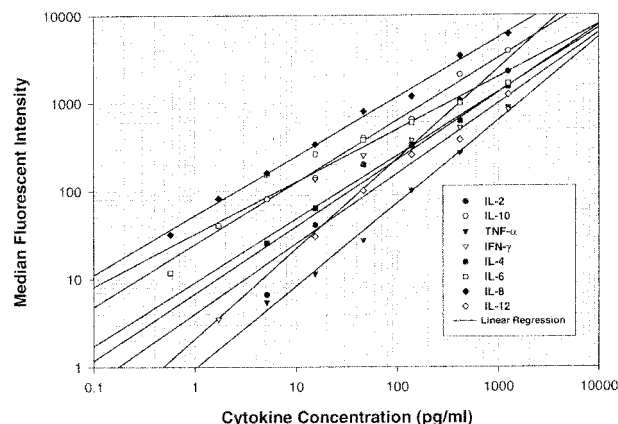


Fig. 1 Standard curves for interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, IL-12, interferon- $\alpha$ , and tumor necrosis factor- $\alpha$  were generated in the multiplexed fluorescent bead immunoassays. Fluorescent intensity was measured on a Luminex 100 with Luminex Firmware 1.7.

available methods but, lacking true reference methods, not all scientists find these comparisons convincing. For example, a number of proteins in serum can interfere with antibody-based immunoassays and skew results, such as soluble receptors, heterophilic antibodies, and serum-binding proteins. These substances and other potential sources of assay interference need to be thoroughly evaluated before any firm conclusions can be reached about the validity of results generated by solid-phase immunoassays whether they are microtiter wells or microspheres. No consensus can be reached about validating the multiplexed assays until these more common complications are resolved.

To illustrate the issues mentioned above, the development and implementation of an eight-plex sandwich immunoassay for human cytokines was presented (1). Initial data were generated with Luminex FlowMetrix beads and software using the FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer (this platform has been discontinued). These multiplexed assays were transferred easily to the Luminex 100 instrument and microspheres with comparable calibration curves and no change in sensitivity (Fig. 1). Cytokine-specific antibody pairs were used as the capture and biotinylated detection antibodies with streptavidin Alexa 488 (FACSCalibur) or PE (Luminex 100) as the reporter. Different antibody pairs were prescreened for their performance with human serum and the assay conditions were optimized for each cytokine. When the assays were multiplexed, false-positive signals were often observed with human sera. These signals resulted from heterophilic or human antianimal antibodies interacting with the capture and detection antibodies and simulating the binding of cytokines. A cocktail of animal sera, similar to the blocking agents used for enzyme-linked immunosorbent assays (ELISAs), was used to block the false positives. The sensitivity of the standard curves for the multiplexed assay did not decrease when the blocking agents were added. To validate the assays, results from the multiplexed

eight-cytokine bead assay and commercial ELISAs that used the same antibody pairs were compared. Correlation between these assay formats for cell culture supernatant samples was 0.974 ( $n = 18$ ) and for human sera, 0.857 ( $n = 28$ ). Sera samples from patients with autoimmune diseases had interfering substances that were not eliminated effectively by the animal sera cocktail, so additional development is needed to make a reliable assay for this type of sample.

The ratio between the biotin-conjugated secondary antibody and streptavidin-PE was crucial to the performance of the bead-based assays, particularly in the no-wash format used with serum samples. For most analytes, a ratio of 1:5 to 1:7.5 total nanograms of biotinylated antibodies to nanograms of streptavidin-PE worked well. However, for some combinations of biotinylated antibodies, a ratio of 1:2.5 was effective (1). Titrating these reagents was necessary for each combination of antibodies. To multiplex many individual assays together, the reading for the highest concentration of the analyte for the standard curves was kept within a range of 3,000–5,000 median fluorescent intensity (MFI) units, so that the detection limits of the photomultipliers were not overwhelmed. The upper limits of the curves were adjusted by changing the concentrations of the biotinylated antibodies.

Development of assays for Dried Blood Spot (DBS) samples instead of serum involves further considerations. Unlike most immunoassays that use 10–50  $\mu$ l of serum, a 3 mm DBS punch contains approximately 1.5  $\mu$ l of serum. In addition to the small amount of serum, matrix effects and filter paper fibers also must be considered. Despite these difficulties, multiplex assays using DBS punches have been developed.

