# The power of multiplexing

The purpose of this paper is to address questions relevant to researchers who are familiar with enzyme-linked immunosorbent assay (ELISA) technology but are considering a multiplex solution such as Invitrogen multiplex assays on the Luminex™ platform to measure multiple proteins simultaneously in one well.

**FAQs:**

1. **What is multiplexing?**
2. **When should I consider multiplex assays on the Luminex platform over ELISA?**
3. **Will Invitrogen multiplex assays give me the same results for each analyte as my current ELISA tests?**
4. **How much do multiplex assays cost, and how does the cost compare to ELISAs?**
5. **How difficult is it to learn to perform Invitrogen multiplex assays compared to ELISAs?**

To enable researchers to interrogate important biological proteins, well-established gold-standard techniques such as western blotting are useful for qualitative detection while ELISA allows for quantitative measurements. ELISA is a common and widely used method to perform sample analysis and can accurately detect and quantify individual proteins with high specificity and sensitivity. The analysis of selected proteins in, for example, serum, plasma, or cell lysates is key to the study of inflammation, metabolism, cell signaling, cancer, cardiovascular disease, toxicology, neuroscience, and many other research and clinical areas. The ever-growing list of cytokines, chemokines, and other growth and differentiation factors, as well as their complex interactive networks in health and disease, has made it crucial to evaluate them in relevant groups rather than individually. Often, for example, the levels of cytokines or chemokines within a pathway in relation to each other are far more relevant than their presence or absence, or than the absolute levels of individual proteins.

## Question 1: What is multiplexing?

### Multiplex assays for protein quantitation

Several antibody-based assay platforms have been developed as alternatives to ELISA for the simultaneous measurement of multiple proteins in a single sample. The most common and well-established format for such multiplex assays makes use of flow-based technology and ligand (e.g., antibody)-coated beads. Luminex™ systems are based on xMAP™ (multi-analyte profiling) technology combined with single and multiplex bead–based immunoassays. The beads used in xMAP™ immunoassays are dyed with different concentrations of fluorophores to generate bead sets that can be easily discriminated. Individual bead sets are coated with a capture antibody qualified for one specific analyte. The captured analyte from a sample is detected using an analyte-specific biotinylated antibody that binds to the appropriate epitope of the immobilized analyte, plus streptavidin-conjugated R-phycoerythrin (S-RPE) (Figure 1). For detection of the immunoassay sandwich complex, Luminex™ instruments use either light-emitting diodes (LEDs) for excitation of each fluorescent bead combined with a CCD camera for bead and analyte detection, or a flow-based detection system using a red and green laser. High-speed digital signal processors are used to interrogate the data (Figure 2). As each antibody-coated bead is individually identifiable for a specific analyte, multiple beads can be combined to simultaneously measure the levels of up to 500 targets for nucleic acid and typically no more than 50 targets for proteins due to biological interference in a single sample.

Luminex™ xMAP™ assays have increasingly been adopted for sample analysis, and over 7,000 scientific publications are available demonstrating high precision and reproducible results. These studies have covered a broad range of common applications, including:

- Measurement of soluble analytes in serum, plasma, urine, CSF, lavage fluids, and other sample types from human, monkey, mouse, rat, swine, and other species (xMAP assays are also used routinely for cell culture supernatants)
- Basic research—*in vitro* and *in vivo* studies
- Preclinical studies—*in vitro* and *in vivo* models
- Screening batches of sera for the presence/absence of defined markers or mediators
- Quantitative confirmation of data for targets identified from proteomic or genomic analysis

