The Use of Luminex Assays to Measure Cytokines

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INTRODUCTION

Cytokines are small, soluble peptides or proteins that are produced by a variety of cells that participate in the immune response, including T cells, B cells, macrophages, mast cells, fibroblasts, and stromal cells, and are important immune mediators that constitute the communication network of the immune system. Cytokines regulate the responsiveness and maturation of particular cell populations, modulate the balance between cellular and humoral immune responses, and are important determinants of health and disease (O'Shea and Murray, 2008). The cytokine milieu provides important insights into physiological and pathological processes and can potentially serve as biomarkers for various diseases (Paczesny *et al*., 2009; Suh and Kim, 2008). Therefore, determining the relative balance of cytokines is important in elucidating the biological pathways that lead to inflammation, sepsis, and disease because dysregulation of these complex signaling pathways is often the earliest marker of a pathologic response (Bozza *et al*., 2007; Chun *et al*., 2007).

In recent years, the Luminex assay has become an important tool in cytokine detection and quantification because of its capacity to measure multiple different cytokines simultaneously in a single run of the assay with small sample size requirements. Thus, this technology enables patterns of numerous cytokines to be examined within and between experiments, which provides a more inclusive and comprehensive depiction of disease than measurement of individual cytokines—an approach that was not previously possible using conventional technologies such as ELISA (Tighe *et al*., 2013).

ADVANTAGES

- Multiplexing 100 tests in a single well of a 96-well plate provides higher density and higher throughput in less time.
- Requires significantly smaller sample volumes than traditional bioassays.
- Results are as accurate as an ELISA and more reproducible than a microarray.
- Customization of specific probes, including antibodies, receptors, peptides, and oligonucleotides, specific to a user's needs.

LIMITATIONS

- Requires dedicated analysis instruments, which creates high upfront installation costs.
- Analyses are subject to variability because of assay manufacturer, product lot number, and assay execution.
- The presence of human autoantibodies in some biological samples may confound the measurement of cytokines in clinical assays because of nonspecific binding.

GENERAL PRINCIPLES AND TECHNIQUE

Luminex assays enable fast and accurate measurements of cytokines by utilizing hundreds of specially prepared micrometer-scale plastic beads (referred to as microspheres), which are internally dyed with a graded mixture of red or infrared fluorescent dyes (Figure 1A). Varying the degree to which the beads are internally dyed creates hundreds of different fluorescent profiles that can be individually interrogated and classified in a single sample (Tighe *et al*., 2013).

Excitation of each of the unique microspheres by a laser, using modified flow cytometry–based instruments, causes them to emit light at different wavelengths. Microspheres of a single identity are conjugated on their surface with a specific capture antibody for a desired cytokine, which can be differentiated from other microsphere sets by its unique spectral address (Figure 1B) (Dupont *et al*., 2005).

The biological sample of interest is then combined with a mixture of microsphere sets—each set specific for a unique cytokine—and incubated in a 96-well plate (Figure 1C). Using a variety of microsphere sets combined within a single assay, it is possible to carry out up to 100 tests in a single well of a 96-well plate. The biological sample–microsphere mixture is then washed, and a cocktail of detection antibodies specific for the desired cytokines is added (Figure 1D). These detection antibodies are also conjugated with a reporter dye, which provides the microsphere with an additional distinct fluorescent emission signature when it binds the cytokine of interest (Tighe *et al*., 2013). Thus, it is possible to differentiate various cytokines in a given sample based on the color of