The New York State Department of Health, Division of Genetic Disorders Laboratory has developed a multiplexed assay for thyroid-stimulating hormone (TSH) and thyroxine (T<sub>4</sub>; 2). The TSH and T<sub>4</sub> multiplex illustrates the flexibility of the Luminex system because TSH is a sandwich assay and T<sub>4</sub> is a competition assay. Initially, filter paper fibers clogged the system. To minimize this, a filtration step was added to the assay and Luminex Corporation changed the design of the uptake needle to make it more difficult for debris to be introduced into the instrument. Subsequently, the multiplexing has been expanded to include a 17-hydroxyprogesterone (17-OHP) competitive assay. As expected, the assay conditions were altered to account for analyte characteristics. At the time of the workshop, the three-plex assay was working but needed to be optimized.

A human immunodeficiency virus (HIV) protein three-plex assay was developed recently (3). During development of the assay, different amounts of blocking agents had to be tested to optimize the blocking agent and its concentration for the multiplexed assay. Overall, many of the considerations for designing multiplex assays are the same for small and large sample sizes.

Further discussion centered on designing oligonucleotide hybridization assays using dried blood spots. Specifically, an assay to detect point mutations in the hemoglo-

bin beta chain was presented. A published report described the assay to detect HbA (normal), HbS (sickle cell), and HbE (variant; 4). The assay was designed originally to detect the HbC variant as well, but HbC could not be identified. Different primers and PCR product sizes were used, but the changes did not influence the result. Steric hindrance may be the cause as the HbC point mutation changes the first nucleotide of codon 6 and the HbS point mutation changes the second nucleotide of codon 6. Also, coupling oligonucleotides to the microspheres was successful only when reagents were prepared just before the reaction. When reagents were prepared 24 h before the coupling, the target oligonucleotide did not couple to the microsphere.

The workshop participants discussed suspension array assays in comparison to microarrays. The advantages of suspension arrays include a lower signal-to-noise ratio and greater throughput. The initial investment required for suspension arrays is much lower than that required for a complete microarray facility. If a problem occurs, unacceptable microsphere sets may be discarded instead of a complete microarray, allowing more flexibility at a lower cost.

Workshop participants also discussed filter bottom plates and background fluorescence. For assaying a large number of samples that require washing, microtiter plates with a filter bottom are recommended. Many users found that up to 50% of the microspheres are lost during washing, which may be attributed to microspheres lodging within the porous bottom of the plate when the vacuum is too strong. Selecting the ideal vacuum source and porous bottom microtiter plate eliminates this problem. Background fluorescence may increase as more bead sets are added to an assay. In general, the Luminex 100 handles background fluorescence better than the original FlowMetric system. However, a 100-plex may not work in some cases.

A number of companies have licensed Luminex technology and are marketing, or are planning to market, kits for research applications. These kit manufacturers must also address reliability, precision, and accuracy. Manufacturers can address reliability and accuracy by considering the rules followed by clinical laboratories, such as the Clinical Laboratory Improvement Amendments (CLIA), and by building in quality control and quality assurance upfront. Quality control and quality assurance for diagnostic applications are especially important with multiplexing because more variables are involved than with a single analyte assay. Kit producers also must consider cost. If the cost of the kit is restrictive, many users will be forced to find alternative technology.

Instrument validation, calibration curves, and a uniform documentation of performance characteristics are also important for manufacturers and laboratories. A common graphic display using a standardized fluorescence intensity scale better ensures the quality of the data and allows for comparison between kits and instruments. These goals are addressed in forthcoming NCCLS (formerly, the Na-

tional Committee for Clinical Laboratory Standards) guidelines (5).

Fluorescence-based multiplexing is an exciting technology with a variety of applications. The ability to use small sample sizes and to perform both immunoassays and oligonucleotide hybridization assays is especially appealing. The use of microspheres as the solid phase of receptor-ligand assays offers significant advantages when they are analyzed by cytometric methods. The fluorescence signal provides sensitive detection as low as a few hundred molecules per microsphere and the event-based detection provides inherent separation of bound and free ligand without any washing steps. These attributes allow the development of homogenous binding assays with attomolar sensitivity.

The discussion at the workshop stimulated many ideas about optimizing current applications and finding new ones. We hope this workshop accelerated further development and improvement in suspension array technology

and that subsequent workshops will enhance the future of such systems in biomedical science and public health.

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