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Research Report

Array-Based ELISAs for High-Throughput Analysis of Human Cytokines

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ABSTRACT

In this report, we describe the development of a mini-array system suitable for high-throughput quantification of proteins. This mini-array is a multiplexed, sandwich-type ELISA that measures the concentration of seven different human cytokines—TNF-α, IFNα, IFNγ, IL-1α, IL-1β, IL-6, and IL-10—from a single sample in each well of a 96-well plate. The mini-array is produced by spotting monoclonal antibodies (mAbs) in a 3 × 3 pattern in the bottom of the wells of 96-well polystyrene plates. Cytokines that are captured by the arrayed mAbs are detected by using biotinylated mAbs, followed by the addition of a streptavidin-horseradish peroxidase (HRP) conjugate and a chemiluminescent substrate. The light produced from the HRP-catalyzed oxidation of the substrate is measured at each spot in the array by imaging the entire plate with a commercially available CCD camera. Here, we demonstrate that these 96-well-plate format mini-arrays have performance characteristics that make them suitable for the high-throughput screening of anti-inflammatory compounds.

INTRODUCTION

The ELISA is an established tool for the quantitative measurement of proteins (6,10,14), and because of the current interest in proteomics, researchers have recognized the value in adapting the ELISA to a multiplexed array format for the development of protein chips (2,4,5,13). However, most of this work has focused on the methods needed to create high-density arrays of antibodies or proteins (1,3,7–9,11), and few reports have described the ability of antibody arrays to quantify the concentration of the target proteins.

We have taken advantage of improvements in robotic "spotting" equipment, chemiluminescent substrates, and charge-couple device (CCD) camera technology to develop a multiplexed ELISA that is capable of sensitive and quantitative detection of seven different human cytokines: TNF-α, IFNα, IFNγ, IL-1α, IL-1β, IL-6, and IL-10. We then demonstrate the utility of this system as a drug discovery screening method by measuring the profile of cytokines secreted from stimulated human cells that have been treated with a compound known to reduce inflammation.

MATERIALS AND METHODS

All of the monoclonal antibodies (mAbs) used for the capture of target cytokines in the array—TNF-α, IFNα, IFNγ, IL-1α, IL-1β, IL-6, and IL-10—the biotin-labeled mAbs used for the detection of the captured target cytokines, and purified cytokine standards are commercially available from Pierce Endogen (Rockford, IL, USA). The mAbs that were spotted in the arrays were diluted to 50 µg/mL in Dulbecco's PBS minus calcium and magnesium (Life Technologies, Rockville, MD, USA). The biotin-labeled detection antibodies were diluted in PBS containing 1 mg/mL BSA (Intergen, Purchase, NY, USA) and mixed together to produce a cocktail of all seven detection antibodies at concentrations ranging from 0.15 to 0.75 µg/mL. The target cytokine preparations were made using the cytokine standards from commercially available ELISA kits. The cytokines were diluted in PBS containing 1 mg/mL BSA and used either individually or mixed together to produce a cocktail of all seven cytokines. Streptavidin-horseradish peroxidase (SA-HRP) conjugate (Pierce Endogen) was diluted to 1:500 000 in PBS just before use. Cell culture media for all experiments consisted of RPMI 1640 plus 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamic acid (all from Life Technologies).

96-Well Microplate Array Spotting

The mAb arrays were spotted in a 3 × 3 pattern in black Maxisorp™ 96-well plates (Nalge Nunc International, Rochester, NY, USA) using the Biochip Arrayer I™ (Packard Instruments, Meriden, CT, USA). The arrayer was programmed to deposit the antibody solutions at 20 nL/spot, and the spot diameter was approximately 0.4 mm. The protein concentration of the mAbs was

typically 50 µg/mL, so that each spot contained approximately 1 ng (approximately 7 fmol) mAb. After spotting, the plates were blocked with SuperBlock® buffer and washed with ELISA wash buffer (both from Pierce Endogen).