**Question 2: When should I consider utilizing multiplex assays on the Luminex platform over ELISA?**

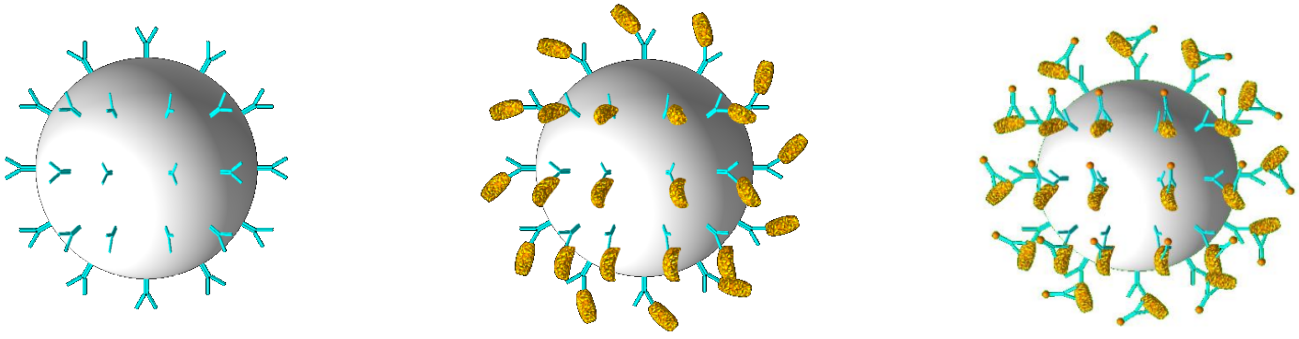
ELISA is a simple and powerful way to quantify specific individual proteins in complex samples. The selectivity of ELISA is achieved through the use of qualified single- or double-antibody sandwich technology, and accurate quantitation is achieved through the use of calibrated standards. ELISAs are performed in a 96-well format with results typically ready after ~4 hours of incubation time. The results obtained with ELISAs are generally reproducible and consistent with biologically relevant sensitivity levels and dynamic ranges.

While ELISA is the gold-standard method of protein analysis, multiplexing methods that enable the measurement of multiple analytes simultaneously in a single sample address a number of specific limitations:

- ELISA allows for the measurement of only one analyte at a time in a given sample, limiting investigators' increasing need to measure multiple targets for a holistic biological understanding of protein interactions.
- The limited sample volume being studied may limit the number of times analyses can be conducted. This is especially true in small animal research, in pediatric testing, and in microplate assays providing limited sample volume. The ability to assay multiple analytes in a single small-volume sample enables more effective use of each sample.
- Difficulties in data interpretation can arise when comparing analyte levels measured by multiple ELISAs, each assay having been performed with different sample aliquots and each subject to systematic errors leading to decreased precision and accuracy.
- Many analytes require assays with broad dynamic ranges to avoid repeat testing or out-of-range values. Multiplex assays can be designed to have large dynamic ranges for all of the analytes, or ranges tailored to various expected analyte concentrations.

**Question 3: Will Invitrogen multiplex assays give me the same results for each analyte as my current ELISA tests?**

Invitrogen multiplex assays, which are based on Luminex xMAP technology, provide a versatile platform that gives users flexibility for multiple analyte detection. Whether users are testing for single or multiple analytes, Invitrogen multiplex assays deliver accurate analytical performance using efficient, easy-to-follow protocols. Each of these assays has undergone the same development, validation, manufacturing, and quality control standardization we conduct for our ELISAs. In addition, the multiplex assays undergo additional testing to ensure that there is no



