Development and Characterization of Human Immuno-Oncology Assays on a Multiplexed Electrochemiluminescence Platform

Jose Rodriguez-Nieves, Christopher Shelburne, David Cheo, Pu Liu, Laure Moller, Jon Buhrman, Svetlana Krepkiy, Simon Kwong, Sripriya Ranganathan, Ilia V. Davydov, Laura Schaefer, Jeff Debad, and Jacob N. Wohlstadter

Meso Scale Discovery, Rockville, Maryland USA

CONTACT INFORMATION: jbuhrman@meso-scale.com

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PURPOSE

Novel developments in cancer immunotherapy, also known as immuno-oncology, have driven increased demand for the sensitive measurement of biomarkers associated with cancer staging, the immune response, and drug targets. The levels of these biomarkers are frequently altered in samples from patients with cancer and can be evaluated by measuring their circulating concentrations. Here, we report the development of assays for 27 immuno-oncology analytes targeting both traditional and emergent cancer biomarkers for use with human samples. They include assays for important checkpoint markers such as PD-1, PD-L1, PD-L2, GITR/TNFRSF18, GITRL/TNFSF18, CD276/B7-H3, CD28, CTLA-4, and HAVCR2/TIM-3.

METHODS

Electrochemiluminescence Technology

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.

- Minimal background, combined with strong response to analyte, yields high signal-to-noise ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.

Immuno-Oncology U-PLEX® Protocol

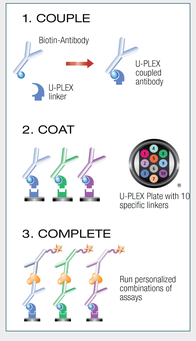
The U-PLEX assay platform uses 10 unique linkers that specifically bind to individual spots, enabling simple and flexible creation of multiplex immunoassays.

Couple and Coat the U-PLEX Plate:

- Add 200 µL of the biotinylated capture antibody to 300 µL of the assigned linker. Vortex. Incubate for 30 minutes.
- Add 200 µL of Stop Solution and vortex. Incubate for 30 minutes.
- Combine each U-PLEX-coupled antibody solution into a single tube and vortex. Add 50 µL of multiplex coating solution to each well.
- Incubate with shaking for 1 hour then wash the plate.

Complete the Assay:

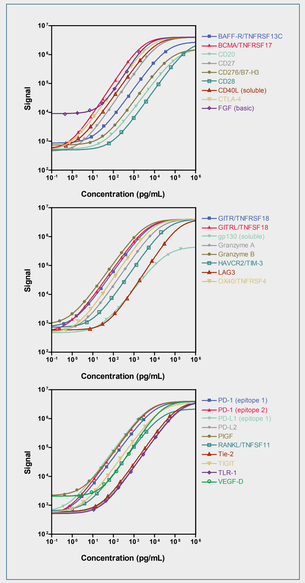
- Add 50 µL of sample, calibrator, or control to each well.
- Incubate the plate for 2 hours, then wash the plate.
- Add 50 µL of detection antibody solution to each well.
- Incubate the plate for 1 hour, then wash the plate.
- Add 150 µL of MSD™ Read Buffer to each well and read the plate.



RESULTS

Assay Characteristics

Calibrator curves, lower limit of detection (LLOD), and upper limit of detection (ULOD), for 27 human immuno-oncology assays are shown below. LLODs were calculated from 3 runs each with >20 blank wells. Control samples for each assay showed expected precision and accuracy, with intra-run CVs less than 10%, inter-run CVs less than 25%, and recoveries largely within 70-130% of target concentrations (data not shown).



Analyte	LLOD (pg/mL)	ULOD (pg/mL)
BAFF-R/TNFRSF13C	1.47	14,000
BCMA/TNFRSF17	0.14	600
CD20	5.38	80,000
CD27	0.77	3,400
CD276/B7-H3	4.89	40,000
CD28	13.9	144,000
CD40L (soluble)	0.33	1,800
CTLA-4	0.12	1500
FGF (basic)	2.03	1,200
GITR/TNFRSF18	0.18	1,300
GITRL/TNFSF18	0.10	1,000
gp130 (soluble)	6.50	188,000
Granzyme A	0.47	3,700
Granzyme B	0.11	750
HVACR2/TIM-3	2.47	9,500
LAG3	7.14	36,000
OX40/TNFRSF4	0.25	1,800
PD1 (epitope 1)	0.34	2,200
PD1 (epitope 2)	0.11	2,200
PD-L1 (epitope 1)	0.11	1,100
PD-L2	5.34	41,000
PIGF	0.21	1,200
RANKL/TNFSF11	1.92	4,000
Tie-2	3.31	29,000
TIGIT	0.48	3,500
TLR1	4.33	37,000
VEGF-D	0.42	1,500

