

# A unique antibody microarray for multiplex analysis of human cytokines

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*Simultaneously measure the levels of 12 cytokines from multiple human sera, plasma, or cell culture supernatants. Sample volumes of 50  $\mu$ l are sufficient for this miniaturized multiplex immunoassay.*

Cytokines are small multifunctional proteins that play critical roles in controlling development and regulating the body's response to disease and infection. The interaction among cytokines and the cellular immune system is a complex, dynamic process that often involves multiple cytokines. A powerful tool to elucidate the biological pathways by which these molecules influence cellular changes is provided by simultaneous detection and measurement of multiple cytokines. Traditionally, enzyme-linked immunosorbent assays (ELISAs) are used to measure cytokines. Although single-analyte ELISA systems provide accurate and sensitive cytokine measurements, they require large sample volumes and significant processing time to obtain information about multiple cytokine levels from the same sample.

Here, we introduce the novel ProteoPlex™ technology platform, which combines the convenience of parallel sample processing with the multiplex data generation capabilities of protein microarrays in a sandwich ELISA format (Figure 1).

The ProteoPlex 16-Well Human Cytokine Array Kit is designed for multiplex detection and measurement of 12 important human cytokines in parallel from up to 15 experimental samples. The combination of advanced surface chemistry, well-characterized antibodies, and a highly sensitive, fluorescence-based detection system provides a robust method for comparing relative cytokine abundance or cytokine quantification from serum, plasma, or cell supernatants (Table 1). The 12 analytes measured in the kit are pro- and anti-inflammatory cytokines important for the study of

immune system regulation, TH1/TH2 differentiation, and diseases such as asthma and arthritis.

The SensiLight™ fluorescent detection system provided with the kit is based on stabilized light-harvesting complexes (phycobilisomes) purified from microalgae. Experiments have shown that SensiLight dyes are often 10- to 100-fold more sensitive than other fluorescent dyes. In our tests, we routinely detect cytokines in concentrations in the low picograms per milliliter range, similar to the best enzymatic amplification systems used in ELISA kits.

Each well on the slide contains a microarray of spotted antibodies with four

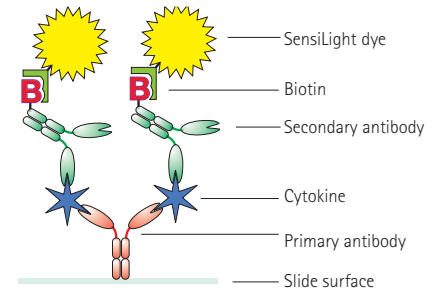


Figure 1. ProteoPlex sandwich detection system

replicate “spots” for each of 12 cytokines plus additional spots for positive and negative controls (Figure 2). The quadruplicate spots provide reliable quantitative data from a single sample. In a typical experiment, 10 samples are processed and compared to a six-point standard curve. The simple procedure is similar to the protocols used for decades in single-analyte immunoassay systems. The 16-well slide format and SensiLight fluorescent

Table 1. ProteoPlex 16-Well Human Cytokine Array Kit specifications

Cytokines:	IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, GM-CSF, IFN $\gamma$ , TNF $\alpha$
Format:	Standard microarray slide format with 16 removable wells; 64 spots/well
Sample Volume:	50 $\mu$ l
Detection Range:	5–2500 pg/ml
Standard Curve Range:	15–800 pg/ml
Reproducibility:	< 20% well-to-well CV
Recovery:	> 80–120% from serum, plasma, and tissue culture supernatants
Assay Duration:	4 hours
Fluorescent Detection System:	SensiLight PBXL-3 (Cy5 wavelengths: 633 nm excitation; 660 nm emission)