Quantification of Cytokines Using the Mini-Array ELISA

The microplate mini-array was used to measure the concentration of the cytokines as follows: each sample (50 µL final volume) was transferred to the wells of the mini-array plate for the capture of the target proteins during a 1-h incubation at 25°C with shaking at 200 rpm. The plate was washed three times with ELISA wash buffer to remove any unbound components. Biotin-labeled detection antibody cocktail was added (100 µL/well) and incubated for 1 h at 25°C with shaking at 200 rpm. Excess biotin-labeled detection antibodies were removed by washing the plate three times with ELISA wash buffer.

SA-HRP conjugate was added (100 µL/well), and the plate was incubated at 25°C for 30 min with shaking at 200 rpm. The excess SA-HRP conjugate was removed by washing the plate three times with ELISA wash buffer. Bound SA-HRP was detected by the addition of 100 µL SuperSignal™ ELISA Femto substrate (Pierce Endogen). The plate was imaged using a FluorChem™ 8000 CCD camera (Alpha Innotech, San Leandro, CA, USA) within 15 min after substrate addition. FluorChem™ software (Alpha Innotech) was used to quantify the signal from each spot in the array.

Standard curves for the assays were run with known amounts of the target cytokines. The signal from each of these standards was used to calculate the picogram/milliliter concentrations of the cytokine targets in the natural samples.

Isolation and Stimulation of PBMCs for Cytokine Induction Time Course

A total of 60 mL blood was drawn and pooled from two healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood using Histopaque®-1077 Hybri-Max® (Sigma, St. Louis, MO, USA). The PBMCs were pelleted by centrifugation at 1000× *g* for 10 min, suspended in 50 mL cell culture media, and then collected again by centrifugation at 1000× *g*. The cells were then suspended in cell culture media at 1×10^6 cells/mL. The cells were stimulated with 10 µg/mL phytohemagglutinin (PHA) and 10 µg/mL Concanavalin A (ConA) (both from Sigma), and then 10-mL aliquots of the cell suspension (1×10^7 cells total) were dispensed into 25-mL tissue culture flasks and incubated at 37°C with 5% CO_2. Samples of media were removed from the cells every hour from 0 to 5 h. The culture media samples were stored at -70°C until analysis.

Stimulation of THP-1 Cells for Anti-Inflammatory Drug Screening

THP-1 cells (American Type Culture Collection, Manassas, VA, USA) were diluted in culture media to a concentration of 8×10^5 cells/mL and then treated with 5 µg/mL lipopolysaccharide (LPS), 5 µg/mL LPS plus 10 µM dexmethasone (both from Sigma), or 5 µg/mL LPS plus 100 µM dexmethasone (control cells in culture media only were also prepared). Aliquots (100 µL) of the treated cells were dispensed into the wells of a 96-well tissue culture plate (5×10^4 cells/well) and then incubated at 37°C with 5% CO_2. After 6 h incubation, the media samples were removed from the plate and stored at -70°C until testing.

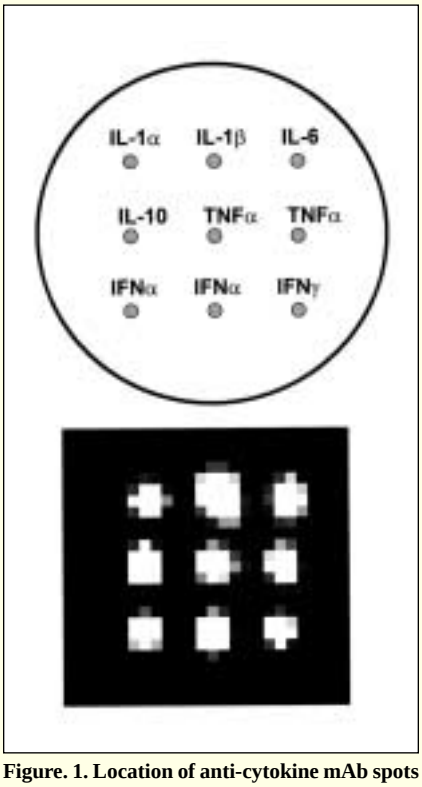


Figure. 1. Location of anti-cytokine mAb spots in the mini-array and CCD camera image of a 3 × 3 mini-array within a single well.