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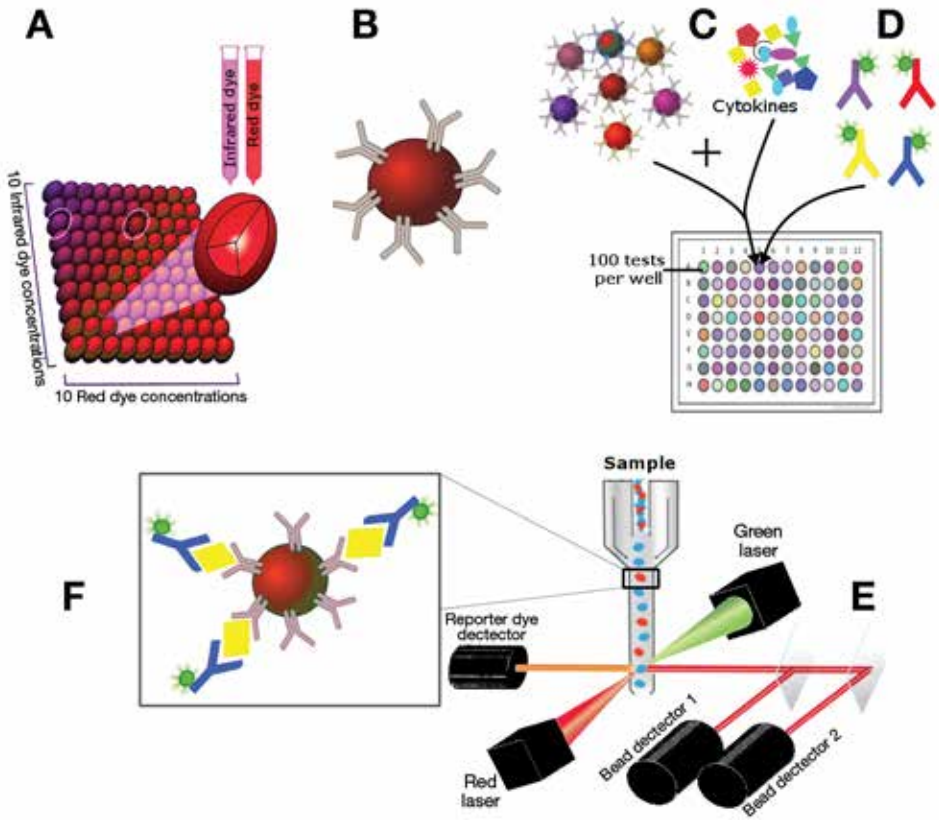

Figure 1. Luminex technique and general principles. (A) Plastic beads (100-μm scale) are internally dyed with a graded mixture of red and infrared fluorescent dyes, providing each microsphere with a unique fluorescent signature. (B) Microspheres of a single identity (i.e., the same fluorescent signature) are conjugated on their surface with a specific capture antibody for a desired cytokine. (C) A mixture of the 100 different microsphere sets is combined with the biological sample of interest in a 96-well plate, incubated, and then washed. (D) Next, a cocktail of detection antibodies conjugated with a reporter dye is added. Notably, a variety of detection antibodies are used, which are specific for different cytokines; however, the reporter dye is the same across all detection antibodies. (E) After another round of washing, the microspheres are analyzed as a single-bead suspension through a flow chamber similar to a flow cytometer. A red laser excites the internal red and infrared fluorescent dyes, which identifies and classifies the bead to one of the 100 microsphere sets. A green laser excites any bound detection antibody–reporter dye complex, and the intensity of this signal quantifies the relative amount of the cytokine bound to the microsphere. (F) Each microsphere traveling through the detection chamber thus has the following components: a capture antibody conjugated to its surface that is specific for a particular cytokine; a variable amount of a specific cytokine that is bound to the capture antibody, which is determined by the concentration of the cytokine in the original sample; and a detection antibody conjugated with a reporter dye that is also specific for the same particular cytokine.

the internally dyed beads themselves, while quantifying their relative abundance via measurement of the intensity of fluorescence of the reporter dye–conjugated detection antibody.

