P010

Analytical Validation of a Multivariate Proteomic Serum Based Assay for Disease Activity Assessments in Multiple Sclerosis

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Background and Objective

- The availability of a validated blood-based assay to quantitatively assess disease activity and progression will significantly advance MS clinical care.
- There are two primary components to validate a test for commercial use in the clinic:
  - **Clinical Validation**: to establish that the test associates with endpoints relevant to assessing the disease. (Study in progress: see P014 ACTRIMS 2021)
  - **Analytical Validation**: to ensure that the test is characterized according to prespecified analytical performance criteria.
- One barrier to having a validated blood-based assay available to neurologists has been the lack of accurate, precise, and robust methods that ensure consistency in reported results over time.
- **Objective:** To analytically validate a blood-based multiplex proteomic immunoassay for MS disease assessments.

Disclosures:
Wayne Hu, Louisa Loh, Hemali Patel, Maria DeGuzman, Michael Becich, Fatima Rubio da Costa, Victor Gehman, and Ferhan Qureshi are employees of Octave Bioscience.
Erika Assarsson, Sandra Ohlsson, Martin Lundberg, Jessica Bergman, and Niklas Nordberg are employees of Olink Proteomics.

Methodology

- A custom immunoassay panel that measures the concentration of 21 proteins was developed using Proximity Extension Assay (PEA) methodology on the Olink™ platform.
- Selection of the 21 proteins was based on associations with clinical and radiographic MS endpoints in several independent discovery studies. Biological pathway modeling and protein network analysis were performed to ensure comprehensive representation of MS neurophysiology (see Chitnis et al., P0063-MS Virtual 2020).
- Two lots of the panel were manufactured for which the critical reagents (paired antibodies used for analyte binding, enzymes used for PCR steps, and protein stocks used for calibration) were varied to the extent possible.
- The two kit lots were subjected to a comprehensive analytical validation protocol to characterize and establish the following parameters in patient serum:
    - Accuracy, precision (inter- and intra-assay), robustness (instrumentation and analysis location), sensitivity (limits of quantification), MS population reference ranges, interference (endogenous interfering substances, heterophilic antibodies, common drugs and DMTs), diurnal variability, and stability of serum samples.

Biomarkers Measured in the Custom Assay Panel	
Analyte	**Protein Name & Alias**
APLP1	Amyloid Beta Precursor Like Protein 1
CCL20	MIP-3 alpha
CD6	Cluster of Differentiation 6
CDCP1	CUB domain-containing protein 1
CNTN2	Contactin 2
COL4A1	Collagen alpha-1(IV) chain
CXCL13	C-X-C Motif Chemokine Ligand 13, BLC
CXCL9	MIG, Monokine Induced by Gamma Interferon
FLRT2	Leucine-rich repeat transmembrane protein
GFAP	Glial Fibrillary Acidic Protein
GH	Growth Hormone, Somatotropin
IL-12B	Interleukin 12B
MOG	Myelin-Oligodendrocyte Glycoprotein
NEFL	NFL, Neurofilament Light
OPG	Osteoprotegerin, TNFRSF11B
OPN	Osteopontin
PRTG	Protogenin
SERPINA9	Serpin Family A Member 9
TNFRSF10A	TRAILR1, DR5 - Death Receptor 5
TNFSF13B	BAFF, B-cell activating factor
VCAN	Versican, Versican Proteoglycan