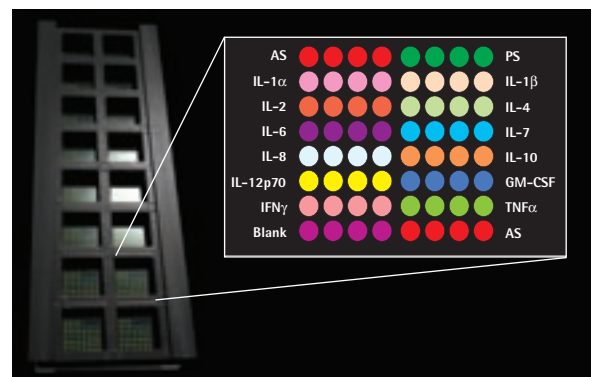


Figure 2. ProteoPlex 16-well array format with microarray layout

Sixteen identical arrays are present on each slide. Key: AS, alignment spots; PS, positive control spots; IL, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; TNF, tumor necrosis factor.

# DISCOVERY THROUGH MULTIPLEX PROTEIN ANALYSIS

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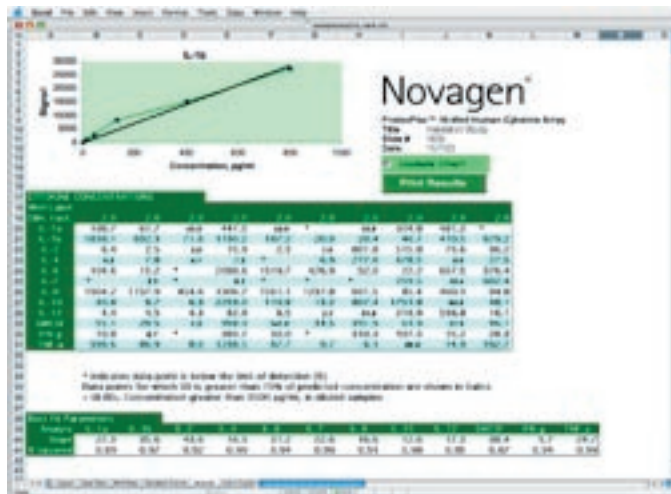
detection system work with a broad range of microarray scanners (see page 5).

The ProteoPlex™ 16-Well Human Cytokine Array Kit is a complete system that includes sample diluent, buffers, cytokine standards, detection antibodies, and SensiLight™ detection reagent. Free Slide Scanning and Analysis Services are also available (Figure 3). Analysis results are returned within a few business days of receiving the slides.

## Validation data

Each batch of kits is tested for key parameters such as standard curve linearity, lower limit of detection (LLOD), and reproducibility. A protocol tests these parameters on a single slide. Known amounts of purified cytokines are added to 10X Sample Diluent diluted to 1X concentration and processed as described in the User Protocol (TB405). To test for linearity and LLOD, we run a dilution series of 0, 5, 15, 44, 133, 400, and 800 pg/ml of each of the 12 cytokines.

As shown in the table in Figure 4, the linearity of each of the 12 standard curves is excellent, with linear regression R<sup>2</sup> values at 0.98 or higher. The LLOD is determined by two methods. First, we look for significant signal-to-noise ratios (2.5 or greater) from cytokines diluted to 5 pg/ml with 1X sample diluent. Second, we extrapolate from the linear regres-



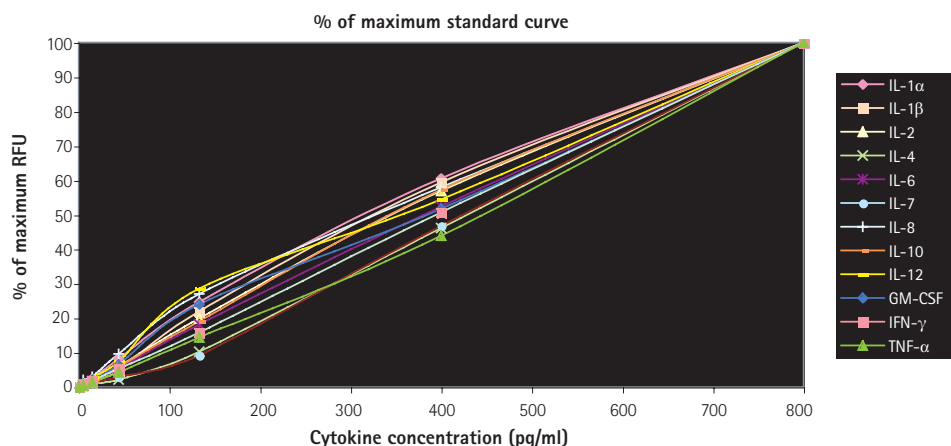
**Figure 3. Scanning and Analysis Service results**