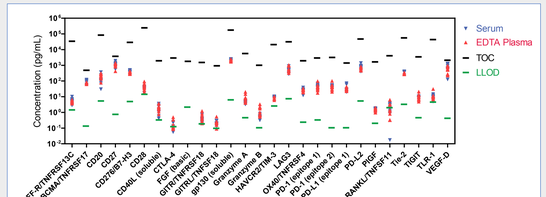
U-PLEX Biomarker Compatibility

Other assays in the U-PLEX product line were tested for compatibility with the new immuno-oncology assays using performance criteria such as dynamic range, sensitivity, sample detection, and non-specific binding between assays of <2%. 84 existing human U-PLEX assays were found to be compatible, creating a human U-PLEX immuno-oncology group with 111 biomarkers (see the table below) that can be used together in multiplexed panels.

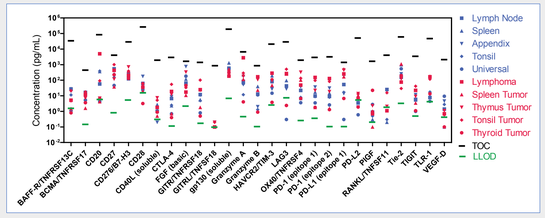
BAFF	EPO	GM-CSF	IL-17 A/F	IL-2Ra	MCP-1	Proinsulin
BAFF-R/TNFRSF13C	FGF (basic)	gp130 (soluble)	IL-17A	IL-3	MCP-2 (CCL8)	PYY (total)
BCMA/TNFRSF17	FGF-23	Granzyme A	IL-17C	IL-31	MCP-4	RANKL/TNFSF11
BDNF	FLT3-L	Granzyme B	IL-17D	IL-33	M-CSF	SDF-1α
CD20	Fractalkine	GRO-α (CXCL1)	IL-17E/IL-25	IL-4	MDC	Tie-2
CD27	FSH	HAVCR2/TIM-3	IL-17F	IL-5	MIF	TIGIT
CD276/B7-H3	G-CSF	I-309 (CCL1)	IL-18	IL-6	MIP-1α	TLR1
CD28	Ghrelin (active)	IFN-α2a	IL-1ra	IL-7	MIP-1β	TNF-α
CD40L (soluble)	Ghrelin (total)	IFN-β	IL-1α	IL-8	MIP-5	TNF-β
C-Peptide	GIP (active)	IFN-γ	IL-1β	IL-9	OX40/TNFRSF4	TPO
CTACK	GIP (inactive)	IL-10	IL-2	Insulin	PD-1 (epitope 1)	TRAIL
CTLA-4	GIP (total)	IL-12p40	IL-21	IP-10	PD-1 (epitope 2)	TSLP
ENA-78	GITR/TNFRSF18	IL-12p70	IL-22	I-TAC	PD-L1	VEGF
Eotaxin	GITRL/TNFSF18	IL-13	IL-23	LAG3	PD-L2	VEGF-D
Eotaxin-2	GLP-1 (active)	IL-15	IL-27	Leptin	PIGF	YKL-40
Eotaxin-3	GLP-1 (inactive)	IL-16	IL-29	LH	PP	

Native Sample Testing

Immuno-oncology assays were evaluated for the ability to detect their respective analytes in human serum and EDTA plasma samples. Sample concentrations (pg/mL) were plotted with the top of curve (TOC) and LLOD for each analyte. Samples were diluted 4-fold except for BCMA/TNFRSF17, gp130 (soluble), HVACR2/TIM-3 and Tie-2 assays where samples were diluted 100-fold. FGF (basic) was not detected in human serum and plasma samples. All other analytes were detected irrespective of the type of matrix.

