A Quantitative Mass Spectrometry Workflow for Highly Multiplexed Measurement of Immunomodulatory Proteins to Support Immunotherapy Clinical Trials

Ons Ousji, Anne Jang, Luca Genovesi, Nick Dupuis, Timon Geib, Gwenael Pottiez and Michael Schirm
CellCarta, Montreal, Quebec, Canada
POSTER: 132

CellCarta

INTRODUCTION

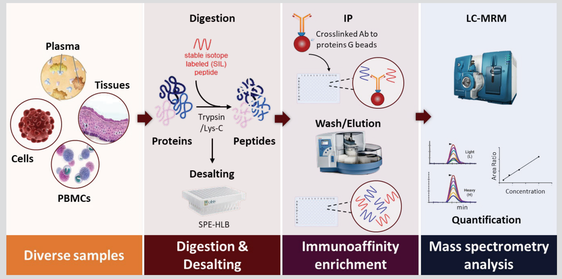
- For a subset of cancer patients, immune checkpoint-directed therapies have proven remarkably effective.
- To benefit additional cancer patient populations, the development of safe and effective novel immunotherapy targets and combination therapies will require a deeper understanding of novel mechanisms of action, and individual drug responses.
- **Central to these advancements:** Reliable, analytically validated assays for accurate measurement of immune-related biomarkers in clinical samples.
- **In response, we present:** Three highly multiplexed anti-peptide IP-LC/MRM assays (IO-1, IO-2, and IO-3) allowing to quantify up to a total of 113 immunomodulatory proteins across a diverse range of matrices.

SAMPLE REQUIREMENTS (≥50 µg of protein)

Biospecimen	PLASMA	BLOOD / PBMCs	Fresh Frozen Tumor	FFPE Section	Core Needle Biopsy
Typical protein yields	60–80 µg/µL	65 µg / 10^6 cells	50 µg/mg wet weight	2.5 µg ($1m^2$ x 5µm)	100-200 µg (16G x 1cm)

The more material is processed the better is the sensitivity of the assay.

WORKFLOW



- Matrices were spiked with stable-isotope-labeled (SIL) peptides, denatured, reduced, alkylated, and digested using Lys-C/Trypsin.
- Immunoaffinity capture involved overnight incubation with anti-peptide antibodies on protein G magnetic beads. Automated washing and elution of peptides from antibodies were performed using the KingFisher platform.
- Enriched peptides were analyzed by LC-MRM analysis. Data analysis was performed using the Skyline and MultiQuant software.

ASSAY MULTIPLEXING CAPABILITIES

List of Antibody Targets

Table 1. Proteins targeted for the multiplexed IO-1, IO-2 and IO-3 immuno-MRM assay panels.