The Scanning and Analysis Service results are delivered in a Microsoft® Excel workbook. This worksheet of the workbook contains the predicted cytokine concentrations for each of the unknown samples, statistics of the standard curves, and a feature to display each of the 12 standard curves. Other worksheets contain the primary data extracted from the microarray image and data from various steps in the analysis scheme.

sion curve to the cytokine concentration at the point where the measurements become significant. All of the cytokines have significant measurements at 5 pg/ml and most of the extrapolated LLOD values are much lower. With this level of sensitivity and a standard curve that ranges up to 800 pg/ml, the 12 cytokines routinely can be measured from almost any biological sample without concentration or dilution.

To test for reproducibility, nine identical samples are run with each cytokine

at 100 pg/ml. The coefficient of variation (CV) is calculated in percentage from the four spots in each well and from nine sample wells. As shown in the table in Figure 4, the %CV for the measurements from the four spots in each well typically ranges from 3 to 9. The %CV for the measurements from the nine sample wells ranges from 5 to 18. With this level of reproducibility within and between wells, it should not be necessary to run replicate sample sets to ensure reliable measurements.



	IL-1 $\alpha$	IL-1 $\beta$	IL-2	IL-4	IL-6	IL-7	IL-8	IL-10	IL-12	GM-CSF	IFN- $\gamma$	TNF- $\alpha$
Linear regression R <sup>2</sup>	0.98	0.99	0.99	0.99	1.00	0.99	0.98	0.99	0.98	0.99	1.00	1.00
Spiked LLOD (pg/ml)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Extrapolated LLOD (pg/ml)	0.88	0.22	0.15	0.16	0.19	0.70	5.40	0.03	0.57	0.27	2.59	1.11
9-well %CV	9.0	10.0	9.0	13.0	10.0	11.0	9.0	5.0	18.0	10.0	10.0	7.0
Mean 4-spot %CV	3.1	4.7	5.3	7.5	5.0	9.3	6.8	5.6	5.4	4.1	5.5	5.5

**Figure 4. Standard curve data generated from serial dilutions of Human Cytokine Standard Mix 1**

**Application example**

The ProteoPlex™ 16-Well Human Cytokine Array Kit was used to measure cytokine production during T-regulatory-1 (Tr-1) cell development. Tr-1 cells are CD4<sup>+</sup> T lymphocytes that are defined by production of IL-10 and the suppression of T-helper cells (1). The development of Tr-1 cells requires activation of CD3, a membrane-bound, five-

polypeptide complex that transduces the activation signals to the cytoplasm of T cells, and CD46, a widely expressed transmembrane glycoprotein that inhibits complement activation on host cells (2–4). Crosslinking CD46 through its physiological or pathogenic ligands initiates signaling events in several types of human cells (5–8).

Two ligands were used in the Tr-1 cell phenotype induction experiment: antibodies against CD46 and an alternate ligand for CD46, the streptococcal M5 protein (9). Human CD4<sup>+</sup> lymphocytes were purified from whole blood and sorted according to their CD45RA/RO profile. Naive T cells (CD45RA<sup>+</sup>/RO<sup>-</sup>) and high responding T cells (CD45RA<sup>+</sup>/RO<sup>+</sup>) were pooled and plated at  $1.5 \times 10^5$  cells per well in plates coated with monoclonal anti-CD3 plus either anti-CD46 monoclonal antibody or streptococcal M5 protein. The cells were

**Table 2: Cytokine measurements\* from Human CD4<sup>+</sup> T lymphocytes**

	48 h			72 h		
	CD3	CD3/CD46	CD3/M5	CD3	CD3/CD46	CD3/M5
IL-10 <sup>a,b</sup>	-	++	+	-	++++	++
IL-2 <sup>b</sup>	++	+	++	++	+	+
IFN- $\gamma$ <sup>b</sup>	-	+	-/+	-/+	++	+
GM-CSF <sup>a,b</sup>	-	++	+	-	++++	+++

\* Activation conditions were established in triplicate. Results represent two independent experiments.