After another round of washing, the microsphere–cytokine–reporter compound is analyzed as a single-bead suspension through a flow chamber equipped with excitation lasers and electronic detection apparatus that measures the intensity of fluorescence of the microsphere–cytokine–reporter compound, similar to a flow cytometer (Figure 1E). Specifically, when a microsphere passes through the detection chamber, a red laser excites both the red and the infrared internal dyes inherent to each particular bead set, which allows the proper classification of the bead to 1 of the 100 microsphere sets. A green laser then excites any reporter dye associated with the binding of the cytokine of interest. Notably, the amount of reporter bound to the microsphere is dependent on the concentration of the particular cytokine in the original biological sample (Figure 1F). The emitted light from the dyes is measured and used for quantitative analysis of each microsphere–cytokine–reporter conjugate.

APPLICATIONS

Luminex assays have revolutionized our ability to rapidly assess the relative concentration of various soluble factors that are important biological mediators of health and/or disease. Many rheumatologic, inflammatory, infectious, and genetic conditions are modulated by the differential expression of anti- and proinflammatory cytokines, chemokines, and growth factors, and elucidation of these complex signaling pathways will likely augment our capacity for early diagnosis and drug discovery. For example, Luminex profiling of epidermal explants from patients with systemic sclerosis was recently utilized to identify increased levels of profibrotic CCN2 (a connective tissue growth factor) and proinflammatory protein S100A9

(Nikitorowicz-Buniak *et al*., 2014). These data suggest that the epidermis of these patients provides an important source of the profibrotic and proinflammatory factors that contribute to the fibrosis and inflammation seen in the disease, and CCN2 and S100A9 emerged as potential markers of disease severity and serve as possible targets for therapeutic intervention.

The use of Luminex assays for basic science discovery, preclinical/translational studies, and clinical trial immune monitoring has rapidly increased in recent years. In particular, Luminex assays have become widely utilized for biomarker analysis of small-volume samples. In solid-organ and vascularized composite allotransplantation (such as hand and face transplantation), for example, Luminex assays have been utilized to predict high-risk antibodies, positive complement-dependent cytotoxicity crossmatch, and immune monitoring of antibody-mediated rejection and inflammatory cytokines (Campbell, 2013; Schneeberger *et al*., 2013).

Although the focus of this article is on the use of Luminex assays to quantify relative cytokine expression patterns, the possible applications of this technique extend far beyond cytokines. Indeed, it is possible to conjugate the surface of microspheres with antibodies, receptors, peptides, and oligonucleotides, thereby facilitating performance of immunoassays, receptor–ligand assays, enzyme assays, and DNA assays, among others, which meets the needs of a wide variety of applications including protein expression profiling, gene expression profiling, molecular basis of infectious disease, and HLA testing. In recent years, multiplex PCR and Luminex technology have been used to identify high-risk mutations in cyclin-dependent kinase inhibitor 2A/cyclin-dependent kinase 4 (CDKN2A/CDK4) among genomic DNA from melanoma-prone families (Figure 2) (Lang *et al*., 2011). The Luminex assay quickly and accurately genotyped CDKN2A/CDK4 mutations in a large number of samples, demonstrating its utility as a sensitive, cost-effective, and reliable method for the screening of mutations associated with an increased risk of melanoma. The ability to analyze a variety of biological samples including tissue and cell lysate, saliva, sputum, serum, plasma, and urine adds to the versatility of the Luminex platform as a useful screening modality.

COMPARISON WITH ELISA AND MICROARRAY FOR CYTOKINE PROFILING

Luminex has several distinct advantages over similar bioassays, such as ELISA and microarray. Conceptually, the analysis of each microsphere is essentially an ELISA on a bead (Dupont *et al*., 2005). This enables multiplexing of up to 100 different analytes in a single well of a Luminex assay because each microsphere serves as an individual test, thereby providing higher throughput than ELISA. The three-dimensional structure and small surface area of the microspheres allow for