IO-1 Panel		IO2- Panel		IO-3 Panel	
Gene	Target Protein Name	Gene	Target Protein Name	Gene	Target Protein Name
ANXA1	Annexin A1	ARG1	Arginase-1	ADA	Adenosine deaminase
ARG2	arginase, type II	BAX	Apoptosis regulator BAX	B2MG	Beta-2-microglobulin
ATM	Serine-protein kinase ATM, Ataxia telangiectasia mutated	BCL10	B-cell lymphoma/leukemia 10	BAD	Bcl2-associated agonist of cell death
C10orf54	chromosome 10 open reading frame 54	BTK	Tyrosine-protein kinase BTK	CASP3	Caspase-3
CCL5	C-C motif chemokine ligand 5	BTLA	B- and T-lymphocyte attenuator	CBG	Corticosteroid-binding globulin
CD14	CD14 molecule	CD166	CD166 antigen	CD20	B-lymphocyte antigen CD20
CD163	CD163 molecule	CD38	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1	CD276	CD276 antigen
CD274	Programmed cell death 1 ligand 1 CD274	CD4	T-cell surface glycoprotein CD4	CD44	CD44 antigen (CDw44)
CD33	CD33 molecule	CD48	CD48 antigen	CD8A	T-cell surface glycoprotein CD8 alpha chain
CD40	tumor necrosis factor receptor superfamily member 5	CSF1R	Granulocyte-macrophage colony-stimulating factor receptor alpha	CRP	C-reactive protein
CD47	CD47 molecule	DPOD1	DNA polymerase delta catalytic subunit	CSF1	Macrophage colony-stimulating factor 1
CD70	CD70 antigen	DRA	HLA class II histocompatibility antigen, DR alpha chain	CTLA4	Cytotoxic T-lymphocyte protein 4
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	ERCC2	General transcription and DNA repair factor IIH helicase subunit XPD	CXCL9	C-X-C motif chemokine 9
CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	FOXO1	Forkhead box protein O1	DNL1	DNA ligase 1
CX3CL1	C-X3-C motif chemokine ligand 1	G3P	Glyceraldehyde-3-phosphate dehydrogenase	EIF2A	Eukaryotic translation initiation factor 2A
CXCL10	C-X-C motif chemokine ligand 10	HGFL	Hepatocyte growth factor-like protein	G3P	Glyceraldehyde-3-phosphate dehydrogenase
CXCL13	chemokine (C-X-C motif) ligand 13	HLAE	HLA class I histocompatibility antigen, alpha chain E	GRAA	Granzyme A
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1	I2O1	Indoleamine 2,3-dioxygenase 1	IL1RA	Interleukin-1 receptor antagonist protein
FAS	Fas (TNF receptor superfamily, member 6)	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	INGR1	Interferon gamma receptor 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	ITAM	Integrin alpha-M
HAVCR2	hepatitis A virus cellular receptor 2	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	JAK1	Tyrosine-protein kinase JAK1
ICAM1	intercellular adhesion molecule 1	IL16	Pro-interleukin-16 [Cleaved into: Interleukin-16	KI67	Proliferation marker protein Ki-67
ICOSLG	inducible T-cell co-stimulator ligand	ILIA	Interleukin-1 alpha	MSH2	DNA mismatch repair protein Msh2
IL18	Interleukin 18 (interferon-gamma-inducing factor)	IL2RG	Cytokine receptor common subunit gamma	MSH3	DNA mismatch repair protein Msh3
ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1)	ILRA	Interleukin-6 receptor subunit alpha	MSLN	Mesothelin
LGALS1	Lectin, galactoside-binding, soluble, 1 (galectin 1)	ITAX	Integrin alpha-X	MUTYH	Adenine DNA glycosylase
LIME1	Lck interacting transmembrane adaptor 1	KI67	Proliferation marker protein Ki-67	NCAM1	Neural cell adhesion molecule 1
PDCD1LG2	programmed cell death 1 ligand 2	LEG9	Galectin-9	PARP1	42a form) (Poly [ADP-ribose] polymerase 1
PECAM1	Platelet endothelial cell adhesion molecule	LY75	Lymphocyte antigen 75	PAX1	PAX-interacting protein 1
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1	MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1	PCNA	Proliferating cell nuclear antigen
PTGS2	prostaglandin-endoperoxide synthase 2	MGMT	Methylated-DNA--protein-cysteine methyltransferase	PDCD1	Programmed cell death protein 1
PTPNC	Protein tyrosine phosphatase, receptor type, C	MSH6	DNA mismatch repair protein Msh6	PMS2	Mismatch repair endonuclease PMS2
RIF1	Telomere-associated protein RIF1	OSTP	Osteopontin	PTN2	Tyrosine-protein phosphatase non-receptor type 2
STAT1	signal transducer and activator of transcription 1, 91kDa	PERM	Myeloperoxidase	SLAF8	SLAM family member 8
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	PMS1	PMS1 protein homolog 1	SN	Sialoadhesin
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	PVR	poliovirus receptor	TGFB1	Transforming growth factor beta-1 proprotein
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	RB1	Retinoblastoma-associated protein	VEGFA	Vascular endothelial growth factor A, long form
TNFRSF14	tumor necrosis factor receptor superfamily member 14	SIGL7	Sialic acid-binding Ig-like lectin 7		
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	TAP1	Antigen peptide transporter 1		
TNFRSF9	tumor necrosis factor receptor superfamily member 9	TPSN	Tapasin		
TNFSF9	tumor necrosis factor superfamily member 9	XRCC5	X-ray repair cross-complementing protein 5		
VCAM1	vascular cell adhesion molecule 1	ZAP70	Tyrosine-protein kinase ZAP-70		
VTCN1	V-set domain containing T cell activation inhibitor 1				

Up to 50 targets can be quantified in an assay. In cases where only very limited amounts of material are available, the supernatant from the initial antibody capture step can be used for further target enrichments[1]. This sequential immuno-capture feature allows to analyze up to 113 targets from just a single sample.