<sup>a</sup> Culture supernatants were collected at 48 and 72 hours and tested with commercial ELISA kits.

<sup>b</sup> Culture supernatants were collected at 48 and 72 hours and tested with a ProteoPlex 16-Well Human Cytokine Array Kit.

Cytokine secretion amounts: -, no secretion; -/+ very low; +, low; ++, medium; +++, medium strong; +++++, strong; ++++++, very strong

maintained in standard media (RPMI, 10% FCS) with the addition of recombinant IL-2, conditions known to lead to Tr-1 development (4). Cytokine levels were measured in the culture media after two or three days of incubation using the ProteoPlex 16-Well Human Cytokine Array Kit and, for selected cytokines, with commercial ELISA kits.

**Results and conclusions**

The cytokine levels measured by the ProteoPlex 16-Well Human Cytokine Array indicate successful activation of the Tr-1 phenotype as typified by production of IL-10. As shown in Table 2, the response occurred on the third day of activation and also resulted in increased levels of IL-2, IFN- $\gamma$ , and GM-CSF. Activation of CD46 by either monoclonal anti-CD46 or streptococcal M5 proteins was required for the increased cytokine production. The ability of the M5 protein to activate CD46 and induce the Tr-1 phenotype may indicate a role in streptococcal pathogenesis. The increased IFN- $\gamma$  levels may be due to contaminating T-memory cells (Kemper, personal communication). The production of GM-CSF by Tr-1 cells may reveal a previously unknown role in immune system regulation.

**Summary**

The ProteoPlex 16-Well Human Cytokine Array Kit is the first antibody microarray kit that is easy, fast, and complete. A simple ELISA-like protocol enables measurement of 12 different cytokines in quadruplicate from up to 15 samples within a few hours using standard microarray scanners. The kit includes sample diluent, buffers, cytokine standards, detection antibodies, and fluorescent detection reagents. Free Slide Scanning and Analysis Services also are available, with results returned within days.

**ACKNOWLEDGMENTS**

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Scanners and software compatible with the ProteoPlex 16-Well Human Cytokine Array Kit include the GenePix 4000<sup>®</sup>B scanner and GenePix<sup>®</sup> Pro software (Axon Instruments) and the following scanners, DNA MicroArray Scanner, model G2565BA (Agilent Technologies), Typhoon<sup>™</sup> 9210 (Amersham Biosciences), ScanArray<sup>®</sup> Express (PerkinElmer), LS200 Scanner (Tecan), and AlphaArray<sup>®</sup> 7000 Reader (Alpha Innotech Corporation), all with ArrayVision<sup>™</sup> software (Imaging Research). Other scanners from these suppliers with standard red laser should also work. For more information, visit [www.proteoplex.com](http://www.proteoplex.com).

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Product	Size	Cat. No.
ProteoPlex™ 16-Well Human Cytokine Array Kit	1 array	71414-3
	2 arrays*	71414-4
	3 arrays*	71414-5

\* The 2-array and 3-array kit sizes are multiples of the 1-array kit.

**Components:**

- 1 ProteoPlex 16-Well Human Cytokine Array
- 1 PBST Buffer Tablet
- 1 ml 10X Sample Diluent
- 1 tube Detection Antibody Cocktail 1, Lyophilized
- 1.6 ml Detection Antibody Diluent
- 1 tube Human Cytokine Standard Mix 1, Lyophilized
- 0.3 ml 10X Standard Diluent, Serum-based
- 1 tube SensiLight™ PBXL-3 Fluorophore, Lyophilized
- 1.8 ml SensiLight PBXL-3 Diluent
- 1.5 ml 200X Final Rinse
- 1 Slide Dryer
- 1 Slide Rinser
- 1 Slide Mailer