

# Automated multiplexed cytokine bead assays

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**Automated multiplexed serum cyto-kine assays result in greater precision by reducing human intervention. This adaptation has increased the sensitivity of the assay by an order of magnitude to the sub-picogram/mL range for the majority of the 24 cytokines measured. Furthermore, the robotic routine has increased the assay throughput and precision while processing cytokine panels capable of discriminating healthy controls from rheumatoid arthritis (RA) patients.**

Adapting complex and high-throughput technologies, such as arrays and bead-based systems, to robotic platforms is increasingly important in the translation of medical research advances into practical clinical utility. One such rapidly expanding field of medical research is that of cytokines. Together with chemokines and growth factors, cytokines are critical mediators in both healthy and diseased immune systems. Reproducible, quantitative testing for aberrant quantities of these molecular signalers may correlate with clinical data that measure the disease state or monitor the efficacy of a treatment regimen. Such measurements could also potentially serve as diagnostic or prognostic indicators, and the molecules being measured are the focus of several new pharmaceutical efforts to treat a range of autoimmune disorders [1, 2, 3].

Recent, bead-based techniques offer the ability to measure several of these proteins in parallel from a single sample. One method for the separation of bead classes uses the light signatures from two internally doped dyes [4, 5]. By coupling unique sets of captured antibodies to individual bead classes, multiple reaction surfaces for immunosandwich assays can co-exist in the same well, separable by their light address. Commercially-produced kits and protocols for intra-laboratory fabrication of multiplex bead assays are available [6, 7].

The sensitivities reported for the current protocols are based on a manual routine, which is prone to human errors such as inadvertent cross-contamination and undetectable pipetting variation. However, the protocol includes several processes that can be controlled more accurately by automated routines. For example, inter-assay differences in the microtitre plate setup times, incubation periods, and mixing conditions can introduce variation into the protocol. Many automated systems are capable of tracking samples and interfacing with secure databases that provide the additional advantages of data quality assurance, security, and reliability [8].

In this report, we describe a strategy used to adapt an inflammatory cytokine monitoring panel (ICMP) [9, 10] to an automated platform, improving not only the quantitative capability of the protocol, but also the process of generating, analysing, and storing data from this multiplexed high throughput assay.

## AUTOMATED PLATFORM AND LABWARE

The ICMP protocol was set up on the Biomek FX automated liquid handling system. This system reports liquid handling errors of  $\pm 1\mu\text{L}$ ; the exact timing of its actions is programmable within the controller software. The system is equipped with a 96-channel head (which also houses a gripper arm) capable of transferring reagents to an entire microplate, and a Span-8 head for well-specific single sample pipetting ("cherry-picking"). The automation system was fitted with special equipment and labware for the ICMP. A vacuum filtration system (Millipore Cat. No. MSVMHTS00) used for bead washing was connected to a microprocessor-controlled valve unit with a regulator in order to toggle the vacuum on and off at a pre-determined vacuum strength (10.18 in-Hg) via software commands. Vacuuming the reaction through a filter plate provides both the means of terminating one phase of the protocol and washing in between phases of the protocol. The correct vacuum filtration system was essential for obtaining efficient, clog-free evacuation of the assay filter plate. Filter plates with a hard polystyrene skirt were used (Millipore Cat. No. MSBVS1210) to ensure correct placement of the plate on the vacuum collar and consistent sealing during unattended operation. A stacker carousel helped maintain a clean working surface by preserving space on the robotic deck, giving the flexibility to run multiple ICMP assays in parallel to increase throughput.

## MULTIPLEXED CYTOKINE PROFILING PROTOCOL

The ICMP is an immunosandwich assay that occurs on the surface of 5.6  $\mu\text{m}$  polystyrene microspheres [7]. Different reagent sets, either bought commercially or synthesised in-house, are compatible with the automation routine. The routine involves three separate reactions: primary antibody capture, secondary antibody binding, and fluorescent labelling. Each reaction step takes place in a filter plate and is preceded and followed by a washing protocol. A generic protocol depicting the robotic tasks in the automated protocol was programmed into the Biomek software and is publicly available (see supplementary materials). In addition, there is a detailed explanation of the protocol along with the labware utilised on the Biomek FX deck for these routines.

