

Validation of Novel Ultrasensitive Assays for Detection of Phosphorylated Tau (pT181 and pT217) in Human Plasma, Serum, and Cerebrospinal Fluid

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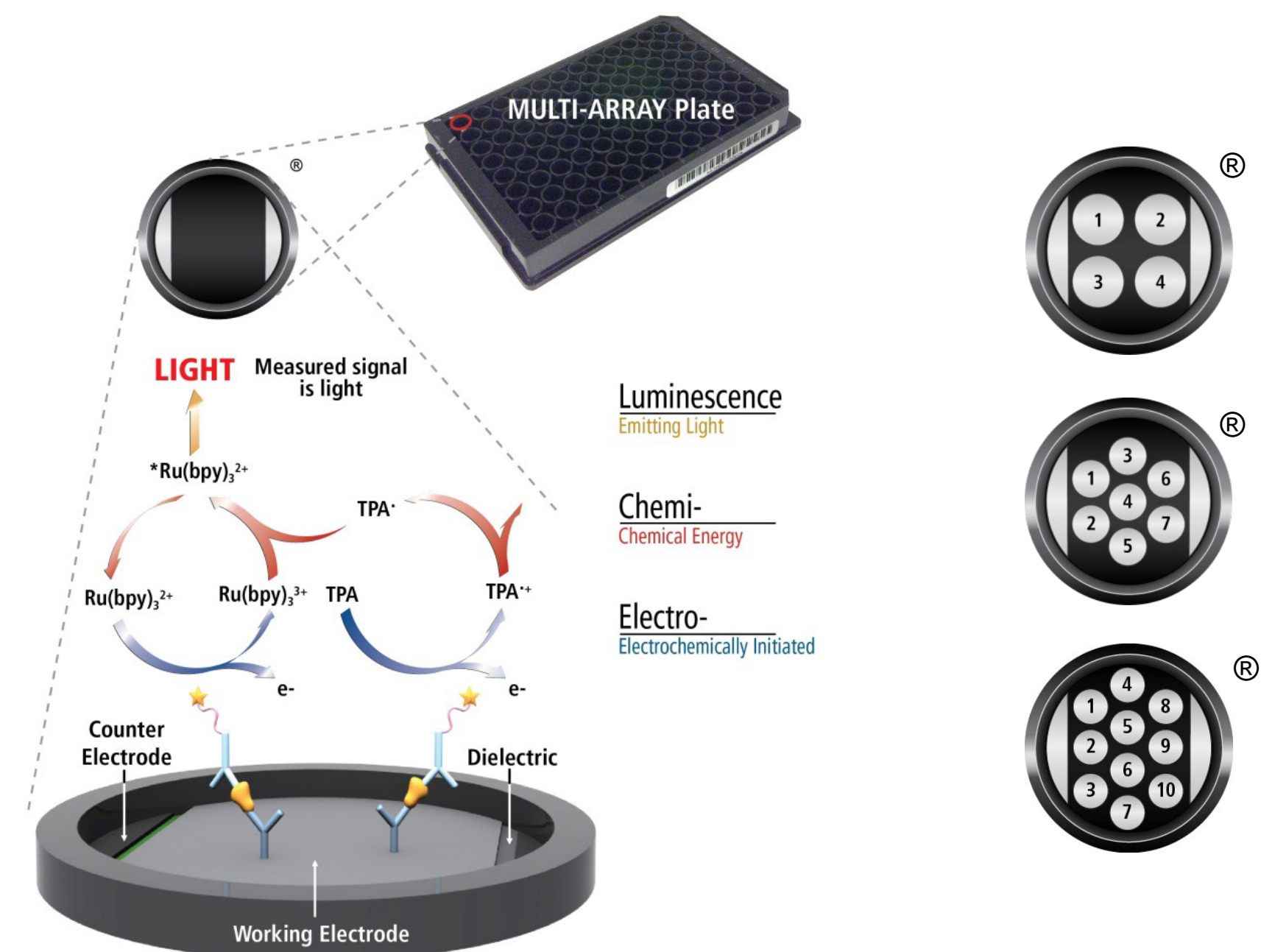
1 Abstract

Aggregation of phosphorylated tau (pTau) into neurofibrillary tangles is closely associated with Alzheimer's disease (AD). Hyper-phosphorylated tau in the brain has been imaged by classical biochemical techniques like immunohistochemical staining, but *in vivo* histological investigations are not possible. Measurement of circulating levels of tau phosphorylated at unique sites holds promise for research regarding early AD detection and potential treatment, as well as selection, classification, and monitoring in clinical trials. Moreover, blood-based biomarker detection assays can simplify sample acquisition further by removing the need for an invasive spinal tap, which is necessary for collecting cerebrospinal fluid (CSF). An accurate, reliable, and ultra-sensitive, blood-based pTau assay should significantly benefit AD research and fuel new understandings in pre-clinical research and screening.

