Quantitation of PD-1, PD-L1, and CTLA-4 in human melanoma using SMC® high sensitivity immunoassays

Anthony Saporita, Joseph Hwang, Matthew Lippold, Laila Faramarzi, Raad Gitan, Wen-Rong Lie, Brooke Gilliam, and Rick Wiese

Introduction

PD-1, PD-L1, and CTLA-4 function as immune checkpoints and play a central role in neoplastic cell immunosurveillance. Due to their function in down-regulating the immune system, they can suppress T cell inflammatory activity, which helps prevent autoimmune diseases. However, they can also prevent the immune system from killing cancer cells. Soluble forms of these cell surface proteins are currently being evaluated as potential biomarkers in the diagnosis, prognosis, and pathogenesis of several forms of cancer, such as melanoma. For this study, we first wanted to establish a baseline from healthy human serum and plasma samples using the MILLIPLEX® Human Immuno-Oncology Checkpoint Protein Panel 1 17-plex. Fifty percent of apparently healthy samples were quantifiable for CTLA-4. To achieve greater sensitivity, we developed the SMC® PD-1, PD-L1, and CTLA-4 high sensitivity immunoassays for the analysis of human serum and plasma from apparently healthy and melanoma samples. SMC® technology delivers ultrasensitive biomarker measurements in comparison to the limits of traditional sandwich ELISA immunoassays for low-abundant biomarker studies. The SMC® PD-1, PD-L1, and CTLA-4 assays consistently achieved LLoQ of 1.74 pg/mL, 0.78 pg/mL, and 0.1 pg/mL, respectively. More importantly, these ultrasensitive SMC® kits allowed quantitation of these cell surface proteins in plasma from both healthy and melanoma samples. Statistical difference in sample levels of CTLA-4 (p-value <0.01) and PD-1 (p-value <0.005) were observed in 12 melanoma samples as compared to 12 healthy samples. The integration of MILLIPLEX® multiplex assays and SMC® high sensitivity assays in a comprehensive workflow enables profiling of multiple analytes and a deeper understanding of immune response dynamics by accurately measuring low-abundant proteins, thereby providing a powerful non-invasive research tool for evaluating disease conditions.

Methods

SMC® Technology

SMC® immunoassays achieve high sensitivity assay performance while following a workflow like that of a traditional ELISA, as shown in Figure 1A. By combining a unique assay elution step and robust digital counting, researchers achieve improved signal-to-noise ratios over traditional immunoassay methods. The SMCxPRO® instrument provides enhanced quantification at both low and high levels of expression on one complete system.

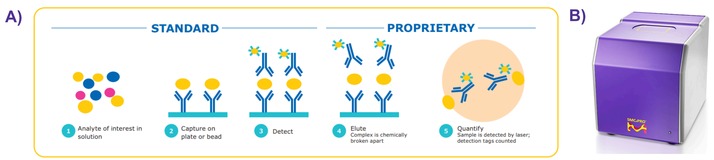


Figure 1: Ultrasensitive SMC® technology for the SMCxPRO® platform.
(A) SMC® immunoassays follow a simple assay protocol. Magnetic beads conjugated to a capture antibody bind to the analyte. A fluorescently-labeled detection antibody then forms a sandwich complex with the analyte and capture beads. Using a proprietary elution step, individual detection antibodies are counted to allow for ultrasensitive measurement.
(B) SMC® immunoassays are available for the SMCxPRO® instrument, which employs a scanning confocal laser to perform digital molecular counting.

MILLIPLEX® Multiplex Assays

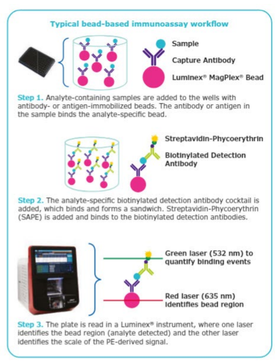


Figure 2: MILLIPLEX® immunoassay workflow
MILLIPLEX® assays use magnetic microspheres (beads) conjugated to capture antibodies. Each set of beads is distinguished by different ratios of two internal dyes yielding a unique fluorescent signature to each bead set, allowing researchers to simultaneously measure the analytes targeted by the capture antibodies. Native protein is analyzed by means of a sandwich immunoassay, pairing the capture beads with a biotinylated detection antibody.

Results

We examined biomarker concentrations in apparently healthy and melanoma plasma samples. While several analytes demonstrated potential as blood-based biomarkers of melanoma, the low abundance of some proteins necessitated the development of assays on a high-sensitivity platform using SMC® technology.

Figure 3: MILLIPLEX® Human Immuno-Oncology Checkpoint Protein Panel 1 17-Plex.
This figure shows the Immuno-Oncology Checkpoint Panel 1 (Cat. No. HCKP-11K) multiplex standard curves for the simultaneous quantification of these 17 analytes.