Antigen-specific capture antibodies are bound to microspheres

Antigen from the test sample is bound to the microspheres

Signal is generated by labeled secondary antibody attachment

**Figure 1. Protocol used with bead-based assays (Luminex Corporation).**

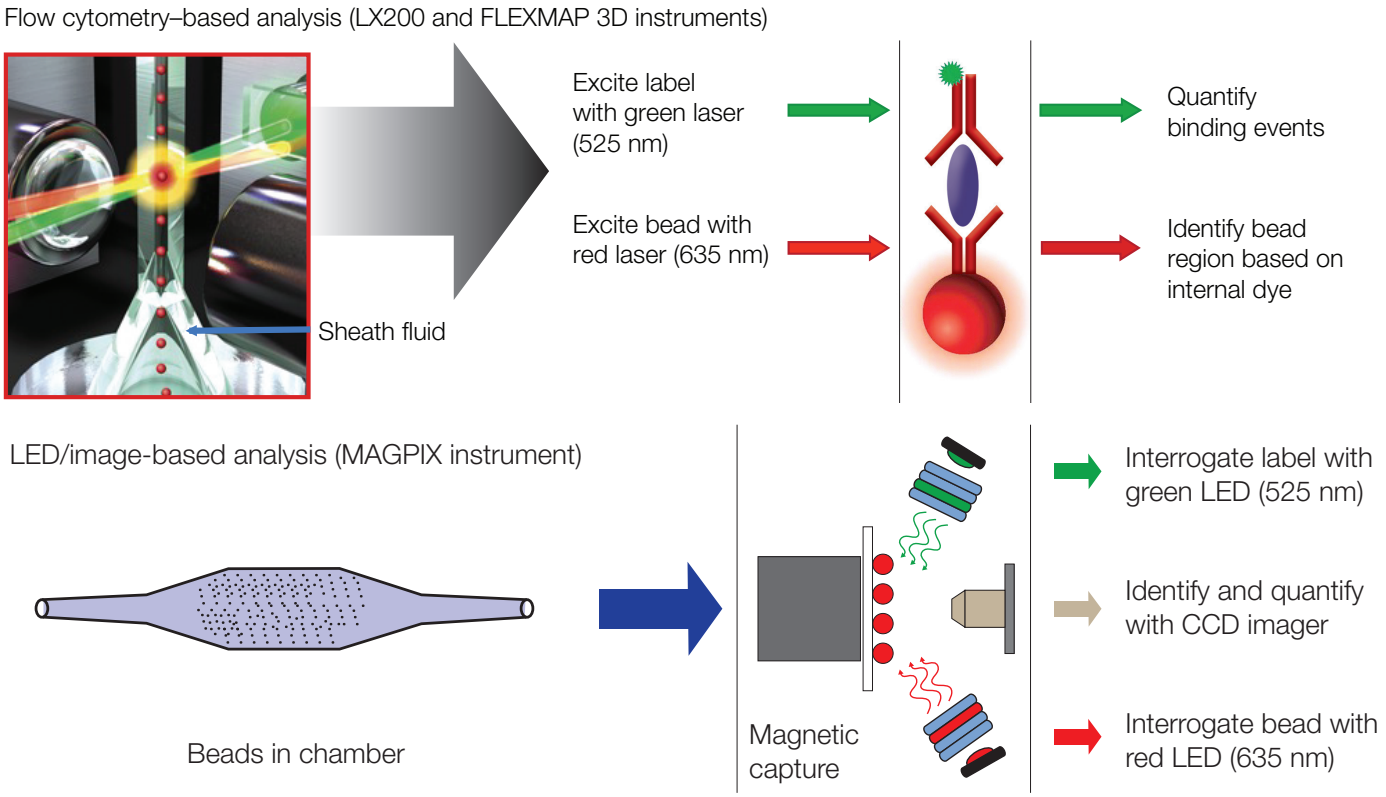



**Figure 2. Luminex xMAP technology uses either light-emitting diodes (LEDs) or lasers to measure the levels of several (typically 5 to 30) targets in a single sample.**

crosstalk between beads and non-cognate analytes. Each Invitrogen multiplex and ELISA assay is fully qualified with the appropriate sample type (i.e., species-specific serum, plasma, and cell culture supernatants), and is evaluated based on the following performance characteristics:

- **Specificity**—each antibody pair analyte is screened to make sure there is no significant cross-reactivity with other analytes in the multiplex test.
- **Recovery**—all of our multiplex assays are tested using native protein in matrices such as serum and plasma, as well as cell culture supernatant. Therefore, both recombinant and natural analytes are detected.
- **Optimal standard curve**—good top and bottom signal, signal-to-noise ratios, and large dynamic range (Figure 3A).
- **Sensitivity**—each analyte is evaluated for analytical sensitivity (detectable signal greater than 2 standard deviations above background).