Here, we describe the validation of ultrasensitive pTau (pT181 and pT217) biomarker assays in human plasma, serum, and CSF. Electrochemiluminescent (ECL) assays were performed on the Meso Scale Discovery S-PLEX platform using 96-well plates, different capture antibodies specific to each phosphorylation site of tau, and a common total tau detection antibody. Validation included assessment of intra- and inter-assay accuracy, precision, specificity, dilution linearity, selectivity, and stability. Both assays demonstrate high sensitivity with limits of detection in the femtomolar/mL range. The specificity of the assays was confirmed using a non-phosphorylated tau protein and tau proteins lacking the phosphorylation sites. All parameters evaluated met the predefined acceptance criteria per the FDA guidelines for analytical method validation. Matched healthy control (N = 15) and AD (N = 15) samples were tested. pTau was detectable in the three matrices, with higher levels of pT181 and pT217 observed in AD samples than in healthy controls. The assays allow for accurate, precise, and ultrasensitive detection of pT181 and pT217 in blood, and they can be used to support research regarding monitoring of disease progression and treatment as well as implementation of biomarker assays for AD.

2 Platform

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.



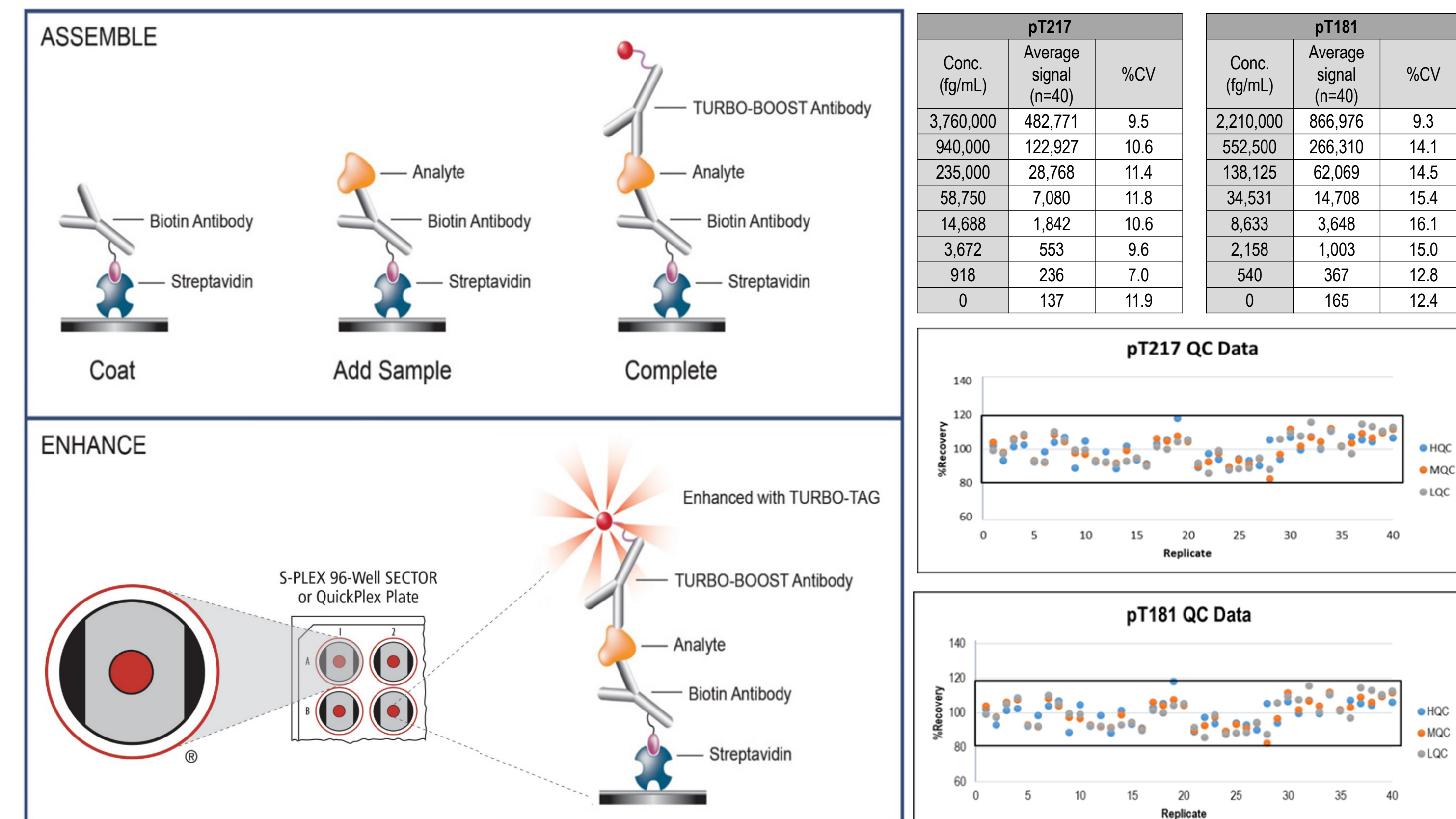
Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background 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.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