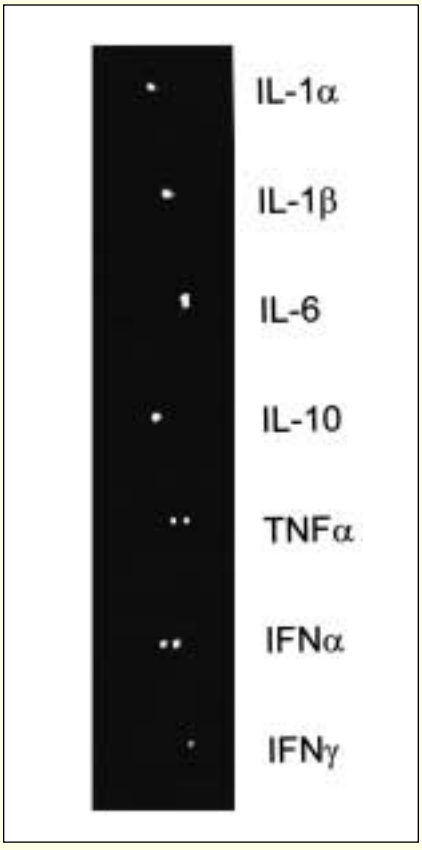


Figure 2. Specificity of the cytokine mini-arrays. A CCD camera image of a mini-array plate tested with cytokines added individually to the wells of the mini-array plate.

RESULTS

Mini-Array Performance

A 3 × 3 array of mAbs that bind TNF-α, IFNα, IFNγ, IL-1α, IL-1β, IL-6, and IL-10 was spotted in the wells of the 96-well microplate according to the pattern shown in Figure 1. TNF-α and IFNα were spotted twice in each array. The intensity of the signal varies for different cytokine targets at the same cytokine concentration; for instance, the IL-1α spot is less intense