## REAGENTS AND SETUP FOR MANUAL VERSUS AUTOMATED PRECISION TESTS

The bead set used in this study was created by coupling capture antibodies from four ELISA kits (from BD Biosciences) to four separate bead sets (Luminex). To preserve inter-assay comparability, the diluent and buffer set for this test was taken from the same commercial kit (Biosource). A stock solution of the four recombinant proteins (TNF- $\alpha$ , IL-7, IL-1 $\alpha$ , IL-1 $\beta$ ) was prepared at 20 ng/mL for each cytokine. Recombinant standards were serially diluted to 10 point standard

curves (using 1:3 dilutions) in replicates of eight, including blanks. Each 88-well setup was performed both manually and by the automated system to compare the intra-assay variability of the two protocols.

### REAGENTS AND SETUP TO DISCRIMINATE RA PATIENTS FROM HEALTHY CONTROLS

A commercially produced Luminex bead kit capable of measuring 24 cytokines, chemokines, and growth factors in serum served as the reagent set. The 13 RA patients used in this study were defined by 1997 American College of Rheumatology (ACR) criteria, while the 31 healthy controls were age and sex-matched. The resulting data were analysed in an effort to discriminate the patient class from healthy controls.

### STATISTICAL ANALYSIS

Precision data were evaluated by comparing the observed concentration to the expected concentration, thereby creating residuals. This resulted in 80 measurements for each cytokine that were used to create an overall variance statistic. These statistics were computed for both the manual and automated assay; they were compared via an F-test with  $p < 0.05$  considered significant. The cytokine data gathered were analysed with discriminant function analysis (DFA) to classify healthy control and RA patients into two groups [11]. (DFA is a data reduction strategy similar to principle component analysis, PCA [12]). Briefly, the DFA maximally separates data sets based upon *a priori* group information (RA vs. control) by creating a linear combination of the variables, denoted as "root". The group differentiation becomes intuitive when the roots are plotted; their relative position visually depicts their relationship to the other group.

### RESULTS

The variance of the fluorescent signals at each serial dilution point ( $n=8$ ) was compared between the robotic and manually processed protocols for four cytokines (TNF- $\alpha$ , IL-7, IL-1 $\alpha$ , IL-1 $\beta$ ). The same set of recombinant standards and reagents were used in both automated and manual assays. The precision of the robotic assay was found to be statistically much higher than the manual assay for all four cytokines measured. The automation routine allowed for higher confidence in measurements near background levels so subsequent experiments were processed by the robot. The robotic routine effectively lowered the detectable limit measured for each cytokine in comparison to the manufacturer's reported sensitivity [Table 1]. This lower detectable limit is partially responsible for the enhanced discriminatory abilities of the protocol. Using the improved cytokine sensitivities, the DFA was able to clearly separate the RA profiles from healthy profiles.

### CONCLUSION

The mechanisation of the bead-based immunosandwich protocol resulted in lower limits of detection and improved assay precision for the analytes measured. Furthermore, these improvements enabled a correlation to be established between measured cytokine panels and the confirmed clinical diagnosis of rheumatoid arthritis in a small test cohort. Difficulties in controlling protocol timing, differences in bead mixing strategies, and the large number of pipetting operations all contribute to the potential for manually-introduced variation. The significance of

Cytokine	Automated Detectable Limit in pg/mL	Manufacturer's Detectable Limit in pg/mL	% Improvement
IL-1 $\beta$	2.36	15	535
IL-1R $\alpha$	1.55	30	1835
IL-2	0.31	6	1835
IL-2R	4.56	30	558
IL-4	0.23	5	2074
IL-5	0.53	3	466
IL-6	0.35	3	57
IL-7	15.1	10	-34
IL-8	0.14	3	2043
IL-10	4.92	5	1.6
IL12p40p70	0.77	15	1848
IL-13	0.75	10	1233
IL-15	0.42	10	2281
IL-17	0.45	10	2122
TNF $\alpha$	2.97	10	237
IFN $\alpha$	18.5	15	-19
IFN $\gamma$	0.39	5	1182
GM-CSF	3.41	15	340
MIP-1 $\beta$	3.1	10	223
IP-10	2.36	5	112
MIG	0.11	4	3536
Eotaxin	1.58	5	216
RANTES	0.41	15	3558
MCP-1	1.36	10	635

Table 1. The detectable limit of recombinant standard in pg/mL was determined by interpolating the lowest concentration from a five point logistic curve, which was at least three times the standard deviation above the zero-standard (background). The use of an automated robotic procedure enabled significant improvement in detectable limits.

adapting complex routines to robotic platforms partly lies in the reduction of such variations introduced into the protocol by these mechanisms.

### SUPPLEMENTARY MATERIALS

Supplementary information, appendices, and a video capture of the Biomek FX are available online at: <http://microarray.omrf.org/publications/2005/cockrum/Automation>. We would like to acknowledge and thank Parima Pathipvanich for creating the webpage.

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