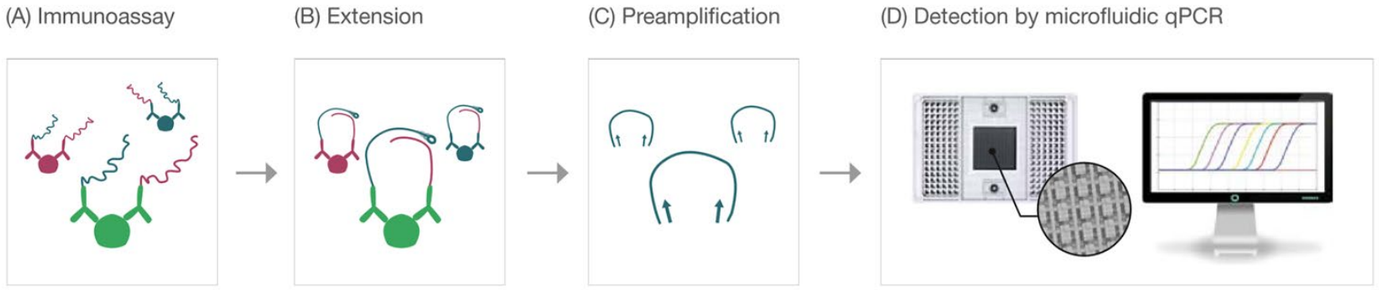
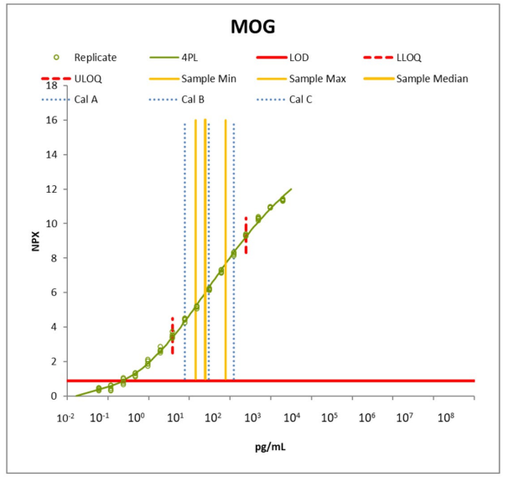
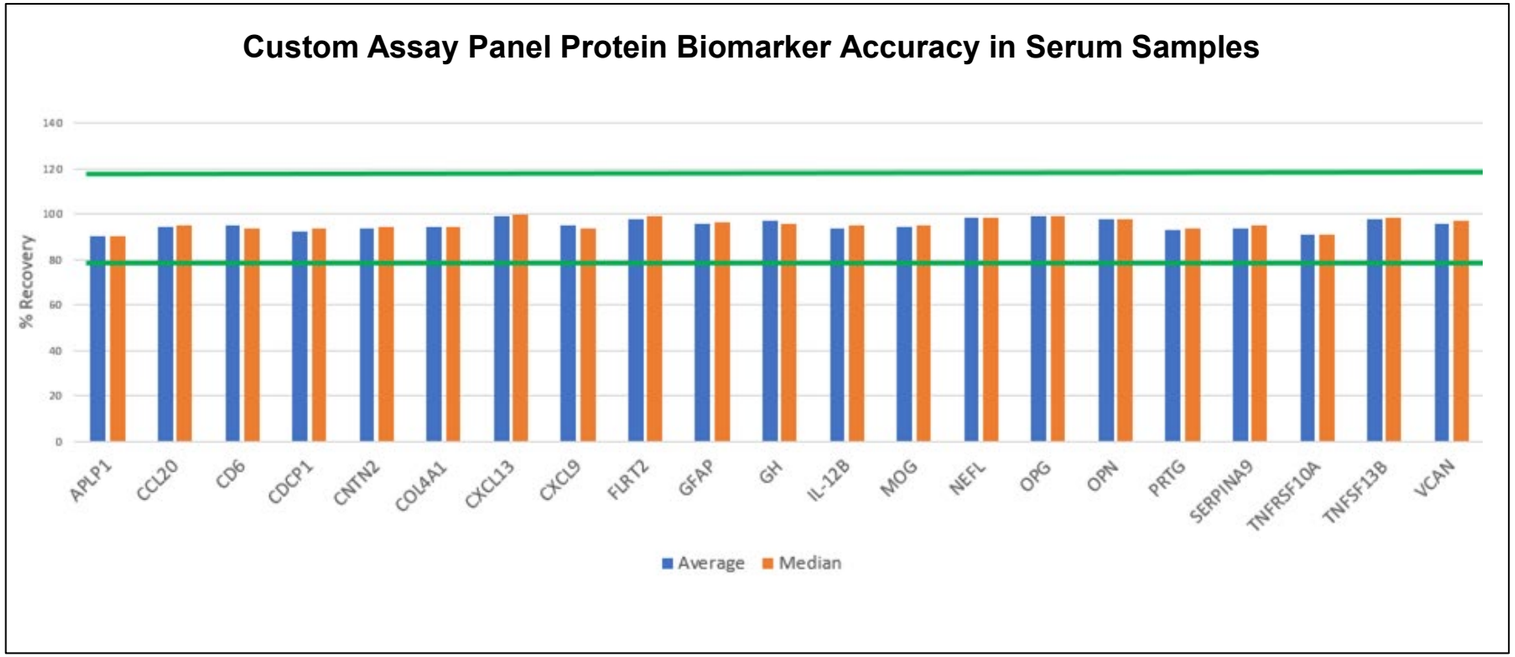


Fig 1. Overview of the PEA technology. (A) 92 Antibody pairs, labelled with DNA oligonucleotides, bind target antigen in solution. (B) Oligonucleotides that are brought into proximity hybridize, and are extended by a DNA polymerase. (C) This newly created piece of DNA barcode is amplified by PCR. (D) The amount of each DNA barcode is quantified by microfluidic qPCR.



