

Development of an 8-plex Cytokine Assay for the AVANTRA™Q400 Biomarker Workstation, a Novel, Automated Multiplex Immunoassay Platform from Decision Biomarkers

Susan Myerow, Laurie Livshin, Cynthia Lessard, Al Girard, Tonya Black, and Peter Maimonis.
Decision Biomarkers, Inc., 150 Bear Hill Rd., Waltham, MA 02451

Abstract

Decision Biomarkers, Inc. (DBI) has developed a novel, automated, immunoassay platform to simultaneously evaluate multiple biomarkers in a single reaction vessel. Biomarker panels are used for many research and clinical indications. We have generated data from a multiplex assay measuring a panel of 8 cytokines: IL-1 β , IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, and TNF α . The heart of the system is a microarray of the biomarker specific monoclonal antibodies spotted onto a glass slide which has been coated with an ultra-thin, two-dimensional film of nitrocellulose. This microarray is assembled in a plastic biochip (MAX BIOCHIP™): a self-contained device with a series of microfluidic channels, valves, sensors, and reagent chambers containing dried, biotinylated detection antibodies, streptavidin-conjugated DyLight™, and wash buffer. The biochip is processed in the AVANTRA™Q400 biomarker workstation following injection of 200 μ l of sample (100 μ l of plasma diluted in 100 μ l of diluent). The instrument initiates, in sequence, the flow of sample, detection antibody, label, and buffer, across the array. The end result is in a fluorescent labeled complex that is then imaged by an internal CCD camera. Spot intensity is correlated with analyte concentration by instrument software. "Master" curves" electronically stored in the instrument are used to calculate analyte concentrations through a 2-point calibration scheme. Each cytokine assay has a 3-4 log dynamic range and individual analytical sensitivities ranging between 2.7 and 20 pg/ml. Both the intra- and inter-assay precision results yielded mean CV's consistently <15%. Reproducibility was demonstrated by overlapping curves following replicate standard evaluation. The unique surface chemistry, marker specific reagents, and enhanced biochip microfluidics and instrument automation, all combine to produce a robust and easy to use multiplex immunoassay.

Currently in development are a 10-plex angiogenesis panel and a 12-plex cardiac panel that will soon be available. Additionally, assays for numerous other biomarker panels are slated for future development and custom assay development is also available.

In summary, DBI has developed a sensitive and precise, multiplex immunoassay for 8 cytokines that can run on its new, automated platform, the AVANTRA™Q400 biomarker workstation.

Introduction

DBI has developed a novel immunoassay system, the AVANTRA™Q400 biomarker workstation. It simplifies the user interface and reduces instrument size, eliminating the need for expensive robotics. At the heart of this platform is a biochip, a self-contained reaction cassette for assay processing. This disposable cassette contains all of the reagents required to perform a multiplex immunoassay, with a capacity of analyzing 20 biomarkers per biochip. Within the assay chamber, all relevant parameters are controlled by the AVANTRA™, including incubation times, reagent flow rates, assay temperature, and detection protocols. The overall system is capable of automatically performing multiplex immunoassays with minimal investigator intervention.

Materials and Methods

Reagents

MABs specific for IL-1 β , IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, and TNF α , and their complementary, biotinylated antibodies, were purchased from R&D Systems, (Minneapolis, MN), Biologend, (San Diego, CA), PeptoTech (Rocky Hills, NJ), Diaclone (Besançon, France), and BD Biosciences (San Jose, CA). Streptavidin-DyLight was purchased from Pierce (Rockford, IL).

Spotted microarrays

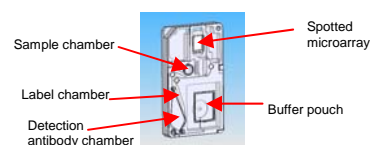
Standard glass microscope slides were first treated with tantalum oxide to provide an adhesive layer to the surface. Treated slides were then coated with a thin film (< 1 μ thick) of nitrocellulose (NC), dried, and exposed to corona treatment.

A Piezoarray System (Perkin Elmer) was then used to spot 300 pl of individual capture MABs in a rectangular array on the NC surface. The slides were then dried, blocked and assembled into biochips.

Biochip

The MAX BIOCHIP™ (Fig. 1) is an injection-molded piece of black polycarbonate. It incorporates a self-enclosed pouch containing phosphate-buffered saline with 0.05% Tween-20 (PBS-T). Three chambers are present; one holds a cocktail of dried, biotinylated, detection antibodies, a second chamber holds dried, DyLight labeled streptavidin, and a third holds injected sample. All chambers are connected by a series of channels (average cross-sectional area of 0.25 mm²) interspersed with a succession of sensors and valves to detect and direct fluid flow. The reagents are solubilized by buffer and sequentially flow across the array in the reaction chamber. Spent reagents flow into 2 waste receptacles. All liquid is self-contained within the biochip.

Figure 1. Schematic diagram of a MAX BIOCHIP™



Instrument

The AVANTRA™Q400 biomarker workstation (Fig. 2) contains all the electronics and mechanics necessary to direct fluids through the biochip at the proper time and in the proper sequence. No fluid flows through the instrument itself. A heating element maintains the reaction temperature at 37°C. The instrument also contains a cooled CCD camera that images the array at assay completion. A PC workstation connects to the instrument to process data.