MRM Chromatograms of the 3 Assays

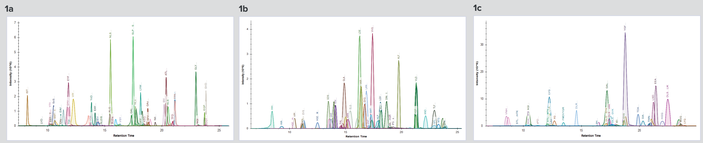


Figure 1. MRM chromatograms of detected peptides from IO-1 (a), IO-2 (b) and IO-3 (C).

Assay Specificity

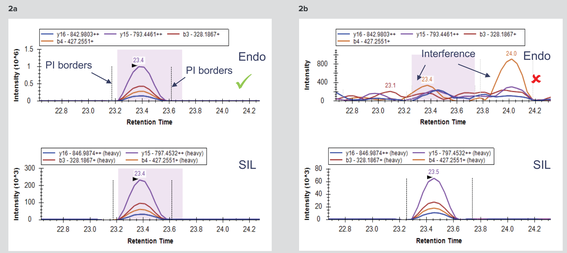


Figure 2. MRM is a highly specific method due to the two selection steps (Precursor → Fragment ion). a) Elution time and transition ratio matches between SIL and endo peptide indicating that the target peptide was successfully detected. b) Transition ratio and elution time do not match between heavy and light transitions. The endo signal is from an interference.

LINEARITY, SENSITIVITY, PRECISION AND ACCURACY

Response Curve

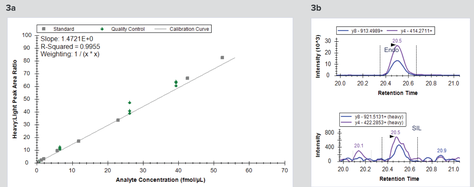


Figure 3. (a) Standard curve generated by spiking SIL peptide standards into plasma at different concentrations covering 3 orders of magnitude for peptide ATLLEEQLPLGK (Q9BQ51IPD1L2) (b) Chromatogram at the LLOQ level (0.05 fmol/µL).

Table 2. Three P&A runs over the quantitative range.

	Calibration Curve											Quality Controls				
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10	STD11	QC1	QC2	QC3	QC4	QC5
Nominal	**0.05**	**0.11**	**0.21**	**0.42**	**1.26**	**1.94**	**5.88**	**11.81**	**23.00**	**42.63**	**52.50**	**0.07**	**0.28**	**3.94**	**17.72**	**39.38**
Run 1	0.05	0.11	0.22	0.40	1.14	2.20	5.94	12.43	21.84	43.61	48.95	0.09	0.28	3.98	16.70	37.70
												0.07	0.31	4.09	16.30	36.82
												0.09	0.29	4.12	17.72	35.10
Run 2	0.05	0.11	0.21	0.45	1.26	1.83	5.48	12.52	24.95	42.35	48.93	0.09	0.28	4.04	16.34	31.15
												0.07	0.29	3.70	15.24	34.72
												0.06	0.27	3.51	15.47	39.34
Run 3	0.06	0.10	0.17	0.42	1.30	1.97	5.47	12.41	25.13	44.68	53.33	0.07	0.27	4.17	15.76	35.96
												0.05	0.26	3.88	18.40	40.00
												0.05	0.26	4.02	17.59	38.76
Mean	0.05	0.11	0.20	0.42	1.23	2.00	5.63	12.45	23.97	43.55	50.40	0.07	0.28	3.95	16.61	36.62
% CV	11%	5%	13%	6%	7%	9%	5%	0%	8%	3%	5%	23%	6%	5%	7%	8%
% Bias	1%	2%	-5%	1%	-2%	3%	-4%	5%	4%	2%	-4%	5%	0%	0%	-6%	-7%

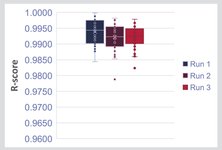


Figure 4. Distribution of R-score values for all IO-1 panel peptides.

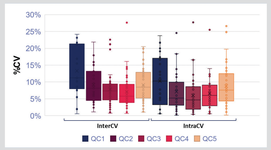


Figure 5. Intra-day and inter-day (n=3) precision for all IO-1 panel peptides.