3 Method

S-PLEX® assays use either S-PLEX 96-well SECTOR™ or QuickPlex® plates that are coated with streptavidin. These plates provide high sensitivity, consistent performance and excellent inter- and intra-tot precision. S-PLEX kits are supplied with biotinylated capture antibody, a TURBO-BOOST® conjugated detection antibody, a calibrator, assay and antibody diluents, and S-PLEX specific reagents.

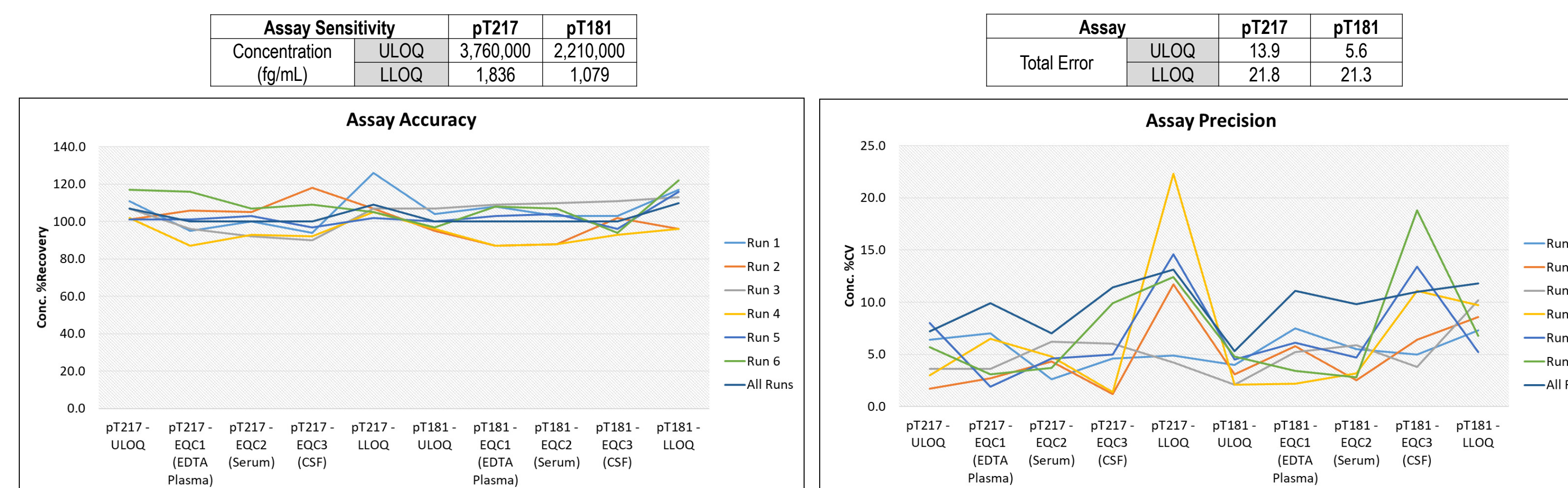
The S-PLEX assay protocol is simple, robust and executed in three steps: Assemble, Enhance, and Read as illustrated below. Representative calibration curve, high, mid, and low quality controls (HQC, MQC and LQC) data for pT217 and pT181 assays across 20 runs by multiple analysts over multiple days are shown and demonstrate S-PLEX assay robustness.