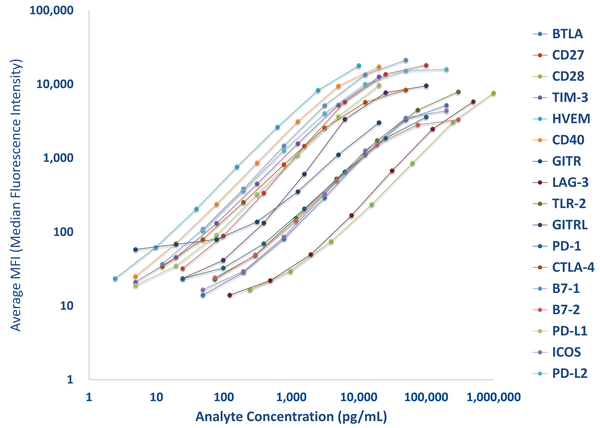
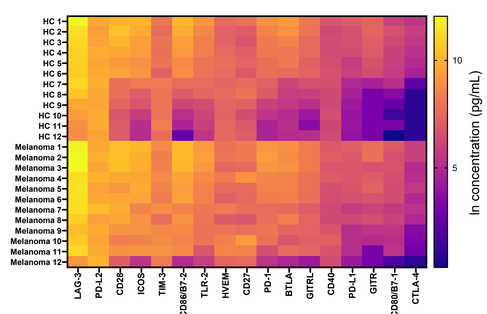


Figure 4: Plasma sample measurements using MILLIPLEX® Human Immuno-Oncology Checkpoint Protein Panel 1.
A cohort of 12 apparently healthy and 12 melanoma human plasma samples were measured with the MILLIPLEX® assay to determine the concentration of 17 analytes associated with immune checkpoints. The heat map below shows the relative concentrations of each analyte in each sample. Three proteins (CD27, CD40, and TIM-3) showed significant increases in melanoma plasma versus healthy controls by unpaired t test (p=0.0179, p=0.0095, p=0.0002, respectively). Significant differences were not observed by the MILLIPLEX® assay for three immune checkpoint proteins of particular interest: CTLA-4, PD-1, and PD-L1. In the case of CTLA-4, samples values were below the limit of detection for nearly half of the healthy controls (7 of 12 samples detectable). To achieve more sensitive detection for these three immune checkpoint proteins, assays were developed using ultrasensitive SMC® technology.



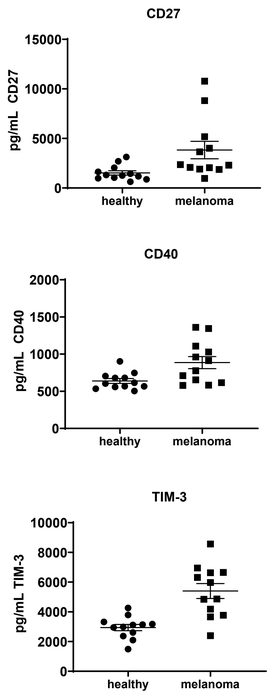
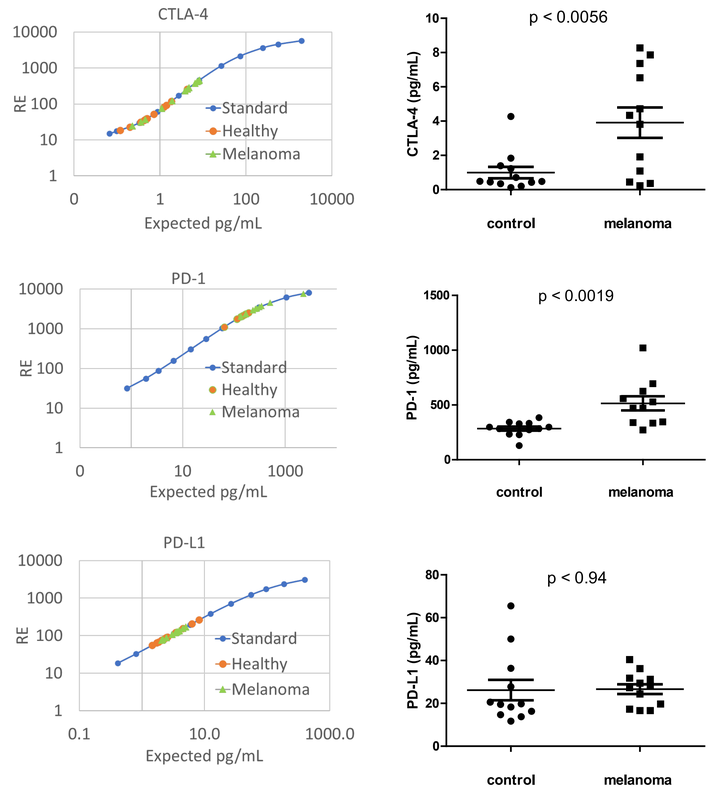


Figure 5: Plasma sample measurements using SMC® high-sensitivity immunoassay kits.
SMC® kits were run according to vendor protocols. Concentrations of CTLA-4, PD-1, and PD-L1 in 12 apparently healthy and 12 melanoma human plasma samples were determined using the SMC® CTLA-4 (Cat. No. 03-0220-00), PD-1 (Cat. No. 03-0207-00), and PD-L1 (Cat. No. 03-0208-00) kits. Standard curves were generated using the SMCxPRO® software and analyte concentrations were plotted on the standard curves. Sample data was analyzed using paired T-test.



Summary

Fit-for-purpose immunoassays give researchers the flexibility to investigate a broad selection of proteins related to various diseases. Here we used MILLIPLEX® technology to screen 17 proteins associated with immune checkpoints, revealing 3 targets (CD27, CD40, and TIM-3) increased in melanoma plasma samples versus healthy controls. Further investigation with high-sensitivity SMC® technology, an ultrasensitive, high-performance immunoassay platform enabling the measurement of previously undetectable proteins, revealed increases in 2 additional analytes in melanoma samples (CTLA-4, PD-1).

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