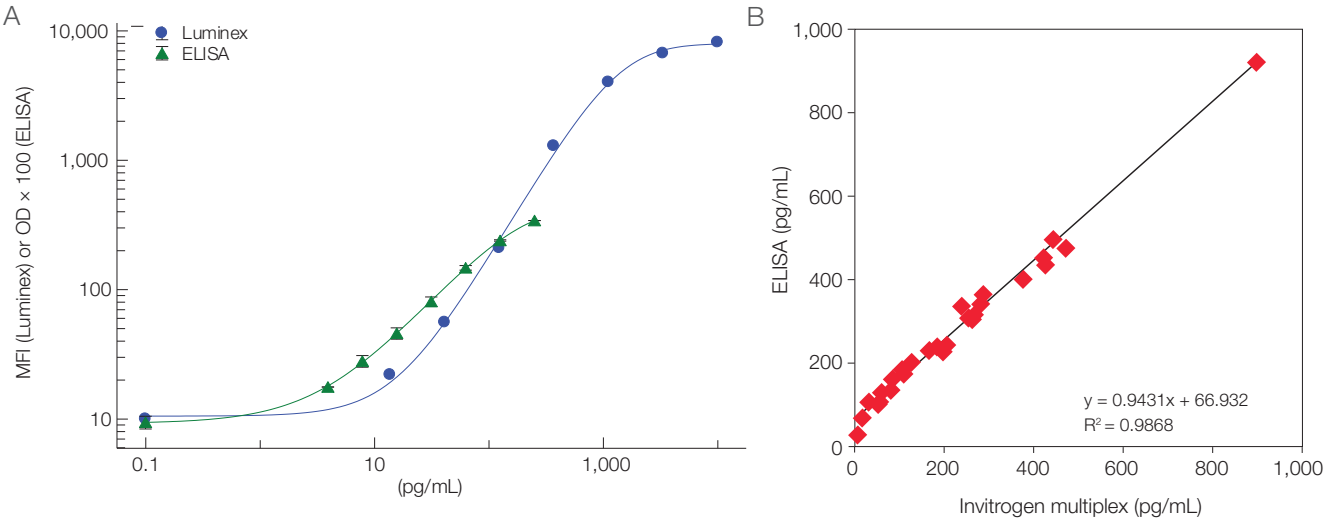



**Figure 3. Dynamic range and correlation to ELISA. (A)** Standard curves were generated for human IL-1ß using an Invitrogen multiplex assay and ELISA. The dynamic range of the Invitrogen multiplex assay is much larger than the ELISA. **(B)** Murine GM-CSF in cell culture supernatant was tested by ELISA (y-axis) and in an Invitrogen multiplex assay (x-axis). Correlation of values over 3 logs of sample dilution was 0.9868.

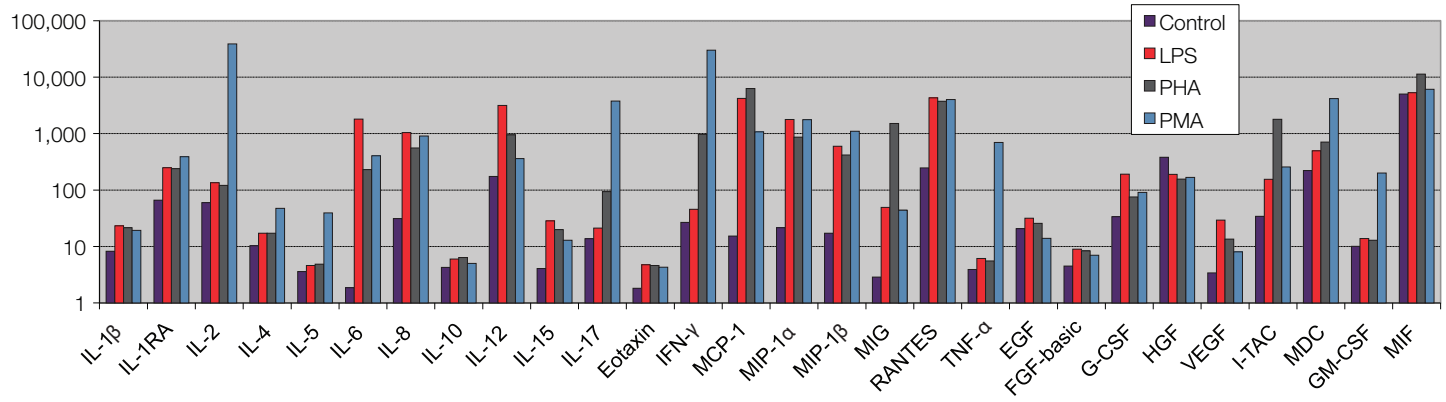



**Figure 4. The power of multiplexing.** Rhesus monkey cells were cutured with medium (control), lipopolysaccharide (LPS), phytohaemagglutinin (PHA), or PMA plus A23187. Supernatant fluids were assayed using the Invitrogen™ Monkey Cytokine Magnetic 29-Plex Panel (Cat. No. LPC0005M).