A multiplexed standard curve was prepared along with 3 levels of Calibrators (High Mid, Low) to cover the range of sample response in the MS population. The 4PL standard curve is re-established for each lot of reagent kits during manufacturing. The calibrators and a blank are run on each assay plate. The signal obtained from the assay (NPX) is converted to absolute concentration (pg/mL) using the calibrators referenced back to the "gold" standard curve. These concentrations are then used as inputs into algorithms corresponding to Disease Activity and Biological Pathway Scores.

Accuracy



Biomarker	APLP1	CCL20	CD6	CDCP1	CNTN2	COL4A1	CXCL13
Min Conc Tested (pg/mL)	8,290.48	5.16	76.54	51.09	874.38	806.75	27.95
Max Conc Tested (pg/mL)	16,253.97	178.16	385.51	784.54	2,493.08	2,064.13	51.84
Minimum % Recovery	79	78	84	78	82	83	89
Maximum % Recovery	99	107	103	104	100	102	110
Average % Recovery	91	95	95	93	94	95	99
Median % Recovery	91	95	94	94	95	94	100

Biomarker	CXCL9	FLRT2	GFAP	GH	IL-12B	MOG	NEFL
Min Conc Tested (pg/mL)	18.81	64.59	29.79	48.36	19.14	11.08	3.62
Max Conc Tested (pg/mL)	225.09	197.43	80.43	384.19	320.35	62.56	27.79
Minimum % Recovery	83	88	84	82	84	86	83
Maximum % Recovery	105	106	111	124	103	104	112
Average % Recovery	95	98	96	97	94	95	99
Median % Recovery	94	99	96	96	95	95	98

Biomarker	OPG	OPN	PRTG	SERPINA9	TNFRSF10A	TNFSF13B	VCAN
Min Conc Tested (pg/mL)	702.90	16,580.79	72.08	11.88	4.31	2,831.99	341.51
Max Conc Tested (pg/mL)	1,709.29	37,544.64	147.53	351.15	27.76	8,552.18	512.12
Minimum % Recovery	87	84	84	83	78	86	83
Maximum % Recovery	109	104	100	103	104	105	101
Average % Recovery	99	98	93	94	91	98	96
Median % Recovery	99	98	94	95	91	98	97

- **Accuracy** is defined as the **closeness of a result to the true value**.
- Accuracy for each analyte was determined by mixing serum samples at different ratios and evaluating the percent recovery of the observed concentration relative to the expected concentration.
  - Sample mixing enables the accuracy assessment to be performed using endogenous protein (vs. a recombinant protein source).
  - Expected concentrations (i.e the true values) were calculated by applying the targeted ratios of the unmixed samples.
- 4 individual samples were blended at various ratios:
  - Ratios of sample blends for mixtures with 2 samples were 25%:75%, 50%:50% & 75%:25%
  - Ratios of sample blends for mixtures with 4 samples were 25%:25%:25%:25% & 40%:10%:40%:10%
  - Samples were selected from an internal MS Cohort (n=64) to target both High and Low concentrations (relative to the MS population)
  - n = 20 mixed samples per each biomarker
  - Acceptability Criteria: Median % recovery for each biomarker must be between 80% - 120%. All analytes passed the specification.

Inter and Intra-Assay Precision

Analyte	Inter & Intra-Assay %CV with Expected Concentrations				
	PC ID ->	PC MS 1	PC MS2	PC RA	PC NM
APLP1	Intra %CV	9	9	4	7
	Inter %CV	9	8	8	9
	pg/mL	10,296	11,560	11,868	11,868
CCL20	Intra %CV	6	8	5	7
	Inter %CV	9	7	9	9
	pg/mL	6.9	9.2	13.7	11.8
CD6	Intra %CV	6	8	5	8
	Inter %CV	8	8	7	9
	pg/mL	89	108	137	112
CDCP1	Intra %CV	8	8	4	7
	Inter %CV	9	9	9	10
	pg/mL	78	125	208	72
CNTN2	Intra %CV	6	7	4	8
	Inter %CV	7	7	6	8
	pg/mL	1,120	1,643	1,554	1,256
COL4A1	Intra %CV	7	19	8	47
	Inter %CV	15	20	59	27
	pg/mL	1,104	1,334	1,601	1,387
CXCL13	Intra %CV	6	8	7	7
	Inter %CV	8	7	8	9
	pg/mL	52.8	42.9	65.3	46.8

Analyte	Inter & Intra-Assay %CV with Expected Concentrations				
	PC ID ->	PC MS 1	PC MS2	PC RA	PC NM
CXCL9	Intra %CV	6	8	5	7
	Inter %CV	11	10	11	11
	pg/mL	31.0	62.6	112.3	27.5
FLRT2	Intra %CV	7	9	5	8
	Inter %CV	8	9	8	9
	pg/mL	103	110	139	116
GFAP	Intra %CV	7	10	8	9
	Inter %CV	18	16	15	18
	pg/mL	70	126	148	77
GH	Intra %CV	7	8	5	7
	Inter %CV	9	7	8	9
	pg/mL	823	595	1,010	366
IL-12B	Intra %CV	7	9	6	8
	Inter %CV	9	7	8	9
	pg/mL	109	122	118	71
MOG	Intra %CV	4	7	5	6
	Inter %CV	6	6	7	8
	pg/mL	21.9	22.8	26.0	17.8
NEFL	Intra %CV	10	13	8	11
	Inter %CV	11	9	8	12
	pg/mL	7.6	15.6	20.6	6.5

