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Neurofilament Light (NfL) is a Bona-fide Blood Based Biomarker for Neurodegenerative Diseases

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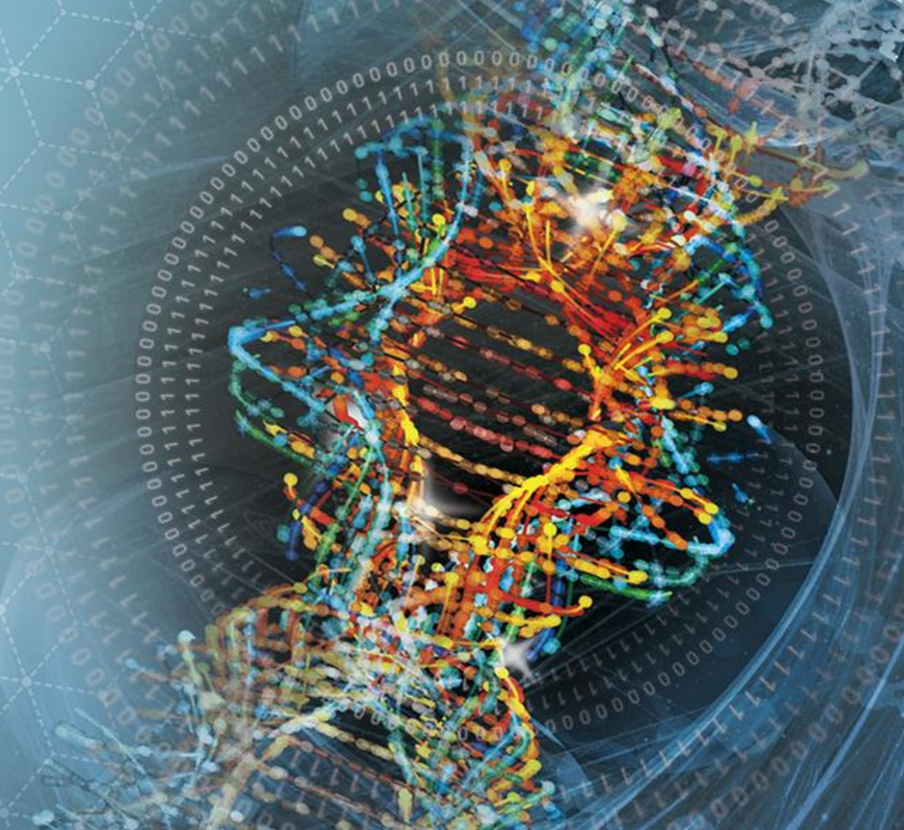

PURPOSE

Structural changes in brain portraying pathophysiology germane to neurodegeneration occurs late in the disease. Early detection of neuronal loss and appropriate therapeutic intervention is quintessential to ameliorate clinical symptoms. Neurofilament Light (NfL) is one of the scaffolding proteins that provides structure and support to axonal filaments. Injury to neurons from direct trauma or progressive neurodegeneration leads to break down of axonal filaments releasing NfL into cerebrospinal fluid (CSF) and eventually into blood. Elevated NfL levels in CSF and blood has been attributed to several neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease (1, 2, 3, 4). Given the biomarker potential of NfL in identifying the severity of the disease and determining the efficacy of novel therapeutics, we sought to develop an ultrasensitive digital ELISA assay for NfL quantification.

METHOD

NfL levels in CSF are elevated incrementally as the disease exacerbates, however only trace amounts, typically in the sub-picomolar range are found in circulation after dilution of limited NfL from CSF into approximately five liters of blood. Therefore, conventional ligand binding assay platforms do not meet the aforementioned sensitivity requirements and an ultrasensitive platform is the sole choice to quantitate NfL in blood. Hence, we resorted to SiMoA (Single Molecule Array) based digital ELISA assay platform on HD-1 analyzer from Quanterix corporation. In this single molecule bead based assay, positive immunocomplexes are labeled with β-galactosidase enzyme which then cleaves the substrate Resorufin-β-GalactoPyranoside (RGP). Resorufin fluorescence emanating from isolated positive immunocomplexes is captured through time lapse molecular imaging. This particular approach ensures detection of pulsatile responses from positive immunocomplexes amidst the thunderous noise (4). Average Enzymes per Bead (AEB) is computed from analog and digital read outs of the signal, which is then interpolated to quantitate NfL concentration in clinical samples. Specifically, our assay has a calibration curve range of 500 - 0.167 pg/mL, has an MRD of 4 and is sensitive enough to detect ~0.5 pg/mL of Neurofilament Light in human plasma. We assessed intra and inter assay accuracy and precision, parallelism, selectivity, freeze-thaw stability, thawed matrix stability, room temperature stability and long-term stability to support rapid NfL quantification in clinical samples.