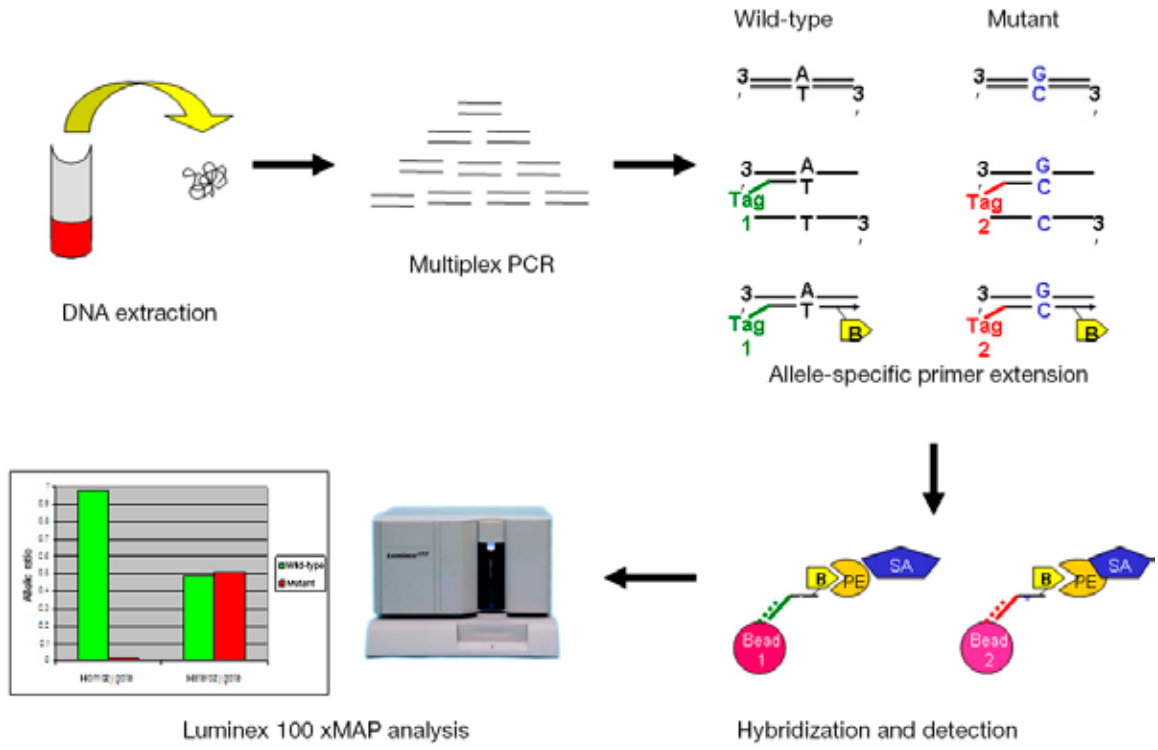

Figure 2. The use of multiplex PCR and Luminex to screen for genomic variation in familial melanoma. Regions of interest (e.g., CDKN2A) are initially amplified by multiplex PCR. Individual probes with the interrogative terminal codon are linked to a unique 24-mer Tag sequence and subjected to allele-specific primer extension (ASPE). The ASPE extension step uses biotinylated deoxycytidine triphosphates, thereby introducing a biotin-labeled moiety into the extended fragment. The 24-mer Tag/biotinylated ASPE fragment is then specifically captured by a color-coded bead adorned with an anti-Tag, which fully complements the 24-mer Tag sequence. Streptavidin-conjugated phycoerythrin (SA-PE) is then introduced to detect an intensity signal. In summary, the bead color defines the address—i.e., the individual single-nucleotide polymorphism (SNP)—and the PE fluorescence intensity defines the amount of the specific SNP fragment. Reprinted with permission from Lang *et al*. (2011).

faster reaction kinetics and shorter assay times than ELISAs and microarrays, which are limited by solid-phase kinetics. Luminex assays also offer the flexibility to customize and attach specific probes to uniquely colored microspheres, easily expanding and readily adapting to specific mutations in specific populations as they are identified, which has important implications for its use as a screening tool. Notably, Luminex requires small sample volumes of only 50 µl per well, greatly diminishing the sample volume required compared to ELISA. Thus, more data are generated from the same sample volume.