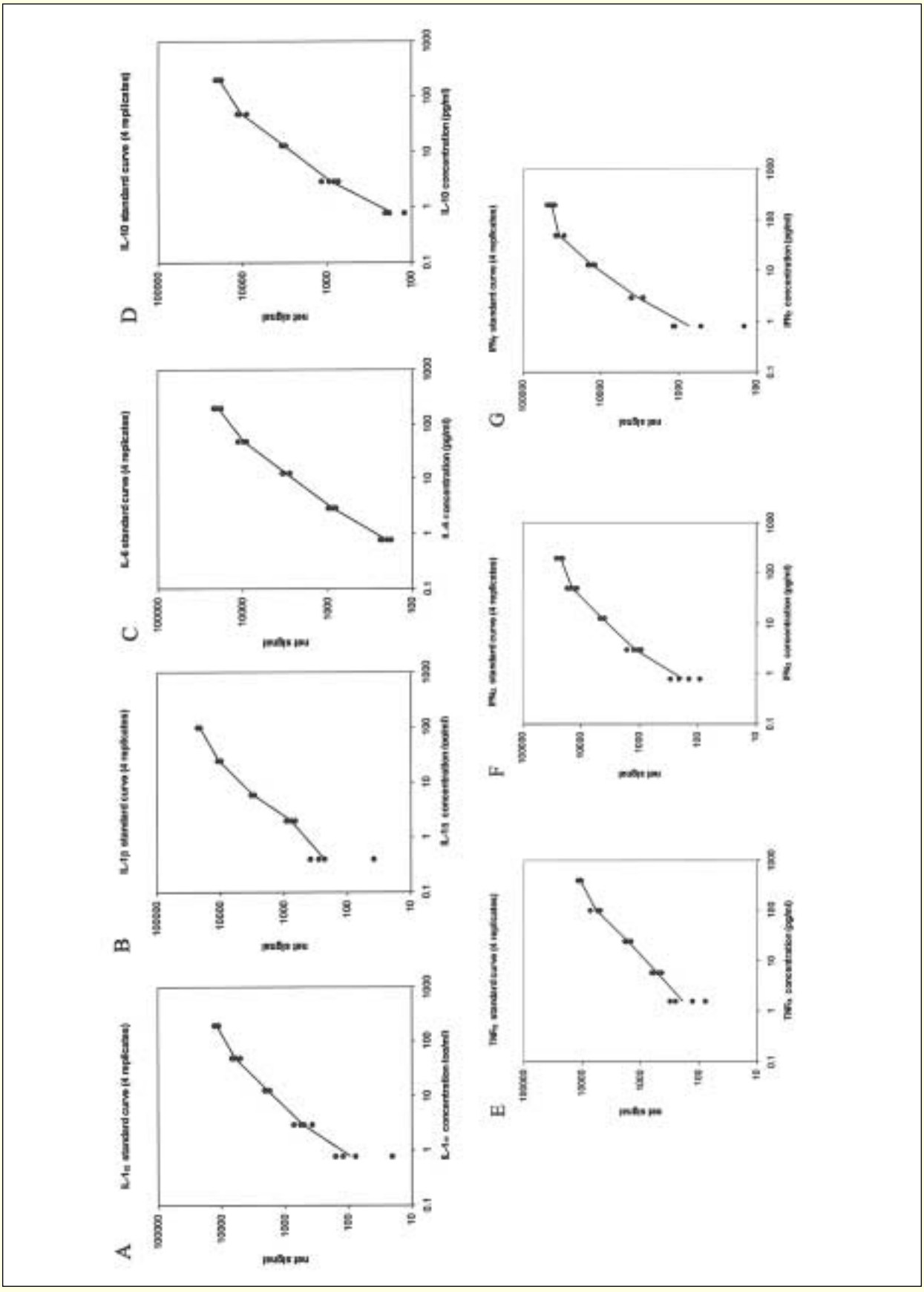


Figure 3. Standard curves for the cytokine mini-arrays. (Panels A–G) Dilutions of a mixture of all seven cytokines were added to the mini-array plate. The concentrations of the cytokine standards were 0.8–200 pg/mL for IL-1α, IL-6, IL-10, IFNα, and IFNγ; 0.4–100 pg/mL for IL-1β; and 1.6–400 pg/mL for TFN-α. The assay was performed with four replicates of the standard curve; the filled circles represent the signal from each spot in the four wells, and the line represents the average signal from the four replicates. Panel A, IL-1α; panel B, IL-1β; panel C, IL-6; panel D, IL-10; panel E, TNF-α; panel F, IFNα; and panel G, IFNγ.

Table 1. Intra-Plate Reproducibility of Cytokine Mini-Arrays

Target Cytokine	Average Signal	SD	% CV
IL-1α	2206	176	8
IL-1β	2053	269	13.1
IL-6	1743	162	9.3
IL-10	2263	194	8.6
TNFα	2916	274	9.4
IFNα	2328	254	10.9
IFNγ	1039	97	9.4

Values obtained from the CCD camera image shown in Figure 4. The mini-array plate was tested with all seven cytokines—TNF-α, IFNα, IFNγ, IL-1α, IL-1β, IL-6, and IL-10—added together to 16 wells of an individual mini-array plate.