4 Results

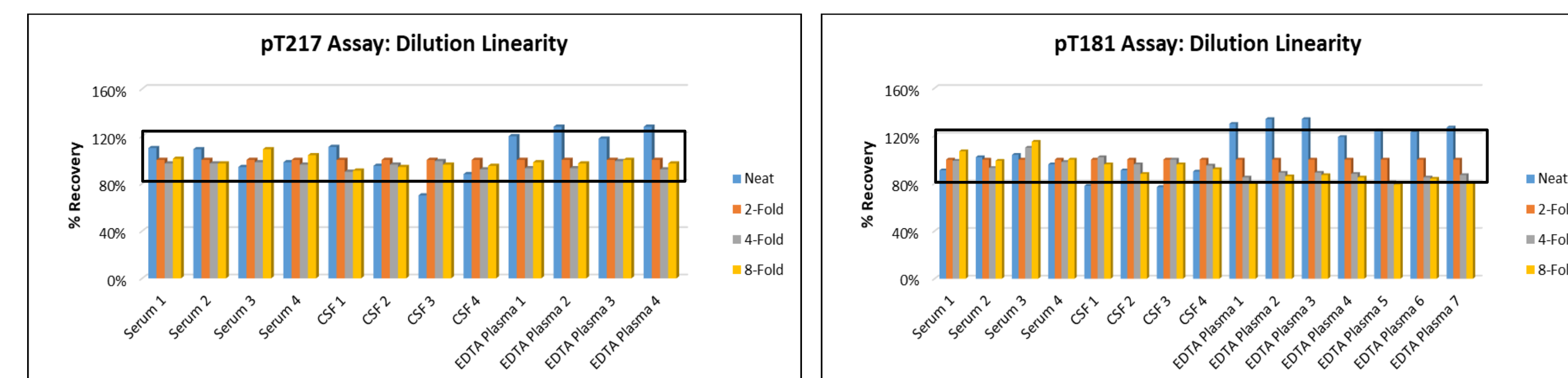
a. Accuracy, Precision & Sensitivity

Inter-assay (between runs) and intra-assay (within runs) accuracy and precision were assessed by performing 6 runs on 4 different days by 4 different analysts. Each run consisted of calibration curve, diluent QCs (HQC, MQC, LQC), matrix QCs (EQC1-EDTA plasma, EQC2-Serum, EQC3-CSF), and upper and lower limit of quantitation samples (ULOQ, LLOQ) tested in duplicate. The pT217 and pT181 assays demonstrated good accuracy and precision. Inter-assay and intra-assay accuracy and precision for diluent and matrix QC samples were $\pm 20\%$ and $< 20\%$, respectively. Samples prepared at the ULOQ and LLOQ exhibited within and between run precision of $\leq 25\%$, accuracy of 75-125% and total error (measurement of bias) of $\leq 40\%$. LLOQ represents assay sensitivity.



b. Dilution Linearity & Prozone Effect

Good dilution linearity (%recovery range $\pm 20\%$) was observed between neat to 8-fold dilutions in CSF and serum, and 2- to 8-fold dilutions in EDTA plasma across the two assays. A slight matrix effect was observed in neat EDTA plasma samples.

