

Development of a 3-plex Cytokine Assay for the AVANTRA™Q400 Biomarker Workstation, a Novel, Automated Multiplex Immunoassay Platform from Decision Biomarkers

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Abstract

Decision Biomarkers, Inc. (DBI) has developed a novel, automated, immunoassay platform to simultaneously evaluate multiple biomarkers in a single reaction vessel. As initial proof of principle, we have generated data from a multiplex assay measuring a panel of 3 cytokines: IL-1 β , IL-6, and TNF- α . The heart of the system is a microarray of marker 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 polyclonal antibodies, streptavidin-conjugated Cy3, and wash buffer. The array containing biochip is processed in the AVANTRA™Q400 biomarker workstation following injection of 100 μ l (diluted 1:2) of a plasma sample. The instrument initiates, in sequence, the flow of plasma, detection antibody, label, and buffer, across the array performing a traditional immunoassay that results in a fluorescently labeled complex. An internal CCD camera then images the labeled array and output is correlated with analyte concentration by instrument software. Typical standard curves for each biomarker are shown below (each point is a mean of 3 replicates). These "master" curves are electronically stored in the memory of the instrument and are then used to calculate analyte concentrations by means of a 2-point calibration scheme. Each cytokine assay has a dynamic range from 0 up to 5000 pg/ml and an individual analytical sensitivity ranging between 5 and 20 pg/ml. Both the intra- and inter-assay precision yielded mean CV's consistently <10%. 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 is an 8-plex cytokine assay that will be available in 2Q2006; additionally, a number of assays for numerous other biomarker panels are slated for development in 2006. Custom assay development is also available. In summary, DBI has developed a sensitive and precise, multiplex immunoassay for 3 cytokines that can run on its new, automated platform, the AVANTRA™Q400 biomarker workstation.

Introduction

Historically, biomarkers have been tested individually to ascertain their value, with the traditional ELISA being the workhorse of analysis. However, as the concept of evaluating biomarker panels gains acceptance, multiplexing capability will become the critical parameter in biomarker validation and testing. New technology capable of measuring multiple biomarkers in a single reaction vessel is still in its infancy and is still not without its limitations. Lack of automation is the overriding characteristic, necessitating extensive hands-on time and effort, thereby reducing reliability and driving up overall costs.

To address some of the shortcomings of current multiplex biomarker testing, DBI has developed a novel immunoassay system, the AVANTRA™Q400 biomarker workstation. It simplifies the user interface while reducing instrument size and eliminates the need for expensive robotics. At the heart of the 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 theoretical limit of analyzing 40 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.

Materials and Methods

Reagents
MABs specific for TNF α , IL-1 β , and IL-6, and their complementary, biotinylated polyclonals (PABs), were purchased from R&D Systems, (Minneapolis, MN). Streptavidin-Cy3 was purchased from GE HealthCare, (Piscataway, NJ). Superblock® was purchased from Pierce (Rockford, IL).

Spotted microarrays

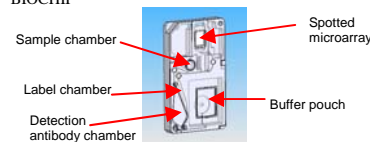
Standard glass microscope slides were first treated with tantalum oxide to provide an adhesive layer to the surface. Treated slides were then coated with a thin film (< 1 μ thick) of nitrocellulose (NC), dried, and exposed to corona treatment.

A Piezoarray System (Perkin Elmer) was then used to spot 300-400 pl of individual MABs in rectangular arrays on the NC surface. The slides were then dried, blocked with Superblock, cut into ~1 cm² pieces, and assembled into the biochip.

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 holding a cocktail of dried, biotinylated, detection PABs, a second chamber holding dried, Cy3-labeled streptavidin, and a third acting as a receptacle for the 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 reagent flow continues toward the reaction chamber, which contains the spotted, NC coated array. Following the reaction, spent reagents flow into 2 large waste receptacles. All liquid is self-contained within the biochip. The only handling of reagents is during sample injection.

Fig. 1. Schematic diagram of a MAX BIOCHIP™



Instrument

The AVANTRA™ (Fig. 2) contains all the electronics and mechanics necessary to direct fluids through the biochip 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 CCD camera that images the array at assay completion. Images are processed by a PC workstation connected to the instrument.

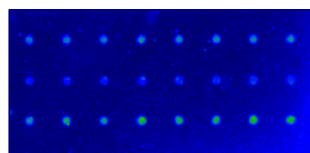


Fig. 2 Two AVANTRA™ instruments linked together.

Assay

Arrays are spotted with MABs specific for each cytokine at initial concentrations of 0.5 mg/ml and arranged in rows of 8 for each analyte. The sample is first diluted 2x in PBS and injected into a port in the biochip, from where it is pumped by the instrument across the array. The biotinylated MABs are then solubilized by the buffer flow and directed over the array, followed by the Streptavidin-Cy3. A final buffer wash completes the assay in just over 60 minutes. The array is then imaged with a CCD camera incorporated in the instrument. The image is sent to a PC and analyzed for fluorescent intensity using in-house developed software. Fig. 3 is the TIF file of an actual image taken by instrument of a fully developed multiplex assay.

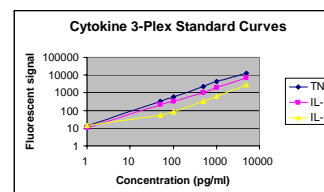
Fig. 3. AVANTRA™ image of a cytokine 3-plex assay for a 5000 pg/ml standard (enhanced color)



Results

A series of multi-standards, each containing all 3 cytokines, was evaluated to generate master curves. Standards were diluted serially from a stock cocktail of analytes, each starting at 5000 pg/ml, prepared in a diluted goat plasma matrix.

Fig. 4. Standard curves for a triplex cytokine assay. Each point represents the mean of 4 data points from 4 separate biochips.



Precision

Precision for each standard level, in replicates of 6, was also evaluated. The mean and %CV for representative standards of each cytokine is shown.

Table 1a. TNF α

pg/ml	5000	500	50
Mean	27692	9738	3310
SD	1397	302	91
CV	5.0	3.1	2.8

Table 1b. IL-1 β

pg/ml	5000	500	50
Mean	16820	6119	3150
SD	971	432	75
CV	5.8	7.1	2.4

Table 1c. IL-6

pg/ml	5000	500	50
Mean	7960	4127	2908
SD	280	167	30
CV	3.5	2.6	1.1

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 the mean. Initial sensitivity for the 3 cytokines ranged in value from 1.5 pg/ml (IL-1 β) to double digit values for the remaining analytes. Continued refinements to the assay are progressively improving the analytical sensitivity, with the goal of achieving <10 pg/ml for all 3 cytokines.

Conclusions

Using standard immunoassay formats, we have developed a 3-plex cytokine assay using our biochip arrays. Our 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. 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

- Decision Biomarkers, Inc. has developed the AVANTRA™Q400 biomarker workstation, an automated, multiplex immunoassay platform using novel surface chemistry, microfluidic technology and fluorescent detection
- We have demonstrated proof of principle of our platform by developing a 3-plex cytokine assay
- Intra & inter-assay precision consistently yields < 10% CV's
- Good analytical sensitivity
- Assay dynamic ranges span 3-logs for each marker
- The instrument will allow for a "sample to results" process without any manual intervention
- We are now developing additional assays for this system and can begin custom assay development with new collaborators



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

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