Table 2. Inter-Plate Variability of Cytokine Mini-Arrays

	IL-1α Signal	Il-1β Signal	IL-6 Signal	IL-10 Signal	TFN-α Signal	IFNα Signal	IFNγ Signal
Plate 1	3860	10119	12671	7265	3964	5854	7864
Plate 2	3602	10163	10683	7161	3850	4934	5906
Plate 3	3190	8668	11596	6780	3781	5112	6474
Plate 4	3412	8167	11586	6039	3742	5204	6214
Average	3516	9279	11634	6811	3834	5276	6614
SD	246	880	704	481	84	348	749
%CV	7.0	9.5	6.1	7.1	2.2	6.6	11.3
SD	IL-1α pg/mL	Il-1β pg/mL	IL-6 pg/mL	IL-10 pg/mL	TNF-α pg/mL	IFNα pg/mL	IFNγ pg/mL
Plate 1	29	54	61	46	78	47	95
Plate 2	26	58	50	42	99	47	98
Plate 3	24	53	51	56	77	40	91
Plate 4	34	57	60	45	81	59	90
Average	28	56	56	47	84	48	93
SD	4	2	5	5	9	7	3
% CV	14.3	3.9	8.6	11.3	10.5	14.2	3.6

Cytokine mini-arrays from four separate plates run in a single experiment in which samples of all seven cytokines—TNF-α, IFNα, IFNγ, IL-1α, IL-1β, IL-6, and IL-10—were added together to the wells (in quadruplicate) of each of the mini-array plates. The signal values were obtained from a CCD camera image, and the reported value from each plate was the average of the four sample replicates from within that plate. A dilution series of cytokine standards was also run on each of the four plates, and this standard curve was used to calculate the concentration (pg/mL) of the cytokines in the samples.

than the IL-1β spot (Figure 1). This is due to the differences in the affinities of the capture antibodies and the biotinylated detection antibodies for their cytokine targets. Another factor that affects spot intensity is the degree of biotinylation of the detection antibodies, as more SA-HRP will bind to detection antibodies that have more biotin labels. The specificity of the mini-arrays was demonstrated by adding samples containing only individual cytokines to different wells of a mini-array plate (Figure 2). In each case, the cytokine in the sample produced a signal only at the spot(s) containing the complementary antibody. There was low background in the absence of target, and no cross reactivity was observed between the different cytokines and their arrayed antibodies.

The sensitivity, dynamic range, and precision of the mini-arrays were evaluated by adding dilutions of a mixture of all seven cytokines to the mini-array plate. The dynamic range for the detection of the target cytokines in the array was demonstrated to be: 0.8–200 pg/mL for IL-1α, IL-6, IL-10, IFNα, and IFNγ; 0.4–100 pg/mL for IL-1β; and 1.6–400 pg/mL for TNF-α (Figure 3, panels A–G). To evaluate the precision of the mini-array assay, the dilution series of cytokine standards was run in four replicates on the plate. The assays had sufficient precision to provide for discrimination between all of the replicates at the different concentrations of target cytokine (Figure 3, panels A–G).

The variability of the arrays in wells within a single plate was determined by adding a cocktail of all seven cytokines to 16 different wells in a mini-array plate (Figure 4 and Table 1). When the signals from the different cytokine spots in the mini-arrays were compared between the 16 replicate wells, the CV (i.e., standard deviation divided by the mean) ranged from 8% for IL-1α to 13.1% for IL-1β.

The variability of the arrays in the wells from different plates was determined by adding a cocktail of all seven cytokines to wells from four different mini-array plates (Table 2). The CV for the chemiluminescent signals produced in the mini-arrays from the different plates ranged from 2.2% for TNF-α to 11.3% for IFNγ. In the same experiment, a dilution series of cytokine standards was also run on arrays from each of the four plates. The signal from these cytokine standards was used to calculate the concentration of the cytokines in the sample wells. The CV for the calculated concentration of the cytokines ranged from 3.6% for IFNγ to 14.3% for IL-1α (Table 2).