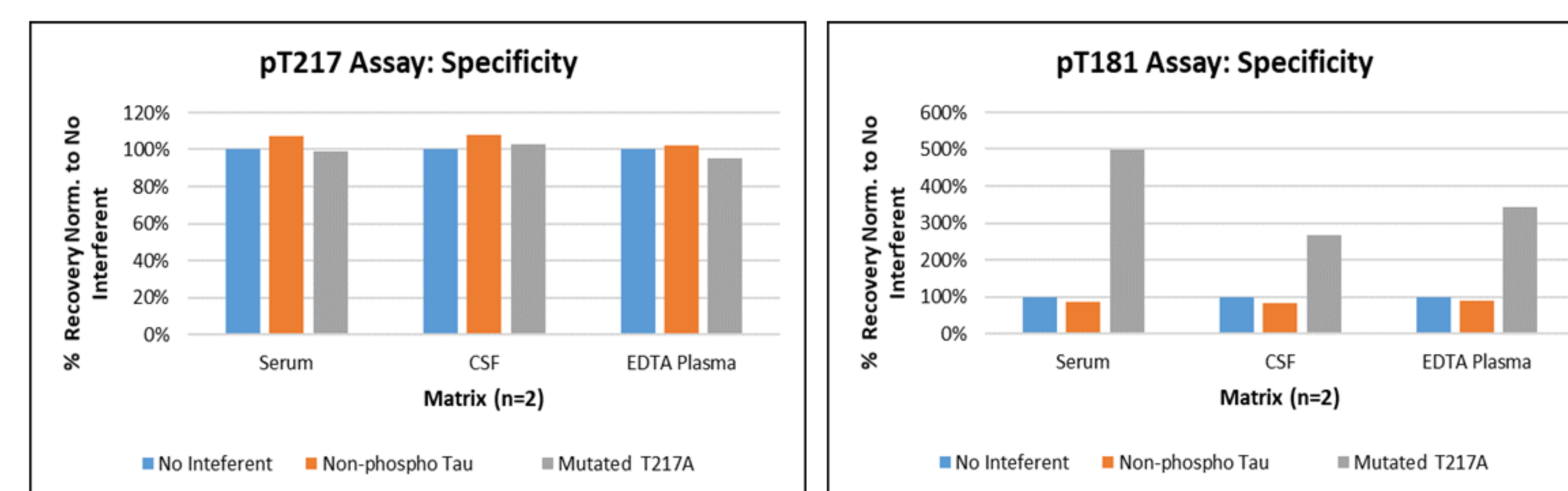


No prozone effect was observed in any of the matrices. Sample concentrations above ULOQ are labeled as ALQ (above limit of quantitation).

Matrix	Dilution Factor	Mean	Mean	Precision (%CV)		%Recovery (Norm. to 4-Fold)	
				Mean	Range	Mean	Range
pT217 Assay	Neat	801,223	4,903,512	1.8	0.5-4.5	ALQ	NA
	2-Fold	412,289	2,320,804	2.0	0.1-5.5	109	104-112
	4-Fold	192,800	1,069,019	1.3	0.2-3.7	100	100-100
	8-Fold	92,839	521,315	1.5	0.2-3.9	96	95-100
	16-Fold	45,085	259,576	1.9	0.4-3.6	97	95-101
pT181 Assay	Neat	648,058	6,560,177	3.1	1.5-5.8	ALQ	NA
	2-Fold	280,464	2,939,944	2.3	1.1-4.8	111	109-117
	4-Fold	121,553	1,319,835	3.9	1.7-7.0	100	100-100
	8-Fold	55,889	624,884	3.1	0.1-8.5	95	935-96
	16-Fold	26,135	322,214	1.9	0.2-3.9	92	89-95