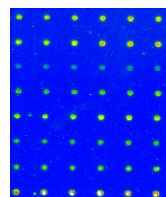
Figure 2. Two AVANTRA™Q400 workstation instruments linked together.



Assay

Arrays are spotted with 8 MABs, at initial concentrations of 0.5 mg/ml, and arranged in rows of 8 for each analyte. The sample is first diluted and then injected into a port in the biochip, from where it is pumped by the instrument across the array. The diluent contains reagents that block potential assay interfering substances. The biotinylated MABs are then solubilized by the buffer flow and directed over the array, followed by the fluorescent label. A final buffer wash completes the assay. The array is then imaged and analyzed for fluorescent intensity using in-house developed software. A TIF file of an actual image of a fully developed multiplex assay is illustrated in Fig. 3

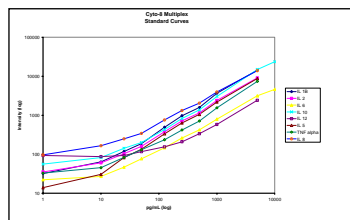
Figure 3. Image of a cytokine 8-plex assay run with a 5000 pg/ml standard



Results

A series of multi-standards, each containing all 8 cytokines, was evaluated to generate master curves. Standards, starting at 5,000 pg/ml, were prepared by serially diluting a stock cocktail of analytes in a diluted goat plasma matrix.

Figure 4. Standard curves for an 8-plex cytokine assay



Precision

Precision was evaluated by replicate analysis of identical samples. Both spot to spot and biochip to biochip precision were calculated. The mean and %CV for the low end of the standard curve, 25 pg/ml, is shown in Table 1 below.

Table 1. Precision at 25 pg/ml

Spot to Spot		Biochip to Biochip	
Cytokine	%CV	Cytokine	%CV
IL-1 β	5.7	IL-1 β	10.9
IL-2	9.5	IL-2	9.5
IL-5	8.6	IL-5	9.6
IL-6	5.3	IL-6	10.3
IL-8	3.9	IL-8	14.0
IL-10	4.3	IL-10	8.5
IL-12	2.4	IL-12	7.4
TNF α	4.3	TNF α	17.5

Analytical sensitivity

Analytical sensitivity was ascertained by evaluating replicates of the zero standard, calculating the mean and SD of those values, and determining the concentration equivalent to 2 SD's above those means. Representative results are shown in Table 2.

Table 2. Analytical sensitivity

Cytokine	pg/ml
IL-1 β	3.0
IL-2	4.4
IL-5	8.7
IL-6	18.3
IL-8	3.5
IL-10	10.3
IL-12	4.6
TNF α	15.3

* Continued refinements are progressively improving the analytical sensitivity, with the goal of achieving <10 pg/ml for all 8 cytokines.

Assay Interferents

To minimize assay interference from rheumatoid factors present in human plasma, a novel diluent, with various blocking agents added, was developed. A human plasma sample with high levels of rheumatoid factor (3090 IU/ml) was tested with old and new diluent. Table 3 shows that the interference seen from rheumatoid factor is eliminated using the new diluent

Table 3. Diluent effect on high RF sample

Cytokine	Old Diluent (pg/ml)	New Diluent (pg/ml)
IL-1 β	24	4
IL-2	12	0
IL-5	65	4
IL-6	914	28
IL-8	0	5
IL-10	0	4
IL-12	8	13
TNF α	9	2

Conclusions

Using standard immunoassay formats, we have developed an 8-plex cytokine assay for the AVANTRA™Q400 biomarker workstation. Our proprietary thin-film nitrocellulose coated glass provides a uniform surface that combines the high protein binding capacity of standard nitrocellulose with the low background fluorescence of thin films. Microfluidic delivery minimizes sample and reagent requirements while the instrument maintains constant flow rates and reaction times. Assay performance for each analyte is thereby optimized, with precision, sensitivity, and dynamic range all comparable to those of single analyte ELISA's. There is no interference from rheumatoid factor in the sample. This multiplex assay platform is flexible in that additional analytes can be added to the array on the same biochip. The simplicity of operation of the system lends itself to a variety of biomarker applications, ranging from drug discovery and development to clinical trials. Custom assays can be developed on our iMARK BIOCHIP™ and will take advantage of the unique offering of the AVANTRA™Q400 biomarker workstation.

Summary

- DBI has developed the AVANTRA™Q400 biomarker workstation, an automated, multiplex immunoassay platform using novel surface chemistry, microfluidic technology and fluorescent detection
- Our 8-plex cytokine assay for this system is now available.
- Intra & inter-assay precision consistently yields <15% CV's
- Analytical sensitivities are within standard requirements for measuring cytokine levels in blood
- Assay dynamic ranges span 3.5-logs for each cytokine
- A novel diluent virtually eliminates assay interference from plasma factors
- The instrument allows for a "sample to results" process in 1 hour without any manual intervention



For additional information about Decision Biomarkers, the AVANTRA™Q400 biomarker workstation, or the MAX BIOCHIP™, please contact:

Decision Biomarkers, Inc.
150 Bear Hill Road
Waltham, MA 02451
Phone: (877) 890-2006 x203
sales@decisionbiomarkers.com

Or visit: www.decisionbiomarkers.com