Analyte	Inter & Intra-Assay %CV with Expected Concentrations				
	PC ID ->	PC MS 1	PC MS2	PC RA	PC NM
OPG	Intra %CV	6	9	6	7
	Inter %CV	11	11	10	12
	pg/mL	699	806	1,022	602
OPN	Intra %CV	6	7	6	7
	Inter %CV	10	10	12	13
	pg/mL	15,733	15,415	17,470	10,450
PRTG	Intra %CV	7	8	5	7
	Inter %CV	6	7	6	7
	pg/mL	94	107	108	103
SERPINA9	Intra %CV	11	11	5	6
	Inter %CV	8	8	7	14
	pg/mL	45.1	37.9	60.0	50.0
TNFRSF10A	Intra %CV	9	9	5	9
	Inter %CV	9	9	8	9
	pg/mL	5.1	5.5	7.6	4.9
TNFSF13B	Intra %CV	7	11	5	7
	Inter %CV	10	11	11	13
	pg/mL	4,075	4,019	4,204	3,003
VCAN	Intra %CV	7	7	4	5
	Inter %CV	8	7	7	8
	pg/mL	316	337	448	310

- **Precision** is defined as the **extent to which repeated measurements agree with one another**.
- Intra-Assay Precision (within a single plate) and Inter-Assay Precision (across multiple plates) was characterized for each analyte.The percent coefficient of variation (%CV) was determined using serum pools enabling the assessment to be performed using endogenous protein.
  - Equipment, reagents and location (i.e. R&D vs. Clinical Lab) were varied throughout the experiments to demonstrate the robustness of the method.
  - The serum pools were manufactured to represent different populations including: two separate MS pools (PC MS 1 & 2 with shorter vs. longer disease duration), one rheumatoid arthritis pool (PC RA; an inflammatory disease control), and one healthy control pool (PC NM).
  - These serum pools (n=4) were included on all R&D runs to date during the assay discovery and development process. They were sourced in large volumes, aliquoted, and stored at -65°C. They will also serve as the Process Controls (PC) used to assess acceptability of future analytical runs (run in triplicate on every plate). The standard deviation of repeated measurements is applied to to the expected concentrations to create control tables for this purpose.
- Acceptability Criteria was established as ≤15% for Intra-Assay %CV and ≤20% for Inter-Assay %CV
  - Intra-Assay Precision Experiment: 12 replicates per serum pool analyzed on a single plate
  - Inter-Assay Precision Assessment: Up to 51 values per serum pool analyzed across 51 plates (spread across two manufactured kit lots)
- COL4A1 was found to have unacceptable inter and intra-assay precision across several serum pools. All other analytes passed the established criteria.

Sensitivity and MS Reference Ranges

Analyte	LLOQ (pg/mL)	ULOQ (pg/mL)	Low MS range (pg/mL) 2.5th Percent	High MS Range (pg/mL) 97.5th Percent	Samples (n=928) Imputed at LLOQ Count (%)	Samples (n=928) Imputed at ULOQ Count (%)
APLP1	2,324	142,798	5,400	22,000	0	0
CCL20	0.92	383	1.90	41	0	0
CD6	4.62	3,319	43	240	0	0
CDCP1	24.22	6,795	27	220	19 (2.0%)	1 (0.1%)
CNTN2	44.46	12,374	590	3,000	0	0
COL4A1	30.65	4,573	520	2,900	0	4 (0.4%)
CXCL13	1.91	1,113	22	160	0	0

