SQI 8-plex Quantitative Cytokine White Paper 2016

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Abstract

This report outlines the use of SQI's microarray Ig_plex™ technology to measure pro-inflammatory cytokines from human serum and plasma in a multiplexed configuration. The 8-plex assay demonstrates low end sensitivity and precision equivalent to, or better than current single-plex ELISA assays.

Introduction

Cytokines are small secreted extracellular signalling proteins that play a crucial role in regulating both cell mediated and humoral immune responses (1-4). They are involved in a number of biological processes including inflammation, allergic reaction and the response to infection. Abnormalities in cytokines and their receptors are characteristic of a variety of diseases, notably oncology, Alzheimer's, and autoimmune diseases. The rise in the number of cytokine modulating therapies offer promise for the treatment of various autoimmune disorders, allergies, asthma, cancers, and transplant rejection (5-8). The involvement of cytokines in such a broad range of responses drives the critical need for accurate measurement of cytokines in both research and clinical laboratories. Multiplexed cytokine assays may be especially valuable, as measuring several cytokines at once best mimics the reality of cytokines working in concert during in-vivo immune responses. Furthermore, both animal and patient sample volumes can be limited, especially in epidemiological and clinical trials.

Since most research projects and clinical trials involve the measurement of multiple cytokines, SQI technology presents a cost effective strategy versus single-plex and/or non-automated options. Multiple protein arrays and multiplexing techniques have been developed over the past 20 years to meet the needs of researchers, but none have achieved the ability to multiplex on a fully-automated, high-throughput system, with the consistent data quality that SQI offers. One such technique is the bead-based multiplexed format. Although this method allows researchers to theoretically measure up to 100 cytokines in a manual assay, there have been reports of lower sensitivity (9). Furthermore, the precision and reproducibility of these bead-based methods have not been well defined and remains questionable to date (10-12).

SQI Diagnostics is now offering a fully-automated, multiplexed cytokine panel to address the needs of both the research and the clinical laboratory. Up to 15 capture antibodies of a researcher's choice are reproducibly printed in a planar array in the bottom of each well of

a specialized 96-well plate. After SQI optimizes the general characteristics of the assay, the user can fine-tune his or her preferences on the SQI system to run the assay at their facility. The following Human Cytokine 8-plex microarray panel quantitatively measures the levels of 8 pro-inflammatory cytokines. These 8 cytokines were selected as being the most widely studied and pertinent cytokines available. The selected pairs of antibodies are extensively tested for sensitive detection and cross-reactivity to ensure the specificity of each capture/detection pair. The protein standards used with the kit were precisely calibrated to reference materials for maximum consistency. To demonstrate platform reliability, the assay was tested across multiple instruments, operators and study sites, thus demonstrating the consistency that is expected for longitudinal research projects and clinical trials.

Methods

To build the arrays, anti-Cytokine capture antibodies are dispensed in each well of a 96 well epoxysilane coated glass plates using a non-contact printing technology. The printed plates undergo post-print processes including curing and blocking. The print solutions and blocking solution are formulated to optimize analyte capture, reduce heterophilic interference and minimize non-specific binding to the capture antibodies. The SQI pro-inflammatory cytokine microarray panel includes:

- Interleukin (IL)-1β (IL-1β)
- IL-2
- IL-4
- IL-6
- IL-8
- IL-10
- Interferon (IFN)-γ (IFN-γ)
- Tumor Necrosis Factor (TNF)-α (TNF-α)

Each plate includes an 8-point standard curve for quantitation of the assay. Mixed 8-plexed assay standards made in specially formulated sample diluents have been referenced to WHO/NIBSC international standards and verified for accuracy against single analyte curves. Generated dose response curves over the range of 0.625 to 5000 pg/mL are automatically analysed with a 4 parameter logistic weighted curves to provide best fit and accurate result reporting.