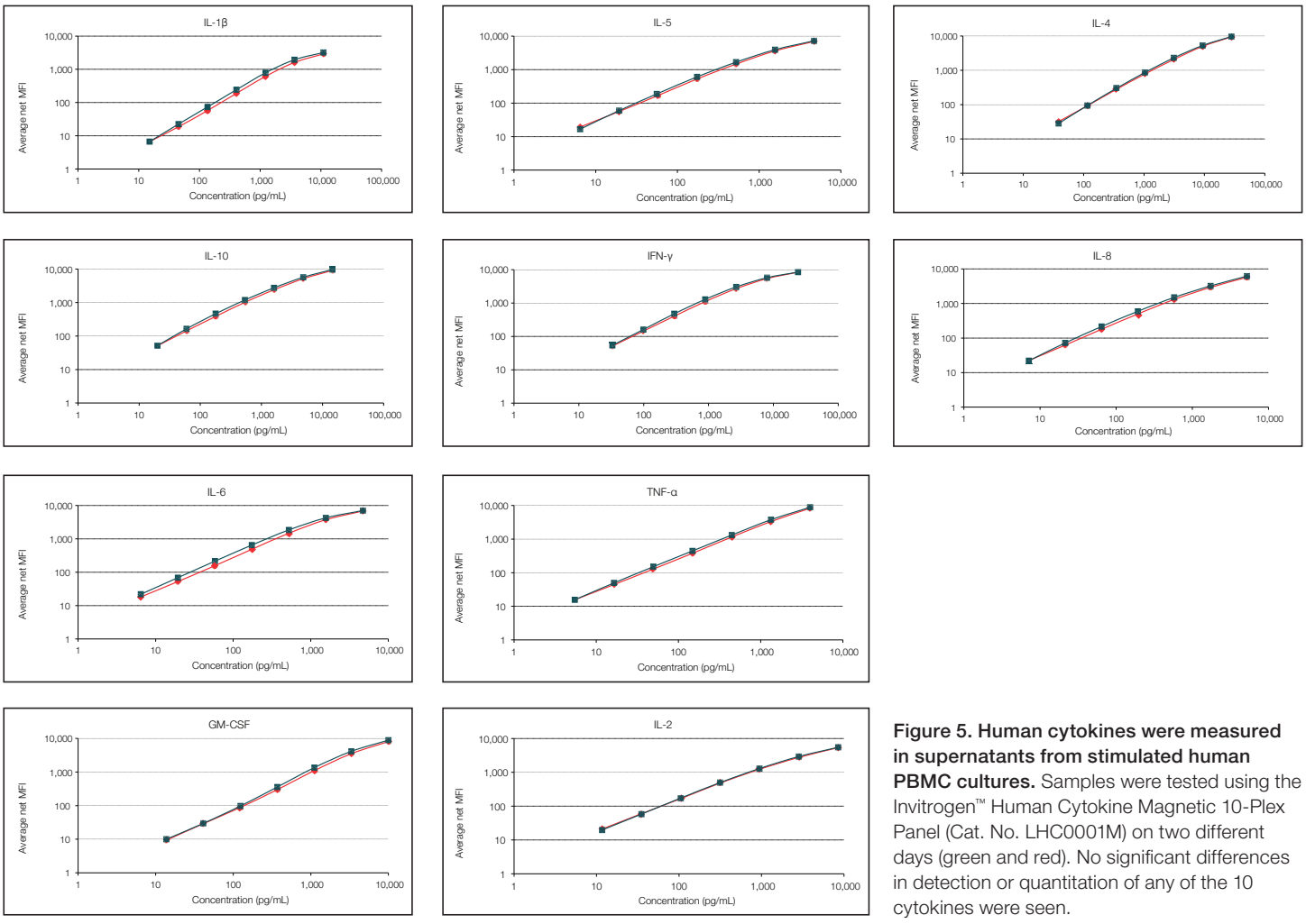



**Figure 5. Human cytokines were measured in supernatants from stimulated human PBMC cultures.** Samples were tested using the Invitrogen™ Human Cytokine Magnetic 10-Plex Panel (Cat. No. LHC0001M) on two different days (green and red). No significant differences in detection or quantitation of any of the 10 cytokines were seen.