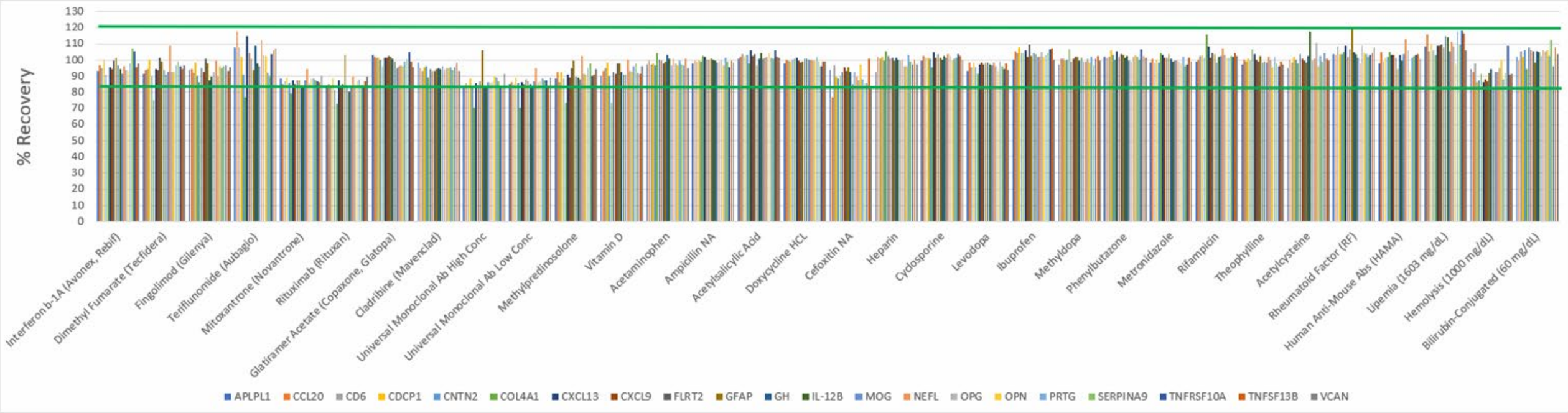
Analyte	LLOQ (pg/mL)	ULOQ (pg/mL)	Low MS range (pg/mL) 2.5th Percent	High MS Range (pg/mL) 97.5th Percent	Samples (n=928) Imputed at LLOQ Count (%)	Samples (n=928) Imputed at ULOQ Count (%)
CXCL9	1.89	1,832	17	250	0	0
FLRT2	35.67	10,107	61	181	0	0
GFAP	12.46	19,583	23	220	0	0
GH	9.63	18,414	20	8,200	2 (0.2%)	7 (0.8%)
IL-12B	0.56	3,044	25	230	0	0
MOG	1.75	577	12	49	0	0
NEFL	3.31	599	4.00	60	10 (1.1%)	0

Analyte	LLOQ (pg/mL)	ULOQ (pg/mL)	Low MS range (pg/mL) 2.5th Percent	High MS Range (pg/mL) 97.5th Percent	Samples (n=928) Imputed at LLOQ Count (%)	Samples (n=928) Imputed at ULOQ Count (%)
OPG	14.58	62,385	410	1,400	0	0
OPN	572.50	157,267	9,300	36,000	0	0
PRTG	3.90	5,921	70	170	0	0
SERPINA9	5.12	9,287	12	150	1 (0.1%)	0
TNFRSF10A	0.48	1,027	2.80	9.20	0	0
TNFSF13B	660.29	130,682	2,000	8,200	0	0
VCAN	8.54	14,674	230	600	0	0

- **Sensitivity** is defined as the assay's ability to **accurately and precisely detect low concentrations of a given substance in a biological specimen**.
- To establish the upper and lower limits of quantitation (LOQ), an LOQ panel was manufactured. For each analyte, 4 levels were targeted near the anticipated upper limit (ULOQ 1-4) and 4 levels near the anticipated lower limit (LLOQ 5-8). The targeted concentrations were based on the shape of the standard curve and location of asymptotes.
- This LOQ panel was run in triplicate over 2 lots (min. of 5 runs per lot) and fit to the standard curve. Accuracy (80%-120% recovery relative to expected concentration) and Precision (Inter-Assay CV ≤ 20%) assessments were used as the acceptability criteria to establish each analyte's LLOQ and ULOQ.
  - Serum samples that recover above the ULOQ or below the LLOQ will be imputed to the established LOQ level for reporting purposes.
- n = 928 MS serum samples were analyzed during the assay development process and used to establish MS Reference Ranges for each analyte. The linear interpolation method per Clinical Laboratory Standards Institute (EP28A3CE) was used to establish the 95% interval (2.5th and 97.5th percentiles).
  - For reporting purposes, the percentile relative to these reference ranges will be presented alongside the protein concentration.
  - To evaluate the impact of these established LOQs, the 928 samples were evaluated to determine the count and percentage of instances where the determined concentration was outside of the LOQ ranges. The assay is highly sensitive, with the maximum percentage of samples requiring imputation at any LOQ being 2.0% (CDCP1 at the LLOQ).