Luminex is unique in that it offers both high-density (multiplexing 100 tests per sample) and high-throughput (can test up to 1,000 samples per day) simultaneously, with results that are highly reproducible, reliable, and as accurate as an ELISA. Although ELISA and real-time PCR have the capacity for high throughput (i.e., it is possible to analyze hundreds of samples per day), they are unable to multiplex more than a few tests at a time. Microarrays, on the other hand, provide high-density screening of several hundred tests at a time, but are limited by their lack of reproducibility needed for high-throughput analyses.

However, several factors may limit the utility and availability of Luminex. First, the Luminex platform requires dedicated analysis instruments that are similar to flow cytometers, which therefore increases the upfront costs associated with this system. Development of newer light-emitting diode–based systems, as opposed to laser-based units, will likely significantly decrease the initial cost to utilize this assay. Luminex assays are also subject to variability because of assay manufacturer, product lot number, and assay execution (Lynch *et al.*, 2014); however, using the same manufacturer in a given assay and maintaining consistent methodology can minimize variability. Additionally, the presence of human autoantibodies in some biological samples may confound the measurement of cytokines in clinical assays as a result of nonspecific binding. Fortunately, several methods are available to remove these autoantibodies while maintaining the integrity of the resultant cytokine profile (de Jager *et al.*, 2005).

CONCLUSION

Luminex is an important tool in the interrogation of the cytokine milieu in physiologic and pathologic processes because it enables patterns of cytokines to be evaluated, thereby providing a more comprehensive depiction of the complex cell signaling network than was previously possible. It provides more robust data delivery with higher throughput and density than traditional methods, but with accuracy comparable to that of ELISA. Luminex requires significantly lower sample volumes and shorter assay times than ELISA, and it can be utilized for a variety of applications beyond cytokine profiling, including protein and gene expression profiling, biomarker identification, and drug discovery. New light-emitting diode–based Luminex instruments offer the promise of reduced installation costs and, therefore, increased availability to potential users.

QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

For each question, more than one answer may be correct.

1. How many distinct cytokines or analytes can be interrogated in a single well of a 96-well plate using Luminex?

A. 1.
B. 5.
C. 50.
D. 100.
E. 250.

2. What type(s) of biological sample(s) can be analyzed using Luminex?

A. Tissue and cell lysate.
B. Saliva.
C. Sputum.
D. Serum.
E. Plasma.
F. Urine.
G. All of the above.

3. Which of the following is *not* true regarding the Luminex platform versus other comparable bioassays?

A. It has the capacity for high-throughput and high-density data delivery with excellent accuracy compared to alternative methods.
B. Luminex requires the use of dedicated analysis instruments similar to flow cytometers.
C. Luminex and ELISA require similar sample volumes for analysis.
D. The three-dimensional structure and small surface area of Luminex microspheres accelerates the reaction kinetics and decreases total assay time.

4. Which of the following compounds can be conjugated to the surface of the Luminex microsphere?

A. Antibodies.
B. Receptors.
C. Peptides.
D. Oligonucleotides.
E. All of the above.
F. None of the above.

5. How does the Luminex system differentiate among various cytokines?

A. A reporter dye is conjugated to a detection antibody that is subsequently excited by the green laser of the Luminex system.
B. Each microsphere set is internally dyed with a graded mixture of red and infrared fluorescent dyes, and excitation of the bead by the red laser emits a fluorescent signature that is unique to each cytokine.
C. Only one cytokine can be tested per well and the user must decide which cytokine to test for in advance and input this information into the analysis software.
D. The fluorescent signal emitted from the internally dyed microsphere and the externally conjugated detection antibody are combined to identify the particular cytokine of interest.

CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

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To take the online quiz, follow the link below:
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SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2015.36.

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