The assay protocol involves incubation with 100 µL of prepared standards, or 1:2 dilution of plasma or serum in sample diluents. The plates are then washed and incubated with a mixed detector antibody cocktail comprising of biotinylated antibodies specific to each cytokine. The biotinylated antibodies are polyclonal or monoclonal antibodies that have been documented to work as non-overlapping pairs with their corresponding capture antibody. The antibody pairs used in this panel were selected by screening pairs from

numerous sources and selecting the pairs that gave the most sensitive and specific interaction. Following another wash step, a fluorophore-labeled streptavidin reporter is added, and after a final wash, the plates are read on a fluorescent scanner. Mean fluorescent intensity units are converted to quantitative pg/mL results via the reporting software.

Results

The performance of the multiplexed cytokine assay was validated using criteria of quantitative range and linearity, recovery, precision and proficiency testing (Tables 1-3).

Using known concentrations of each cytokine, a series of standard curves were generated by plotting the median fluorescent intensity (MFI) signal against concentration. These standard curves are then interpolated to determine the concentrations of unknown samples. The following multiplex standard curves were reproducibly obtained in SQI 8-plex Cytokine assay.

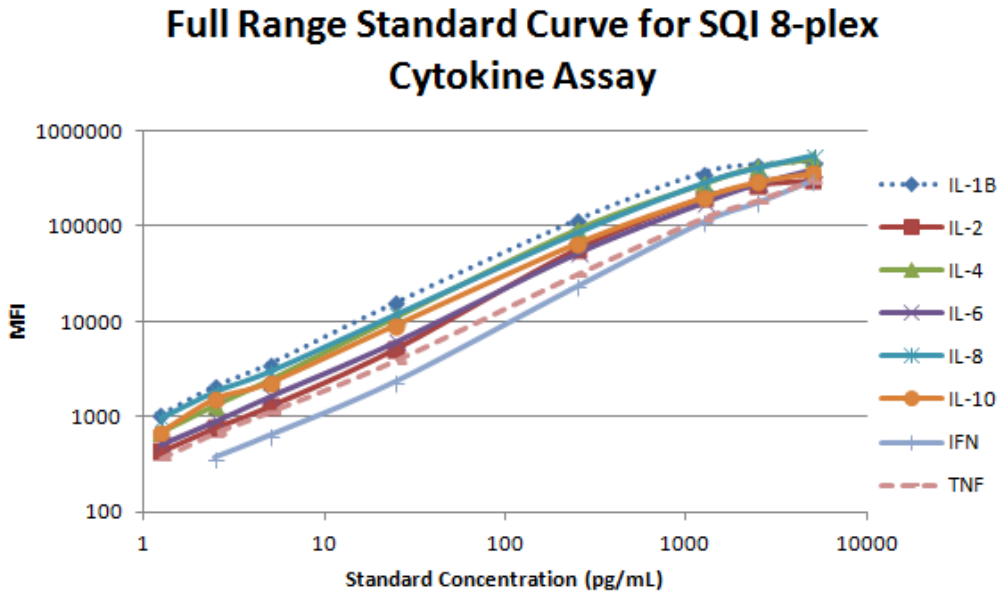

Figure 1: Representative standard curves demonstrating broad dynamic range.

The quality of the curve fit was confirmed using back-calculation of standards, and by spiking sample diluent with cytokine levels that are not included in the standard curve. The overall assay performance (recovery and precision) is summarized in Table 1. Spike recovery was calculated by adding known concentration of cytokines into normal serum or plasma. Assay sensitivity (LOD) for each cytokine was assessed by calculating the value at three standard deviations above the MFI of at least 8 replicates of the 0 pg/mL spike in sample diluents. The accuracy and

precision data at 1:2 sample dilution confirms the use of either serum or plasma specimen matrices for the assay.

To demonstrate a linear dose response in the selected matrix, recombinant standards (2500 pg/mL) were spiked into matrix and then serially diluted. The % linearity for each dilution of the sample is calculated by dividing the observed concentration by the previous concentration/dilution factor. The % linearity seen for each of the cytokines is shown in Table 2.
Proficiency samples (from College of American Pathologists) were obtained for each cytokine except IL-4. The results obtained using SQI assay matched the reported median results from other systems (Table 3).