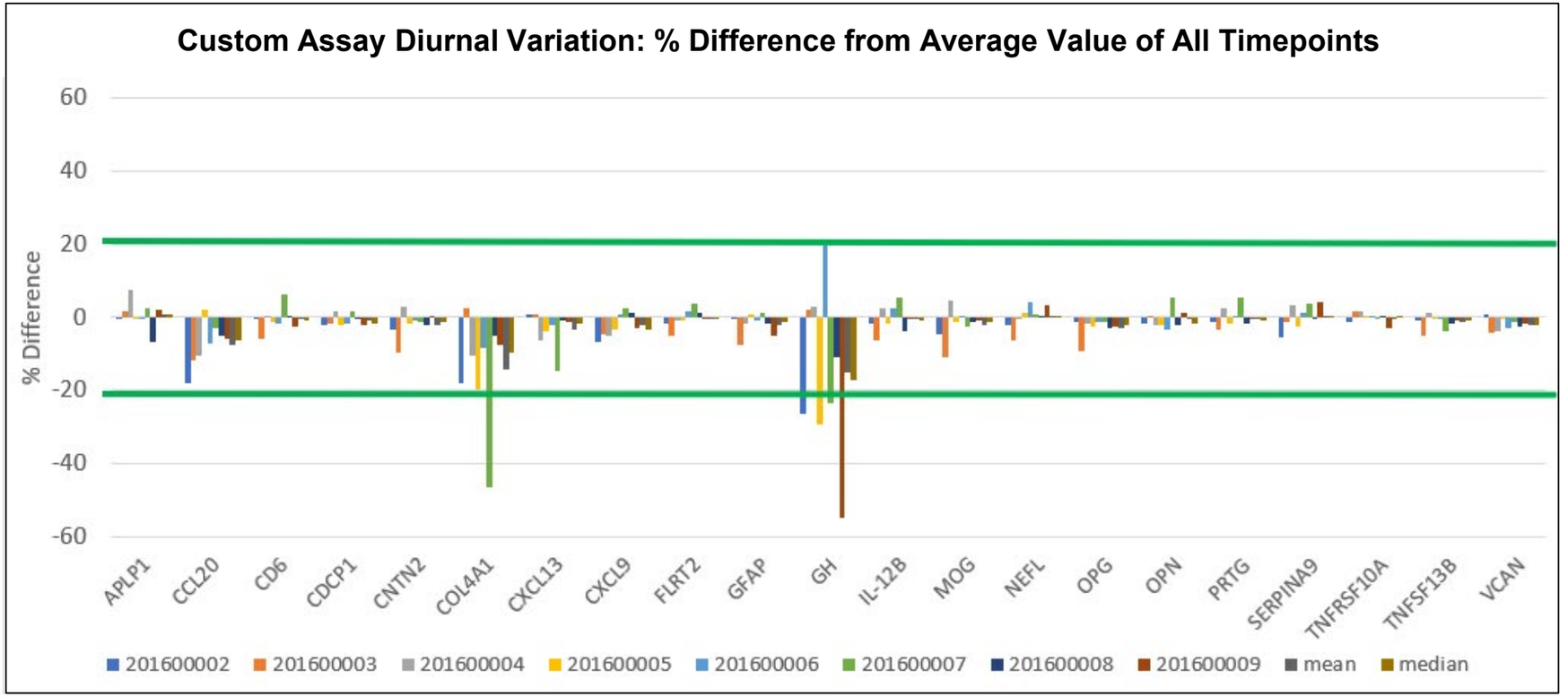
Assay Interference

Assay Interference: MS Therapeutics, Common Drugs, Heterophilic Antibodies and Endogenous Interferents



- **Assay interference** is defined as t**he effect of a substance present in the sample that alters the correct value of the result.**
- To evaluate analytical interference of therapeutics in the assay 4 serum pools were spiked with MS DMTs and common drugs
  - MS Therapeutics/Disease-Modifying Therapies (DMTs): Tested concentrations were targeted at 2X the reported C_{max} (pharmacokinetic results from literature), or the highest possible concentration allowable for spiking with the procured interferent stock.
    - A universal monoclonal antibody standard was used as a surrogate for several DMTs and tested at two concentrations.
  - Components and concentrations for common drugs based on expert panel recommendations and Clinical Laboratory Standards Institute (CLSI EP-7A).
- For Rheumatoid Factor (RF) interference, RF concentrate (Lee Biosolutions) was used to spike 6 serum samples at high and low concentrations.
- For Human Anti-Mouse Antibodies (HAMA) interference, 5 HAMA positive serum samples were mixed at different ratios with MS samples from an internal cohort.
- For endogenous interference 4 serum pools were spiked with Bilirubin, Hemolysate, and Lipids at typical concentrations (Assurance Interference Test Kit)
- Median % Recovery was calculated relative to a corresponding spike control (represents the same alteration of the serum sample without the addition of the interferent) or for HAMA relative to the expected concentrations as determined from the unmixed samples.
- 80% to 120% median recovery across all spikes or sample mixtures was established as the acceptability threshold for the interference assessment.
  - For COL4A1 under recovery was observed for several drugs. Results are likely an artifact of established assay imprecision.
  - For CCL20, Cefoxitin spiked at 660 mg/dL resulted in median percent recovery of 77%. This finding will be further characterized.
  - For all other biomarker/interferent combinations, the median percent recovery was observed to be within 80 - 120%.