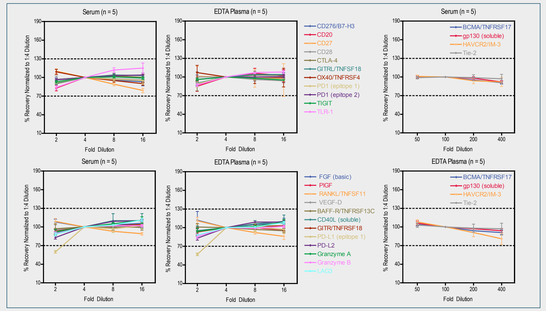


Human tissue lysate samples (5 µg) derived from different normal tissues (blue symbols) and tumor tissues (red symbols) were tested. Sample concentrations (pg/mL) were plotted with TOC and LLOD values for each analyte. All analytes were detected in most or all of the different tissue lysate samples.



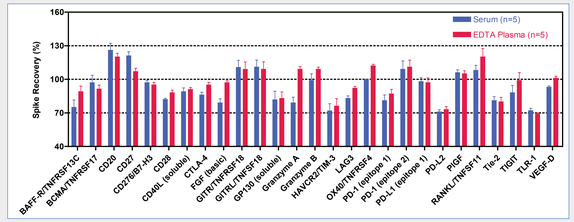
Dilution Linearity

Serum and EDTA plasma samples were spiked with calibrator and diluted 2, 4, 8, and 16-fold before testing. Sample concentrations were normalized to the 4-fold sample dilution. For BCMA/TNFRSF17, gp130 (soluble), HAVCR2/TIM-3 and Tie-2, unspiked samples were diluted 50, 100, 200 and 400-fold. Sample concentrations were normalized to the 100-fold sample dilution. All analytes recovered within 70-130% in each type of sample.



Spike Recovery

Normal human serum and EDTA plasma samples were spiked with calibrator at 3 levels (high, mid, and low). Spike recovery values for the three spike levels were averaged and plotted. Recovery of most analytes was within 70-130% in each sample type.



Assay Specificity

Human immuno-oncology assays were evaluated for interference and competition with homologous and/or related analytes. In addition, the CTLA-4, PD1 and PD-L1 assays were tested for sensitivity to specific therapeutic antibody drugs. Assay interference was evaluated by comparing the recovery of a single mid-range analyte concentration in the presence of a wide range of concentrations of the potential interferent. Competition was evaluated by comparing human serum and EDTA plasma sample concentrations measured in singleplex and multiplex assay formats. Testing with the therapeutic antibody drugs Nivolumab and Pembrolizumab demonstrated that the PD1 (epitope 1) assay is more resistant to these drugs than the PD1 (epitope 2) assay. No unexpected assay interference or competition was observed.

Assay	Interferent	Impact
BAFF-R/TNFRSF13C	BAFF	None
	BCMA/TNFRSF17	None
	TACI/TNFRSF13B	None
BCMA/TNFSF17	APRIL/TNFSF13	None
	BAFF	None
	APRIL/BAFF	None
CD28	CD80/B7-1	None
	CTLA-4	None
Granzyme A	Perforin	None
	Protease Inhibitors	None
CTLA-4	Ipilumab (120 ng/mL)	90%
	CD28	None
	CD80/B7-1	None
GITR/TNFSF18	GITRL/TNFSF18	None
gp130 (soluble)	IL-6	None
HVACR2/TIM-3	Galectin-9	None

Assay	Interferent	Impact
OX40/TNFRSF4	OX40L/TNFSF4	None
PD1 (epitope 1)	Nivolumab (10 µg/mL)	33%
	Pembrolizumab (10 µg/mL)	19%
	PD-L1	None
	PD-L2	None
PD1 (epitope 2)	Nivolumab (10 µg/mL)	99%
	Pembrolizumab (10 µg/mL)	98%
	Atezolizumab (50 ng/mL)	91%
PD-L1 (epitope 1)	PD1	None
	PD-L2	None
PD-L2	PD1	None
	PD-L1	None
PIGF	VEGFR-1/Flt-1	None
RANKL/TNFSF11	Osteoprotegenin	None
TIGIT	CD155	None

CONCLUSIONS

Twenty-seven human assays were developed that provide sensitive and reliable measurements of traditional and emergent biomarkers associated with cancer and cancer immunotherapy. The assays can be used in singleplex and multiplex formats and include therapeutic antibody-sensitive and -insensitive PD-1 assays. Moreover, these assays can be run in combination with 84 additional biomarker assays, enabling researchers and drug developers to simultaneously measure immuno-oncology analytes along with cytokines, chemokines, and inflammatory markers.

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