Simultaneous Quantification of Six Human Cytokines in a Single Sample Using Microparticle-based Flow Cytometric Technology, Roy Chen,1* Larry Lowe,1 Jerry D. Wilson,2 Eric Crowther,1 Kifle Tzeggai,1 Jim E. Bishop,1 and Rudi Varro1 (1 Becton Dickinson Immunocytometry Systems, 2350 Qume Dr., San Jose, CA 95131-1807, and 2 Pharmingen, San Diego, CA 92121; * author for correspondence: fax 408-954-2156, e-mail roy_chen@bdis.com)

The study of cytokines has been of great interest to researchers in many scientific disciplines, including cell biology and immunology. Cytokines form a sophisticated network that serves to modulate a myriad of cellular events. Within such a network, and through complex feedback mechanisms, cytokine functions are mostly interdependent. Because of the extreme complexity of the cytokine network, a simultaneous measurement of multiple cytokines in a single sample represents a desirable and effective approach.

Several methods are available to measure cytokines and their messenger RNAs. To measure cytokines secreted from cells, researchers commonly use conventional ELISA techniques. ELISA methods are generally cost-effective but restricted to measuring one cytokine at a time. Consequently, the ELISA approach requires not only multiple sample aliquots but also repetitive execution of the procedures for each cytokine of interest. The ability of flow cytometry to simultaneously acquire data for numerous particles and subsequently analyze multiple characteristics for each particle makes it a powerful technique for cell sorting and cell analyses. The application is fundamentally built on the high sensitivity of a flow cytometer to discern characteristics either within or on the surface of a cell. Several investigators have constructed microparticle-based immunoassays that use a flow cytometer to simultaneously measuring multiple analytes (1–5).

This report describes the development of a uniform size microparticle-based flow cytometric method for a panel of six human cytokines—interleukin (IL)-2, IL-4, interferon-γ, tumor necrosis factor-α, IL-10, and IL-12—simultaneously measured in a single sample. The sample types included cell culture supernatant and human serum. The assays are two-site “sandwich” immunoassays configured with six pairs of antibodies, two fluorescent dyes, and particles of a uniform size. Particles (7.5 μm) are dyed to six different fluorescence intensities. The dye has an emission wavelength of ~650 nm (FL3). Each particle population of a given intensity represents a discrete population for constructing an immunoassay for a single cytokine. Each particle is covalently coupled with an antibody (Ab) against one of the six cytokines. These Ab-particles, which are unique in their FL3 intensity, serve as a capture for a given cytokine in the immunoassay panel. When these Ab-particles are used as a mixture, one can simultaneously detect six separate cytokines. We configured the assay for each cytokine in each of the two formats below.

Indirect format. The biotin-conjugated “detector” Ab is used to complete the particle-Ab-cytokine-Ab sandwich. Fluorescent dye-conjugated streptavidin is used to report the amount of bound secondary Ab. The fluorescent dye emits at ~530 nm (FL1). The fluorescence intensity measured at FL1 is proportional to the concentration of the cytokines and is quantified from a calibration curve. Direct format. The fluorescent dye is phycoerythrin (PE) which emits at ~585 nm (FL2). A PE-conjugated detector Ab is used to complete the sandwich, and the fluorescence intensity measured at FL2 is proportional to the concentration of the cytokine in the sample, which is quantified from a calibration curve. An important feature of the assay system is that the calibrators, Ab-bead reagent and the second Ab reagent are each made as mixtures for all six cytokines. Therefore, six calibration curves can be obtained from one set of calibrators and six results can be obtained on one test sample.

We used six pairs of antibody to construct the sandwich assay for the six cytokines. The antibody used for capture and the second Ab labeled with biotin (used in the indirect...
asay) or PE (used in the direct assay) are available from PharMingen in San Diego, CA. Polystyrene beads (7.5 μm) functionalized with amino groups (Bang’s Laboratories) were dyed with a fluorescent dye to six discrete intensities. The maximal emission wavelength of the dye is 650 nm (FL3). The bead population with each intensity was used to prepare a given antibody-bead for each of the six cytokines of interest. The Ab-beads were prepared via a covalent linkage based on thiol-maleimide chemistry. Covalent chemistry afforded advantages such as higher assay sensitivity and better storage stability. A FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) was used to generate the data presented here. ELISA assays and reagents kits for the six cytokines of interest (PharMingen) were used as recommended for the method-comparison study.

The Indirect assay used three incubations. Ab-bead reagent (50 μL) was added to 50 μL of sample or calibrator and incubated for 30 min, followed by addition of 50 μL of Ab-biotin reagent and a further incubation for 15 min. The final incubation step involved the addition of 50 μL of streptavidin-Alexa (FL1) and a third incubation for 75 min before data acquisition was performed by a flow cytometer.

The Direct assay included one incubation. A 100-μL mixture of 50 μL each of Ab-bead reagent and Ab-PE reagent for the cytokines was added to 50 μL of sample or calibrator. The 150-μL mixture was subsequently incubated for 120 min before data acquisition with the flow cytometer.

Peripheral blood mononuclear cell samples from individual donors were cultured in RPMI 1640 either with or without lipopolysaccharide stimulation. Aliquots of the individual samples were tested with both the flow cytometric and ELISA methods. In the flow cytometric method, one 50-μL sample was sufficient to provide six results vs 600 μL for ELISA. The bead reagent contained a mixture of Ab-beads for six cytokines. The Ab-PE or Ab biotin reagent contained a mixture of the second Ab components for six cytokines. Each calibrator contained a mixture of equal weights of the six recombinant cytokines; the concentrations of the cytokines in the calibrators were 0, 20, 80, 625, and 2500 ng/L, respectively.

The preliminary performance evaluation of the assays configured in the Indirect format is summarized in Table 1. Similar results were obtained from the Direct assays.

In summary, this microparticle-based flow cytometric method is an efficient method for simultaneously measuring multiple cytokines from a variety of test samples. It has comparable analytical sensitivity and a wider dynamic range than conventional ELISA.

### References


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a Detection limit is defined as a signal 2 SD above the mean of the zero calibrator.
b Intraassay CVs were determined by testing six samples of each of two calibrators (containing all six cytokines) at 625 and 1250 ng/L, respectively.
c Cell culture supernatants were used for comparison with the ELISA method as described in the text.
d IFN, interferon; TNF, tumor necrosis factor.