Diurnal Variability



Sample %CV Across 6 Draws Spanning 12 Days

Sample ID	APLP1	CCL20	CD6	CDCP1	CNTN2	COL4A1	CXCL13
201600002	16	40	6	11	14	50	8
201600003	16	28	19	18	21	27	26
201600004	14	20	11	16	13	50	21
201600005	8	13	8	7	8	57	13
201600006	8	22	5	7	7	19	9
201600007	14	49	18	14	12	109	52
201600008	16	13	4	13	6	21	6
201600009	17	15	6	7	8	20	10
mean	14	25	10	12	11	44	18
median	15	21	7	12	10	39	11

Sample ID	CXCL9	FLRT2	GFAP	GH	IL-12B	MOG	NEFL
201600002	19	9	12	57	6	17	21
201600003	19	21	15	33	21	23	24
201600004	19	13	12	34	12	12	9
201600005	12	7	7	105	7	8	21
201600006	7	8	13	72	5	12	10
201600007	8	13	18	87	10	11	18
201600008	8	4	11	91	11	6	16
201600009	9	4	17	146	6	11	15
mean	13	10	13	78	10	12	17
median	11	8	12	79	9	11	17

Sample ID	OPG	OPN	PRTG	SERPINA9	TNFRSF10A	TNFSF13B	VCAN
201600002	8	6	5	16	8	7	5
201600003	18	14	15	13	13	22	18
201600004	15	21	10	11	15	13	11
201600005	10	6	4	16	7	6	9
201600006	11	9	7	6	4	9	5
201600007	10	12	14	17	13	15	17
201600008	10	7	5	7	11	8	5
201600009	8	6	2	8	12	4	6
mean	11	10	8	12	10	11	10
median	10	8	6	12	12	8	7

- A **Diurnal Variability** study was performed to **characterize fluctuations that can occur in biomarker levels between days** over a relatively short duration.
- This initial study consisted of 8 subjects that had a serum sample collected at 6 time points: **Day 1, Day 2, Day 3, Day 4, Day 5, and Day 12**
- For each timepoint & subject, the % difference of the observed protein concentration was calculated relative to the average concentration determined from all 6 timepoints. Additionally, the %CV was calculated for all 6 time points per patient
- Mean & median % differences for each biomarker/subject were observed to be within ± 20%. Mean and median % CV was found to be <20% for 18 of the 21 biomarkers. A follow up study restricted to the MS population including multiple draws within a single day and additional timepoints beyond 12 days is in the planning stages.
  - A higher level of diurnal variability observed for COL4A1 is likely the result of the established assay imprecision.
  - Mean and Median % CV for CCL20 (chemotactic cytokine) was observed to be >20%. Result will be characterized further in future studies with additional focus on relevance to MS disease endpoints.
  - Growth Hormone (GH) results were found to be more variable than the other 20 biomarkers. GH is a biomarker that has been well established in the literature to have a high degree of ultradian and diurnal variability.