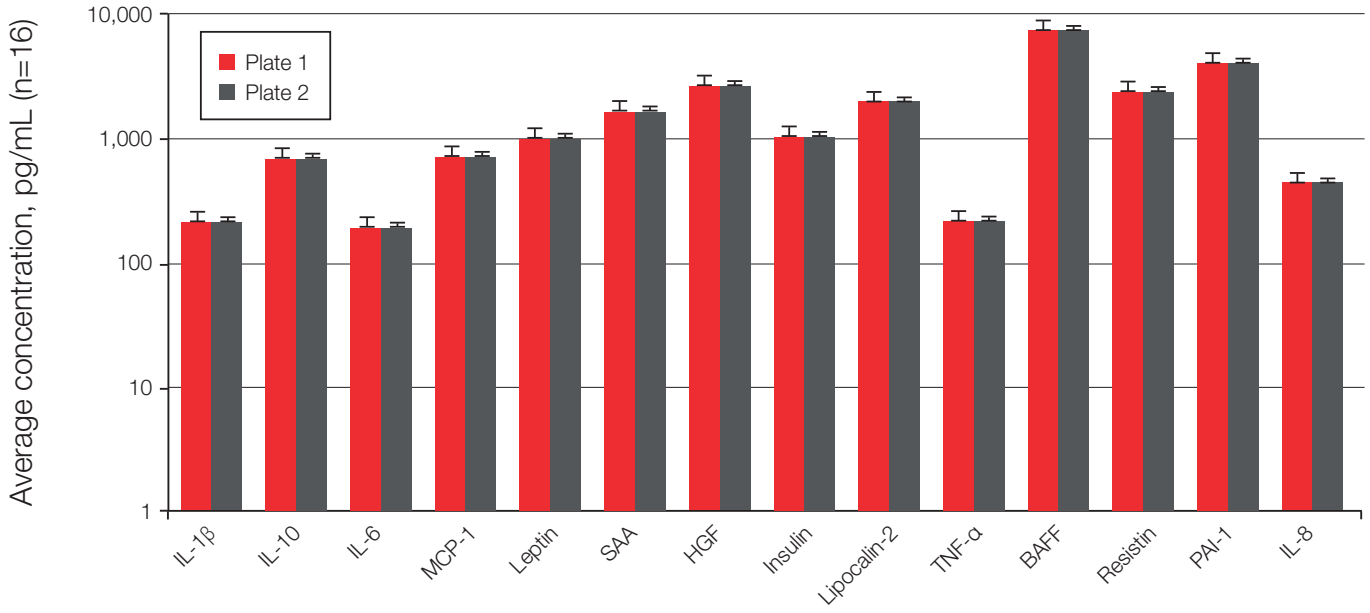



**Figure 6. Intra-assay precision shows CV <10%.** Assay was performed using the Invitrogen™ Human Adipokine Magnetic 14-Plex Panel (Cat. No. LHC0017M).

- **Precision**—multiplex assays have good intra-assay and inter-assay precision (CV <10%), and lot-to-lot consistency. These values are comparable to ELISA tests.

Invitrogen multiplex assays are tested against the matching ELISAs. Analytical comparison of the two assay formats assures that multiplex assays can effectively be used to obtain comparable results. As such, studies that use ELISAs to quantify multiple analytes in samples can be confidently transitioned to the use of Invitrogen multiplex assays and obtain improved analysis efficiency, economy, and performance. An example is shown in Figure 3B.

## The power of multiplexing

Not only does multiplexing with Invitrogen multiplex assays give the same results as ELISA, it does so in a way that lets a single sample serve as the source for many fluorescence-based sandwich assays that are run simultaneously. Figure 4 shows an example of the power of multiplexing. Relative levels of multiple cytokines and chemokines were measured in the supernatant of rhesus monkey peripheral blood mononuclear cell (PBMC) cultures. Cells were untreated or treated with LPS, PHA, or PMA plus A23187. Normalized data (untreated PBMCs set as 1) are shown on a log scale (Figure 4). Data were generated using the Invitrogen Monkey Cytokine Magnetic 29-Plex Panel.