Table 1: Performance Data for SQI Diagnostics 8-plex Cytokine Panel

	IL-1β	**IL-2**	**IL-4**	**IL-6**	**IL-8**	**IL-10**	**IFN-γ**	**TNF-α**
LLOD (pg/mL	0.26	1.01	0.31	0.62	0.73	0.37	2.5	0.77
LLOQ (pg/mL)	0.6	1.25	0.6	1.25	1.25	0.6	2.5	1.25
ULD (pg/mL)	5000	5000	5000	5000	5000	5000	5000	5000
%CV (intra-assay) (25pg/mL) (n=24)	8%	10%	7%	7%	6%	4%	15%	10%
%CV (inter-assay) (25pg/mL) (n=3)	11%	12%	9%	11%	11%	10%	21%	11%
% Recovery in 1:2 serum(tested 5,25,50,250pg/mL)	85-91%	80-103%	80-100%	90-100%	75-90%	80-95%	60-80%	70-85%
% Recovery in 1:2 plasma (Tested 5,25 and 50pg/mL)	85-95%	80-100%	70-90%	90-100%	75-90%	80-95%	60-80%	70-90%
WHO(NIBSC) : SQI	113%	104%	120%	103%	90%	87%	94%	74%
WHO standard product number	86/680	86/500	88/656	89/548	89/520	93/722	82/587	88/786

Table 2: Linearity of spiked cytokines in normal serum starting at 2500pg/mL

	IL1-b β	**IL2**	**IL4**	**IL6**	**IL8**	**IL10**	**IFN-g γ**	**TNF-α**
NS-2.5	103%	116%	98%	110%	120%	100%	n/a	110%
NS-5	91%	110%	105%	120%	144%	107%	84%	99%
NS-25	100%	102%	87%	100%	117%	105%	89%	102%
NS-250	107%	120%	110%	100%	96%	115%	112%	95%
NS-1250	110%	95%	79%	107%	103%	88%	128%	101%
NS-2500	102%	95%	99%	97%	93%	79%	93%	86%

Table 3: Agreement data for Proficiency testing samples

IL-1B	Target	Median	SQI	Agreement
Sample#1	<3.9	2	2	+
Sample#2	<3.9	2	1	+
Sample#3	58	57	54	+

IL-2	Target	Median	SQI	
Sample#1	<31.3	3	4	+
Sample#2	<31.3	0	3	+
Sample#3	500	626	820	+

IL-6	Target	Median	SQI	
Sample#1	<31.3	8	2	+
Sample#2	<31.3	13	1	+
Sample#3	81	84	104	+

IL-10	Target	Median	SQI	
Sample#1	<7.8	4	3	+
Sample#2	<7.8	3	1	+
Sample#3	115	175	316	+

TNF	Target	Median	SQI	
Sample#1	<15.6	3	5	+
Sample#2	<15.6	5	6	+
Sample#3	326	198	166	+

IFN	Target	Median	SQI	
Sample#7	285	157	390	+
Sample#8	588	339	893	+
Sample#9	128	58	152	+

IL-8	Target	Median	SQI	
Sample#4	<31.3	5	2	+
Sample#5	<31.3	4	1	+
Sample#6	547	624	848	+

Conclusion

SQI has developed and validated a quantitative cytokine assay that allows for the accurate measurement of 8 human cytokines in any matrix. The SQI microarray format provides both precision and sensitivity for all 8 cytokines.

Critical SQI assay elements vital to commercially available ultrasensitive multiplex cytokine assays are:

- Ability to determine levels of circulating cytokines in serum and plasma
- Extended dynamic assay range
- Reproducibility across users so as to produce comparable results in different sites using different systems,
- Reproducibility across lots to provide comparable results over time
- Agreement with COP proficiency test samples

SQI's cytokine accurate and precise RUO assay can be run on a fully automated "load and go" SqidLite platform or a semi-automated Sqid-x system. The assay was also designed with end user cost in mind, it is available at a significantly lower cost than other ultrasensitive multiplex assays. The SQI Diagnostic Human Cytokine 8-Plex Microarray is available for clinical evaluation and implementation into high throughput testing.

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