Sample Stability

Average % Difference versus Experimental Control Conditions (-65°C or below for Temperature Storage and "Fresh" Sample for Freeze-Thaw Cycles)																					
Storage	APLP1	CCL20	CD6	CDCP1	CNTN2	COL4A1	CXCL13	CXCL9	FLRT2	GFAP	GH	IL-12B	MOG	NEFL	OPG	OPN	PRTG	SERPINA9	TNFRSF10A	TNFSF13B	VCAN
RT 4H	4	4	-2	-5	0	-5	-1	-2	-4	0	1	-2	-2	-3	-2	-4	-1	-1	0	-4	-3
RT D1	17	2	-4	-1	-3	-3	-7	-4	-1	2	0	-13	0	-6	-4	-5	-6	-3	-2	-7	-3
RT D3	7	-6	-14	-13	-13	-14	-25	-14	-11	-5	-10	-34	-10	-11	-13	-18	-19	-15	-11	-20	-10
RT D7	19	-7	-5	-2	-3	-1	-27	-7	0	9	-6	-40	1	2	-5	-13	-20	-8	0	-10	-1
RT D14	48	8	19	26	24	22	-24	16	26	39	9	-32	23	29	18	1	-13	10	31	19	23
RT D28	134	47	93	114	71	91	7	65	97	142	52	-24	83	110	94	47	-1	43	108	108	72
4°C 4H	-6	1	-10	-10	-8	-10	-5	-6	-10	-7	-6	-7	-7	-11	-6	-6	-6	-7	-7	-10	-6
4°C D1	-8	-3	-11	-10	-10	-11	-6	-10	-10	-9	-9	-11	-9	-9	-9	-16	-8	-11	-11	-14	-11
4°C D3	12	11	-7	-2	-9	-12	-8	-11	-2	-5	2	-14	-1	1	-8	-27	-7	-4	1	-12	-1
4°C D7	31	24	7	6	2	2	-6	-4	11	20	10	-18	13	4	1	-24	-3	2	11	0	9
4°C D14	12	15	-2	1	-3	-2	-4	-6	2	1	7	-9	2	3	-1	-26	-4	0	3	-6	0
4°C D28	25	23	10	10	7	10	-3	-2	13	18	12	-15	12	12	5	-26	-1	3	10	1	7
-20°C D1	5	12	1	0	2	0	6	2	-1	3	4	3	1	-4	1	0	3	1	0	1	0
-20°C D3	9	13	5	7	6	4	12	6	8	11	9	8	6	4	6	5	6	5	9	7	4
-20°C D7	0	6	-1	-3	1	9	4	1	-1	-1	1	0	1	0	-1	-1	0	-1	-2	1	-2
-20°C D14	5	5	1	1	2	3	7	3	1	3	5	3	3	2	2	3	2	1	0	3	2
-20°C D28	10	7	1	1	3	2	5	4	2	9	7	4	3	2	2	-1	2	2	2	2	1
FT Cycle	APLP1	CCL20	CD6	CDCP1	CNTN2	COL4A1	CXCL13	CXCL9	FLRT2	GFAP	GH	IL-12B	MOG	NEFL	OPG	OPN	PRTG	SERPINA9	TNFRSF10A	TNFSF13B	VCAN
FT 1	-6	-7	-1	-1	1	2	-1	-1	-2	-9	-7	-4	-3	1	-1	-3	-3	13	-8	-1	-1
FT 2	6	-2	0	-1	3	3	-5	1	-3	-4	1	0	1	-2	-3	-2	-1	-2	2	0	1
FT 3	-13	-13	-9	-11	-6	-11	-11	-11	-10	-20	-12	-13	-11	-12	-11	-11	-10	-13	-12	-9	-8
FT 4	-5	-10	-8	-8	-1	-5	-10	-5	-10	-23	-15	-7	-7	-12	-6	-7	-9	-11	-8	-4	-4
FT 5	-7	-11	-7	-7	-3	-7	-9	-7	-8	-28	-13	-9	-9	-1	-8	-9	-8	-12	-9	-7	-6

- **Stability studies for serum samples** have been performed to **characterize storage and processing conditions anticipated in a clinical setting**.
- Stability was assessed at 4 temperatures: -65°C or below, -10°C or below (-20°C), 2-8°C (4°C), and room temperature (RT) using 4 MS serum samples.
  - Results from -20°C, 4°C, and RT were compared to the control storage condition (-65°C or below) at the following timepoints:
    - 4 hours (for 4°C, and RT only), Day 1, Day 3, Day 7, Day 14, and Day 28
      - All biomarkers were stable for up to 1 day at RT and 4°C and up to 28 days at -20°C
        - For room temperature: CXCL13, IL-12B, and TNFSF13B decreased beyond -20% at 3 days
        - For 4°C: OPN decreased beyond -20% at 3 days
- Additionally 5 Freeze-Thaw (FT 1-5) cycles (performed at the -65°C or below) were evaluated using 4 MS serum samples.
  - 3 Freeze-Thaw cycles were found to be acceptable using ± 20% difference (average) vs. the control condition (fresh sample) as the threshold.
    - GFAP concentrations decreased beyond -20% for Freeze Thaw cycles 4 and 5
- Specifications for sample transport, processing, and storage will reflect results from this study and an ongoing expanded study (additional timepoints and samples)

Summary and Conclusions

- Performance has been assessed at the individual biomarker level. The custom assay panel has met acceptability criteria satisfying a fit-for-purpose analytical validation for 20 of the 21 proteins.
    - At present, COL4A1 has not met acceptability criteria due to assay imprecision.
- Additional validation experiments have been performed but not presented herein due to space constraints including: Cross-Reactivity (intra-panel and homologous proteins), Incurred Sample Reanalysis (ISR), and Plate Uniformity.
- Upon completion of the clinical validation study, the final Disease Activity and Disease Pathway Algorithms that utilize ensembles of proteins for the reported output will also be assessed for analytical validation parameters.
- A validated multivariate proteomic blood-based assay for objective MS disease assessments can serve as a quantitative, minimally invasive and cost-effective tool to enhance the standard of care for MS patients and their physicians.
    - The results of this analytical validation study will complement the ongoing clinical validation study and provide assurance that the assay has been thoroughly characterized and results are accurate, precise and robust.
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