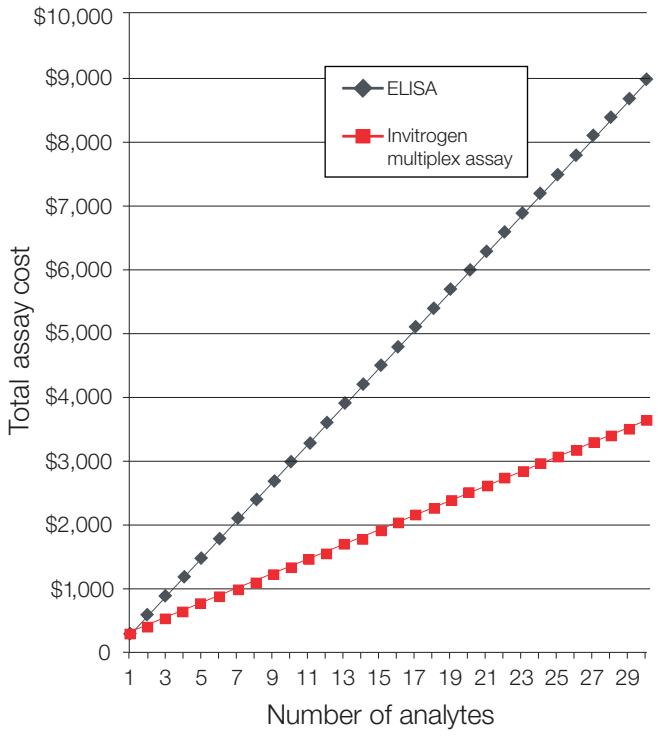



**Figure 7. Cost and time savings for Invitrogen multiplex assay kits vs. ELISAs.** The x-axis shows the number of analytes tested, and the y-axis the total assay cost. The cost advantage of Invitrogen multiplexing assays becomes more and more significant as the number of analytes is increased.

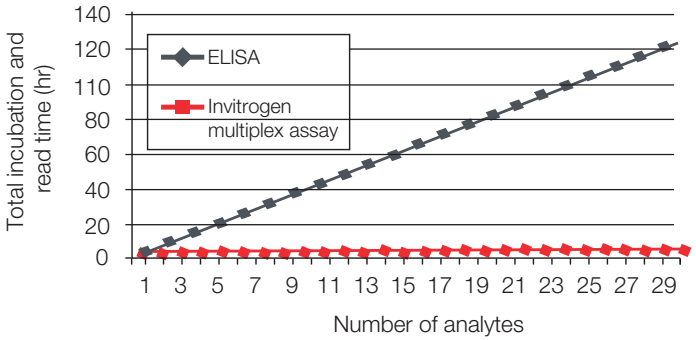



**Figure 8. Time savings for Invitrogen multiplex assay kits vs. ELISAs.** The time savings of multiplexing becomes more significant as the number of analytes is increased.

## Reproducibility of multiplexed assays

Figure 5 compares standard curves of 10 human cytokines run on 2 different days. Green and red lines represent day 1 and day 2, respectively. Data were generated using the Invitrogen Human Cytokine Magnetic 10-Plex Panel.

## Assay precision in multiplexed assays

Figure 6 shows excellent precision in median fluorescence intensity (MFI) with 24 replicates in two different runs.

### Question 4: How much do multiplex assays cost, and how does the cost compare to ELISAs?

Because one multiplex panel provides the ability to measure many proteins with one kit, each kit may appear to cost more than an ELISA test. However, price per target is cheaper if the assay contains two or more targets. Figures 7 and 8 show the cost and time savings of multiplexing.

It should be noted that access to a Luminex instrument (MAGPIX™, LX200™, or FLEXMAP 3D™ instrument) is needed. However, with over 10,000 instruments placed globally, Luminex instruments are now more accessible than ever. Often times, Luminex systems are considered share devices at many institutions or can be accessed through core labs. As for the wash steps, we recommend a handheld magnet (Cat. No. A14179), which is a one-time cost. Alternatively, many ELISA washers can be reconfigured to handle the washing of magnetic particles (through add-on manual or automatic magnets), and the costs for this conversion are nominal.

### Question 5: How difficult is it to learn to perform Invitrogen multiplex assays compared to ELISAs?

## If you can run an ELISA, you can run an Invitrogen multiplex assay for the Luminex platform

Invitrogen multiplex assay kits are used in much the same way as ELISAs (Figure 9). The steps are similar, with the exception that beads are added to the 96-well plate. These beads capture the analyte, instead of having capture ligands attached to the plate. Samples are placed into 96-well microtiter plates. Each kit provides standards of known concentration, so that a standard curve can be established. After incubation on a shaker, the beads are washed by putting the 96-well plate on a flat magnet for 30 seconds, after which the fluid is discarded by flicking the wells. The magnet is removed, and the beads are resuspended in the detection antibody. Another incubation and wash are followed by the addition of S-RPE. The beads are then washed and are ready to analyze. Automated washers with magnets are also available. The magnetic immobilization of the beads allows for thorough washing with very little bead loss.