Detection of Cytokines in Natural Samples

The ability of the mini-arrays to monitor changes in the expression profile of cytokines produced by stimulated human cells was investigated in an ex-

periment in which PBMCs were treated with LPS and samples of the media were removed at 1-h intervals. Analysis of 12.5-µL aliquots of these samples (diluted with 37.5 µL PBS) in the mini-arrays showed that the baseline-level concentration of all of the cytokines at time zero was undetectable (Table 3). All of the cytokines were detectable at low levels 1 h after induction (except for IL-10, which was not detected until 3 h after induction). Five hours after induction, the mini-arrays measured IL-6, TNF-α, and IL-1β at 343, 124, and 40 pg/mL, respectively. The levels of IL-10, IFNγ and IL-1α were much lower at 6, 3.6, and 3 pg/mL, respectively. The concentration of IFNα was not determined because the signal never exceeded the signal of the low standard (0.8 pg/mL) in this experiment.

Mini-Arrays Monitor the Effects of Anti-Inflammatory Drugs

THP-1 cells, a human monocyte cell line, were treated with LPS alone or in combination with the anti-inflammatory drug dexamethasone. After 6 h of culture, 50 µL cell-free media were removed and tested in the mini-array. Upon LPS stimulation, the mini-arrays detected a substantial increase in the amount of TNF-α, IL-1β, and IL-6 (538, 147, and 66 pg/mL, respectively) and a smaller increase in the amount of IL-10 and IFNγ (17 and 10 pg/mL, respectively) (Figure 5). There was no increase in IL-1α or IFNα observed in this experiment. When samples from cells treated with 10 µM dexamethasone were examined, there was a reduction in the concentration of all of the induced cytokines. The dexamethasone effect was dose dependent because a greater reduction in the inflammatory cytokines was observed with 100 µM dexamethasone treatment (Figure 5).

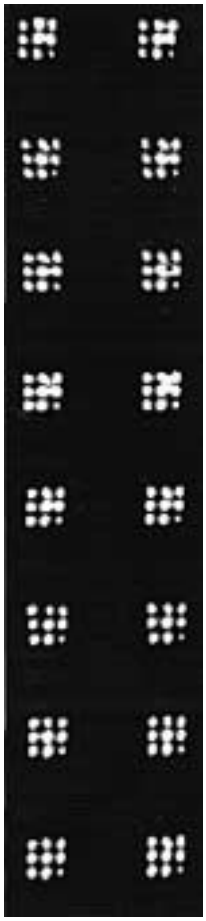
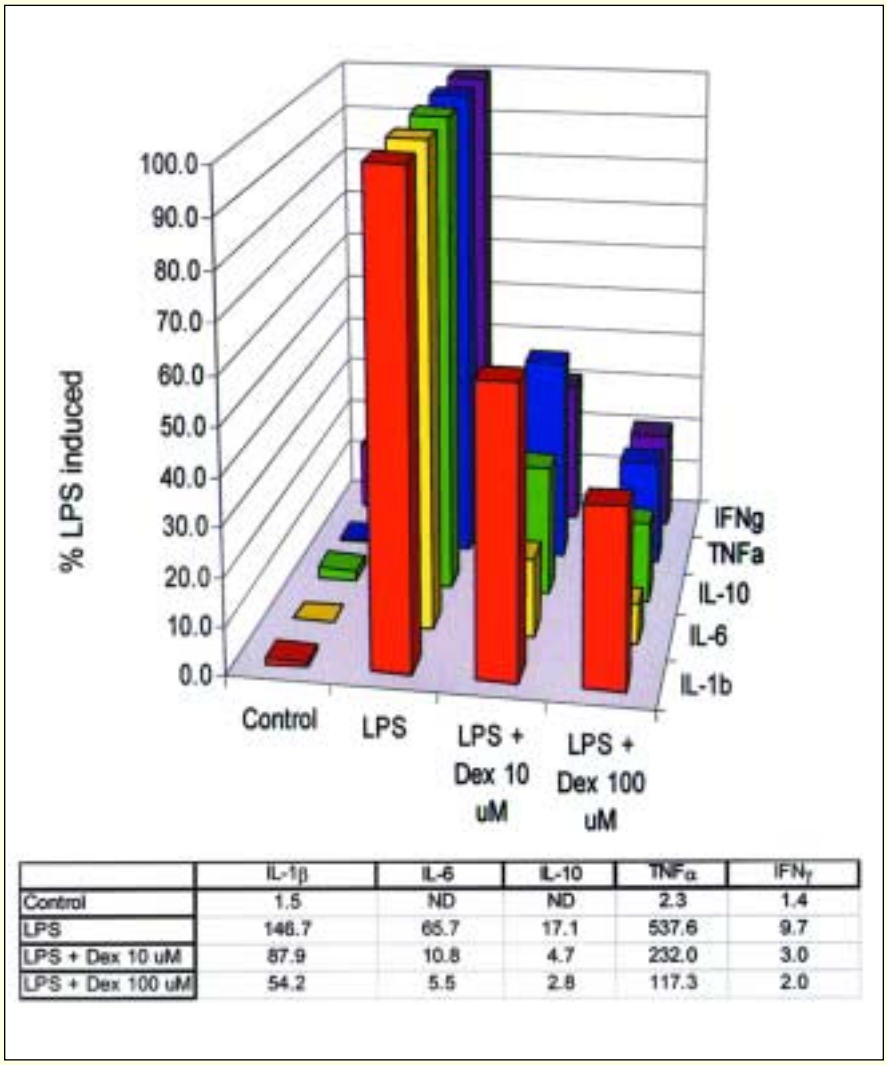