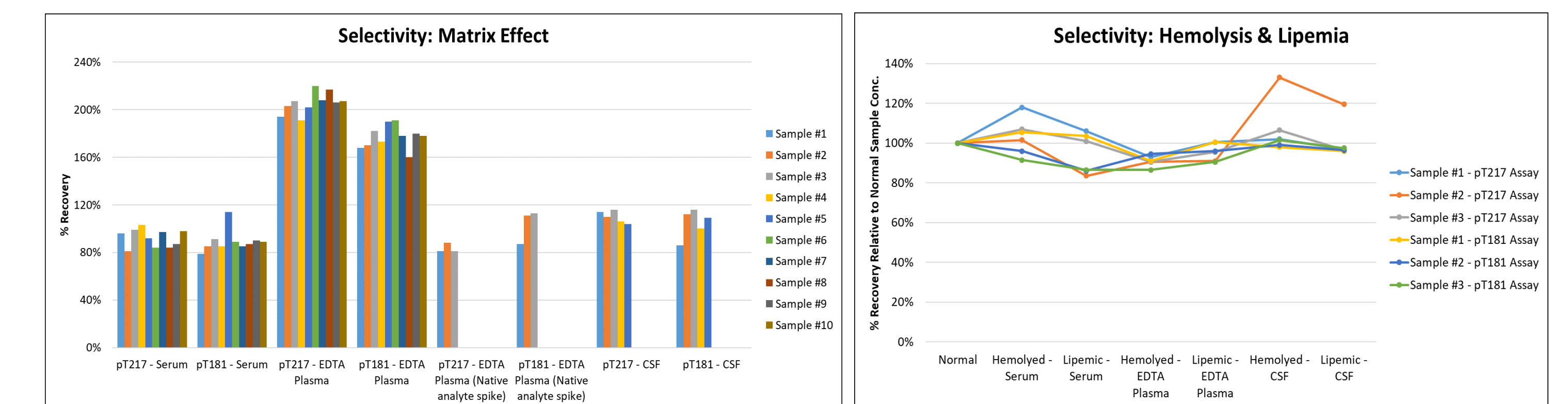
c. Specificity

The pT217 and pT181 assays showed no impact on sample quantitation upon addition of non-phosphorylated tau protein. The addition of tau protein containing a T217 - A217 mutation also did not interfere with the pT217 assay. As expected, over-recovery was observed for the pT181 assay in the presence of the mutated protein, which is partially phosphorylated at the T181 site.



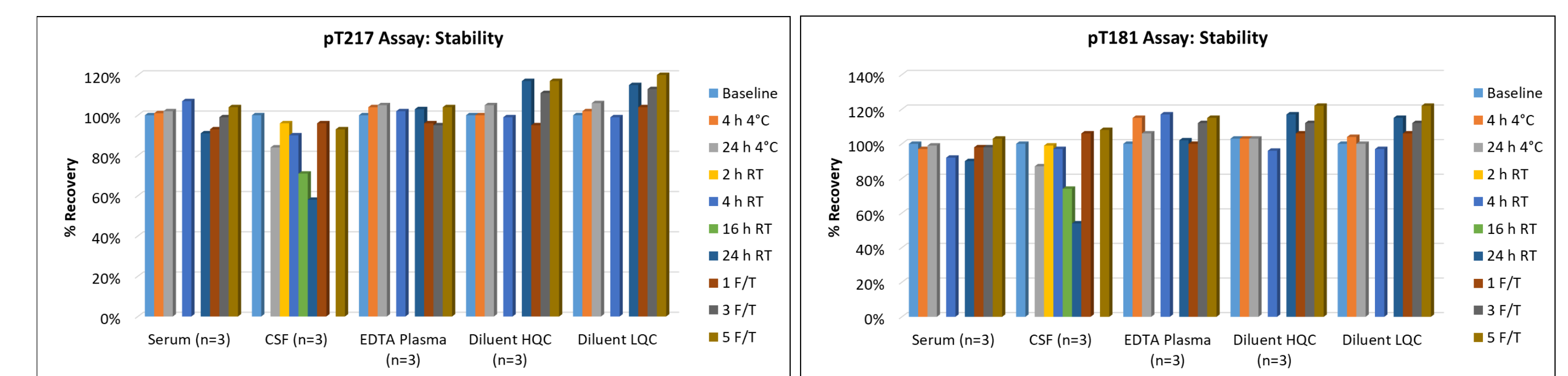
d. Selectivity

Selectivity was assessed by spiking calibrator in human serum (n=10), EDTA plasma (n=10), and CSF (n=5) samples. There was no discernable matrix effect in serum and CSF: $> 80\%$ of samples recovered within $\pm 20\%$ across both assays. Over-recovery was observed in calibrator-spiked EDTA plasma samples due to the nature of the recombinant calibrator. Recovery within $\pm 20\%$ was obtained with spiked native analyte (high CSF sample) in EDTA plasma samples. Both assays are tolerant to hemolysis and lipemia.