## Summary of the benefits of xMAP multiplexing assays

The Luminex multiplex bead-based assays have a number of significant advantages compared with traditional ELISA technology, including:

- **Time savings**—Luminex systems allow testing of up to 500 analytes in a single sample. In the same time it takes to set up one ELISA, multiple analytes can be measured, significantly reducing labor time.
- **Smaller sample volume**—depending on the expression levels, assays require 50 µL of sample or less for multiplex assays, while still obtaining accurate results for all analytes.
- **Broad dynamic range**—provides the ability to reliably detect proteins across a broad concentration range.
- **High throughput**—the Luminex system automatically reads up to 96 samples from a conventional microtiter plate. Combined with the ability to read up to 500 analytes per sample, this provides a high-throughput path to data collection.

Field Application Specialists (FAS) are available to educate researchers on multiplexing, answer questions, and help support all aspects of running and analyzing an Invitrogen multiplex assay. For any questions, please contact us at **LuminexFAS@thermofisher.com**

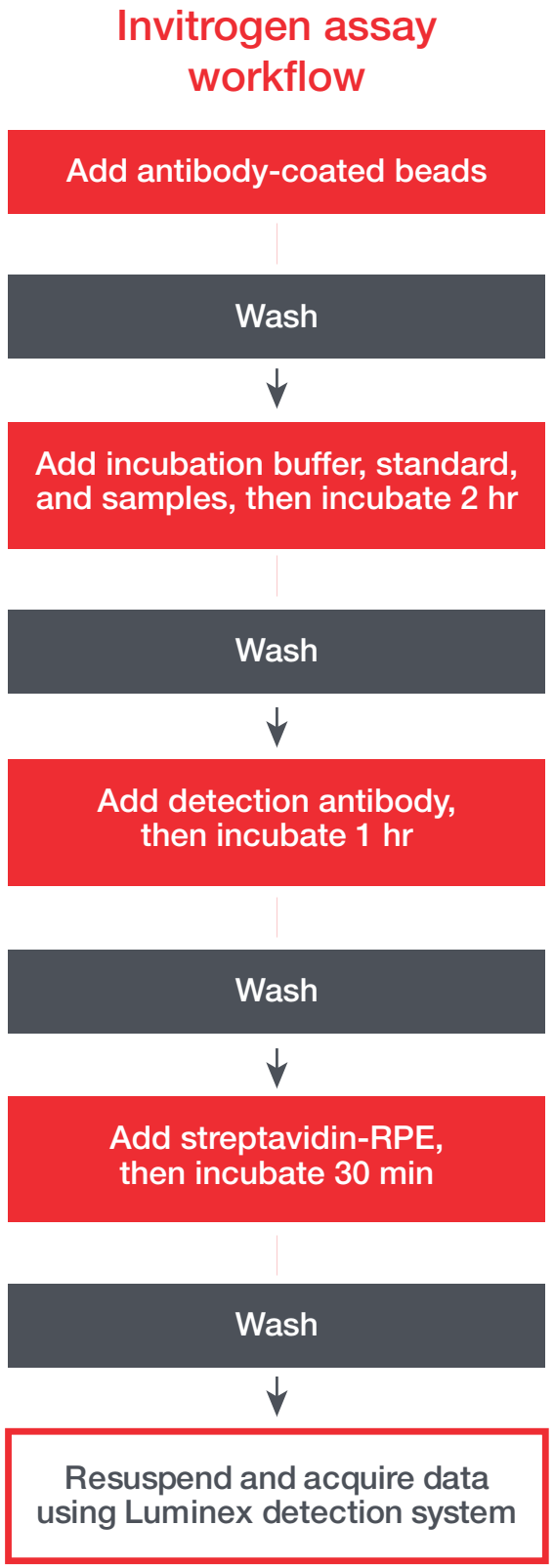



Figure 9. Workflow for using Invitrogen multiplex assay kits.

## Summary

Many researchers have made the easy transition from ELISA to Luminex xMAP technology. Invitrogen multiplex assays further simplify this transition, making multiplexing technology even more accessible and affordable. Experimental results obtained with Invitrogen multiplex assay kits are comparable to ELISAs, and the ability to simultaneously test for multiple analytes in a single sample provides an easy and economical path to greater productivity and more comprehensive studies.

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