Figure 4. Intra-plate reproducibility of cytokine mini-arrays. CCD camera image of mini-array plate tested with the all seven cytokines—TNF-α, IFNα, IFNγ, IL-1α, IL-1β, IL-6, and IL-10—added together to 16 wells of an individual mini-array plate.



	IL-1β	IL-6	IL-10	TNF_α	IFN_γ
Control	1.5	ND	ND	2.3	1.4
LPS	146.7	65.7	17.1	537.6	9.7
LPS + Dex 10 uM	87.9	10.8	4.7	232.0	3.0
LPS + Dex 100 uM	54.2	5.5	2.8	117.3	2.0

Figure 5. Mini-arrays monitor the anti-inflammatory effects of dexamethasone. THP-1 cells (80 000 cells/well) were treated with 100 µL media containing 5 µg/mL LPS, 5 µg/mL LPS plus 10 µM dexamethasone, or 5 µg/mL LPS plus 100 µM dexamethasone (control cells in culture media only were also prepared). After 6 h incubation at 37°C with 5% CO_2, the media were removed from the plate, and 50 µL media samples were analyzed in the mini-arrays. Values plotted represent the picogram/milliliter concentration of duplicates normalized as the percentage of the concentration of the LPS-stimulated positive control cells.

Table 3. Mini-Array Quantification of Cytokines Produced by ConA/PHA-Stimulated PBMCs

Hours after Induction	IL-1α	IL-1β	IL-6	IL-10	TNF-α	IFNα	IFNγ
0	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
1	0.8	0.7	0.8	ND[a]	2.8	ND[a]	ND[a]
2	0.7	2	41	ND[a]	25	ND[a]	1.7
3	1.4	7.5	224	0.7	54	ND[a]	2.5
4	1.5	16	291	1.3	96	ND[a]	2.2
5	3	40	343	6	124	ND[a]	3.6

Cells were stimulated with 10 μg/mL PHA and 10 μg/mL ConA, and samples of media were removed from the cells at 1-h intervals. The media samples (12.5 μL) were mixed with 37.5 μL PBS and analyzed in the mini-arrays. Values listed are the cytokine concentrations (pg/mL) and represent the average of the duplicates.
[a]ND, not detected.