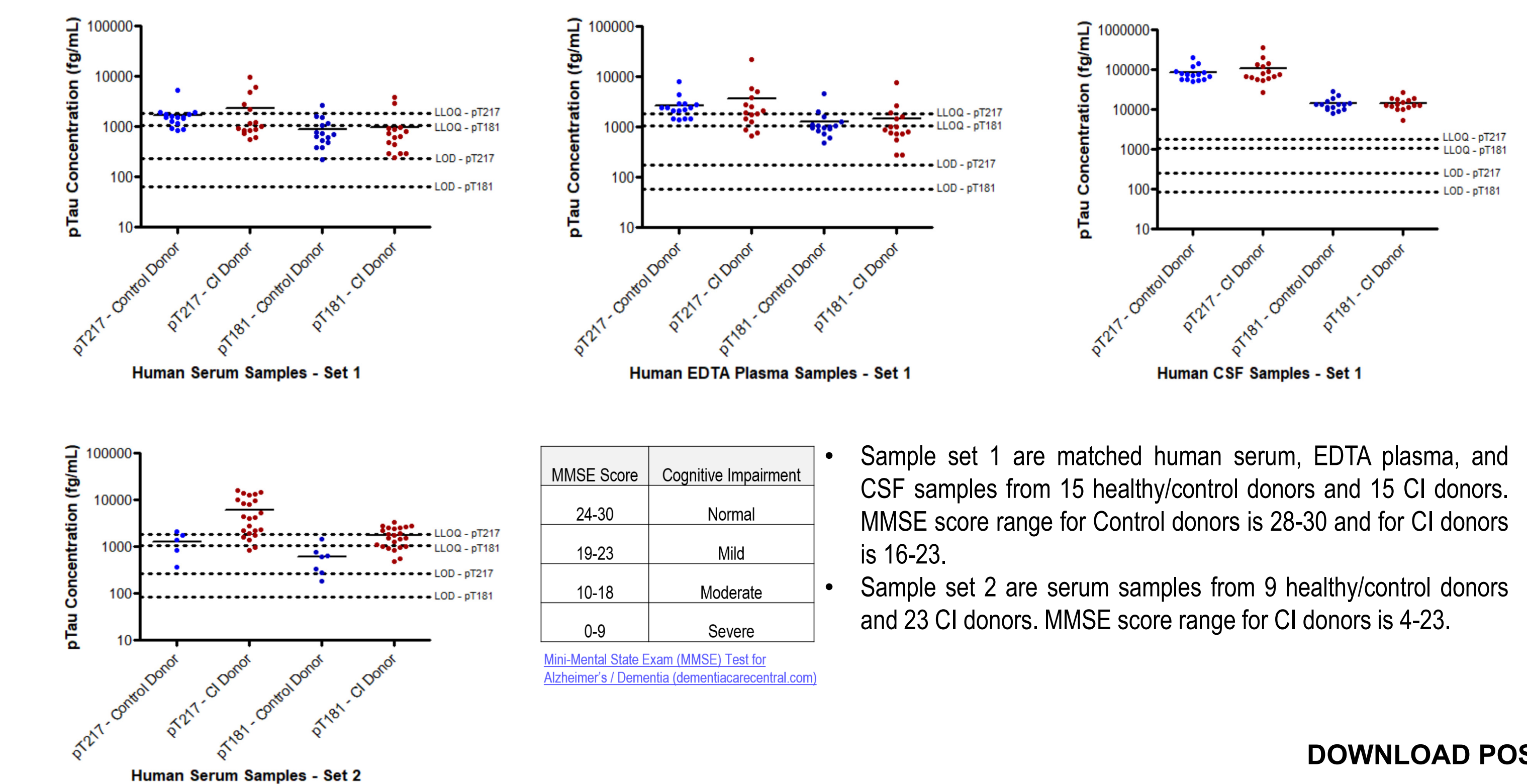
e. Stability

Samples (human serum, EDTA plasma and CSF) were analyzed after being subjected to storage at RT ($23^\circ\text{C} \pm 2^\circ\text{C}$) or $2-8^\circ\text{C}$ for 4 and 24 hours, or subjected to 1, 3 or 5 freeze-thaw cycles. The data indicates that all samples are stable, with percent recoveries within $\pm 25\%$ of baseline sample concentrations and precision $\leq 20\%$, through storage at RT for 24 hours (except for CSF), $2-8^\circ\text{C}$ for 24 hours and up to 5 freeze-thaw cycles. CSF is stable for 4 hours at RT.



f. Sample Testing

Two sets of samples were tested neat in duplicate to evaluate pT217 and pT181 levels in human serum, EDTA plasma, and CSF. Data are shown for all samples with pTau levels above the assay limits of detection. Higher levels of pT217 and pT181 were observed in cognitively impaired (CI) donors with higher MMSE scores compared to healthy donors.



5 Conclusion

Novel ultrasensitive assays were validated for detection of phosphorylated tau (pT217 and pT181). The assays allow for accurate and precise quantitation of pT217 and pT181 in human serum, EDTA plasma, and CSF. The assays were validated in MSD's Bioanalytical Laboratory, which is GLP-compliant and provides preclinical and clinical sample testing services.

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