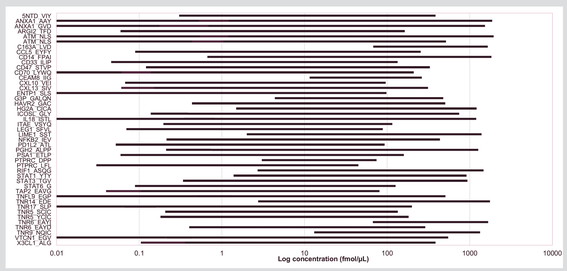


Figure 6. Analytical measuring range of targets for the IO-1 panel, using 10 µL of plasma defined by the lower and upper limits of quantification (LLOQ-ULOQ).

DETECTION RATE OF IO-1, IO-2 AND IO-3 PANELS

Table 3. Detection rates of the IO panels in different matrices using 500 µg of protein amount.

	IO-1		IO-2		IO-3	
Sample ID	# of peptides detected	% of peptides detected	# of peptides detected	% of peptides detected	# of peptides detected	% of peptides detected
Plasma	30	67%	23	53%	21	50%
Cell lines	38	84%	36	84%	33	79%

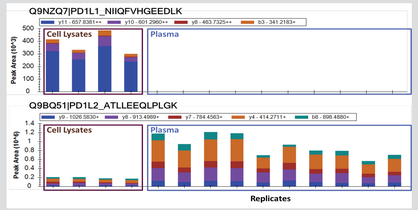


Figure 7. PD1L1 and PD1L2 target peptides detection in different matrices.

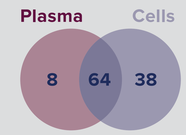


Figure 8. A Venn diagram visualizing the number of distinct and common peptides detected in plasma and in cell lysate from the 3 panels.

STABILITY

	IO-1	
Stability	Median CV	Median %diff
8h at 4°C	0.029	0.2
48h at 4°C	0.025	-0.8
2 freeze-thaw cycles	0.029	3.5
-80°C for 5 weeks	0.031	0.8

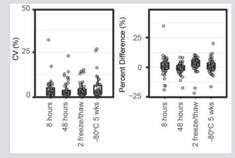


Figure 9. The stability of the enriched peptides was determined by analyzing aliquots of the medium spike level sample used in repeatability studies after storage at 4°C in the autosampler for 8 hours, storage at 4°C for 48 hours, after 2 freeze-thaw cycles, and after extended storage at -80°C for 5 weeks (Whiteaker., *et al.* 2021)[2].

CONCLUSIONS

The multiplexed immuno-MRM assay panels available at CellCarta have undergone validation in accordance with the CPTAC guidelines. The panels presented here allow for the precise and accurate quantitation of up to 113 important immunomodulatory proteins over a wide dynamic range. The approach offers exceptional sensitivity for detecting low-abundance proteins. It provides valuable complementary data to classical methods of tissue-based protein expression and has been published in several renowned research papers investigating a broad range of cancer types. Moreover, our ability to readily multiplex analytes using LC-MRM significantly expands the potential for large-scale protein network analysis.

Table 4. CellCarta immuno-MRM panels.

Immuno-Oncology panel 1 (IO-1)	DNA damage response panel 3 (DDR-3)
Immuno-Oncology panel 2 (IO-2)	Cancer biomarkers and normalization panel
Immuno-Oncology panel 3 (IO-3)	RAS/MAPK panel 1 (RAS-1)
DNA damage response panel 1 (DDR-1)	RAS/MAPK panel 2 (RAS-2)
DNA damage response panel 2 (DDR-2)	

ACKNOWLEDGMENTS

We thank Jeffery Whiteaker and Amanda Paulovich for developing these panels and their invaluable support in its successful implementation at CellCarta.

1 Whiteaker, Jeffrey R., *et al.* "A multiplexed assay for quantifying immunomodulatory proteins supports correlative studies in immunotherapy clinical trials." Frontiers in Oncology 13 (2023): 1168710.
2 Whiteaker, Jeffrey R., *et al.* "Targeted mass spectrometry enables multiplexed quantification of immunomodulatory proteins in clinical biospecimens." Frontiers in Immunology 12 (2021): 4719.

Please send inquiries to:
Timon Geib, Ph.D.
Senior Scientist, R&D Proteomics
E: timon.geib@cellcarta.com