DISCUSSION

The concept of using arrays of antibodies in microarray-based studies of proteins was first described more than 10 years ago when Ekins et al. (4,5) described multianalyte microspot immunoassays that would use very small amounts of antibodies printed as spots in dense arrays. Since that time, the development of biochips for the multiplexed analysis of proteins has become a subject of much interest (13). Much of the effort in the development of protein biochips has focused on high-density devices. MacBeath and Schrieber (9) recently described the printing of an array of more than 10 000 protein spots on a glass slide. They demonstrated how such an array could be used to screen for protein-protein interactions and to identify substrates of protein kinases and the protein targets of small molecules. Other researchers have described arrays containing thousands of proteins (8) or single-chain antibody fragments displayed in phage libraries (3) to be used for the high-throughput screening of antibody libraries.

While these high-density arrays have demonstrated the ability to detect qualitative interactions between proteins, other researchers have focused on methods for the multiplexed quantification of proteins. Mendoza et al. (11) described the printing and detection of antibodies spotted in arrays of 144 spots/well in a 96-well format. The antibodies in the spots were detected by the addition of biotinylated mAbs directed toward the different species of IgG printed in the array, followed by the addition of a SA-alkaline phosphatase conjugate and a precipitating fluorescent substrate. Schweitzer et al. (12) have demonstrated a microarray assay for human IgE using the rolling circle amplification (RCA) reporter system. This "immuno-RCA" method had a sensitivity of 1 pg/mL and may prove to be a useful system for multiplexed protein measurement.

As this review of the recently published work reveals, an array-based method that combines the sensitivity and precision required for high-throughput, multiplexed, and accurate measurement of biologically relevant changes in cytokine levels has yet to be described. Our results show that the mini-arrays described in this report have the desired sensitivity, precision, and reproducibility to provide robust performance in a variety of proteomics applications. In addition, the multi-well format described here makes the application of this proteomics technology feasible for high-throughput drug screening.

The signal amplification provided

by the use of a chemiluminescent substrate provides the sensitivity needed to measure cytokines at concentrations as low as 1 pg/mL. The sensitivity of these mini-arrays compares favorably with the sensitivity of conventional colorimetric cytokine ELISAs. The use of a chemiluminescent substrate also provides a convenient method to collect data from 864 spots by imaging the entire plate with a CCD camera. Because the size of the light image from the spots increases as the concentration of the target protein increases, light from adjacent spots will overlap if the spots are placed too close together. To achieve a wider dynamic range, we found that it is necessary to separate the spots by at least 1.25 mm and limit the number of spots to nine spots/well.

Because the performance of any assay system is limited by its precision and reproducibility, we have developed mini-array ELISAs with performance characteristics that would allow us to see biologically relevant changes in cytokine concentrations. The CV for the signal averaged less than 10% for comparisons of mini-arrays within a single plate or between plates (Table 1 and Table 2). Evidence that this level of performance is acceptable for detecting biologically relevant changes in cytokine expression was demonstrated by the mini-array's ability to measure the induction time course of cytokines secreted from ConA/PHA-stimulated human PBMCs (Table 3). The mini-arrays were able to simultaneously show an increase in cytokine levels during the 5-h incubation that ranged from 0.8 to 346 pg/mL for IL-6 and from 0.8 to 3 pg/mL for 1L-1α.

To demonstrate how the mini-arrays could provide data useful for drug screening purposes, we tested the ability of the arrays to measure changes in the cytokine profiles of human THP-1 cells induced to produce inflammatory cytokines in the presence of a known anti-inflammatory compound, dexamethasone (Figure 5). The treated cells were cultured in a 96-well plate, a format that permits the use of robotic liquid-handling equipment to directly transfer a sample from the wells of the cell culture plate into the wells of the mini-array plate. The mini-arrays were able to detect the LPS-induced increases in the concentration of IFNγ, TNF-α, IL-1β, IL-6, and IL-10 in the media from the treated cells. When media samples from wells containing both LPS and dexamethasone were tested, a reduction in the amount of these cytokines was observed, and this reduction was greater in samples from the cells treated with a greater concentration of the drug.

In conclusion, these cytokine mini-array assays have sensitivity and precision comparable to traditional ELISAs, and we believe that they provide an example of how array technology can be a valuable tool for proteomic analysis.

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