RESULT(S)

Prediluted individual calibrators were obtained from Quanterix corporation. The sensitivity of NfL SiMoA assay was further extended by adding another calibrator below the manufacturer provided LLOQ. Therefore, Neurofilament light calibration curve range was set from 500 pg/mL (ULOQ) to 0.167 pg/mL (LLOQ). The Average Enzymes per Bead (AEB) obtained at respective calibrator concentrations is plotted in Figure 1. Error bars indicate standard deviation of AEB obtained from three independent accuracy and precision runs. Limit of Detection (LoD) computed from blank response is 10 fg/mL, clearly pushing the detection limits into ultrasensitive range.

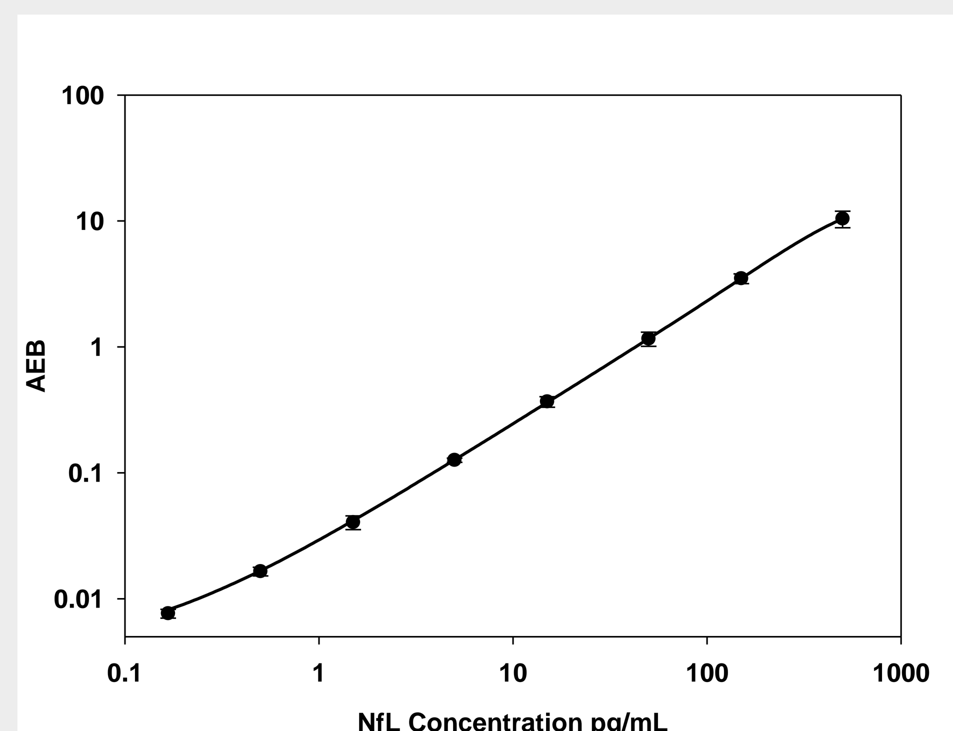

Figure 1: Neurofilament Light calibration curve

Inter-assay precision data for quality controls are reported in Table 1. Buffer controls (QC1, QC2) and K_2-EDTA plasma samples (QCP1, QCP2) were diluted to minimum required dilution (MRD) of 4 and assayed at n = 3 replicates in three independent experiments. Percent coefficient of variation among replicates between different runs was within 10% suggesting good reproducibility of the assay. Pre-diluted buffer controls were obtained from Quanterix corporation. The mean values obtained for QC1 and QC2 fall within the ranges provided in the certificate of analysis indicating accuracy of the assay.

Run ID	QC 1 Dil 4 (pg/mL)	QC 2 Dil 4 (pg/mL)	QCP 1 Dil 4 (pg/mL)	QCP 2 Dil 4 (pg/mL)
1RNBS	3.23	137	9.05	5.77
	3.33	148	9.01	6.31
	3.22	143	8.69	6.85
2RNBS	3.74	136	9.51	6.50
	3.36	140	9.10	7.38
	2.99	147	9.48	6.44
3RNBS	3.77	133	8.57	7.70
	3.56	139	8.74	6.58
	3.26	132	8.57	6.06
N	9	9	9	9
Mean	3.38	139	8.97	6.62
%C.V.	7.55	4.03	3.99	9.20

Table 1: Inter-assay precision of buffer and matrix quality controls

Parallelism experiments in samples reveal critical information regarding the matrix interference, Minimum Required Dilution (MRD) and sensitivity of the assay. Figure 2 represents data from parallelism experiments evaluated in three human K_2-EDTA plasma samples. These samples were diluted to MRD, followed by serial dilution beyond MRD until the signal drops below quantification limits. Percent recovery obtained at higher dilutions is comparable to the calculated concentration obtained at MRD suggesting the specificity of the assay.

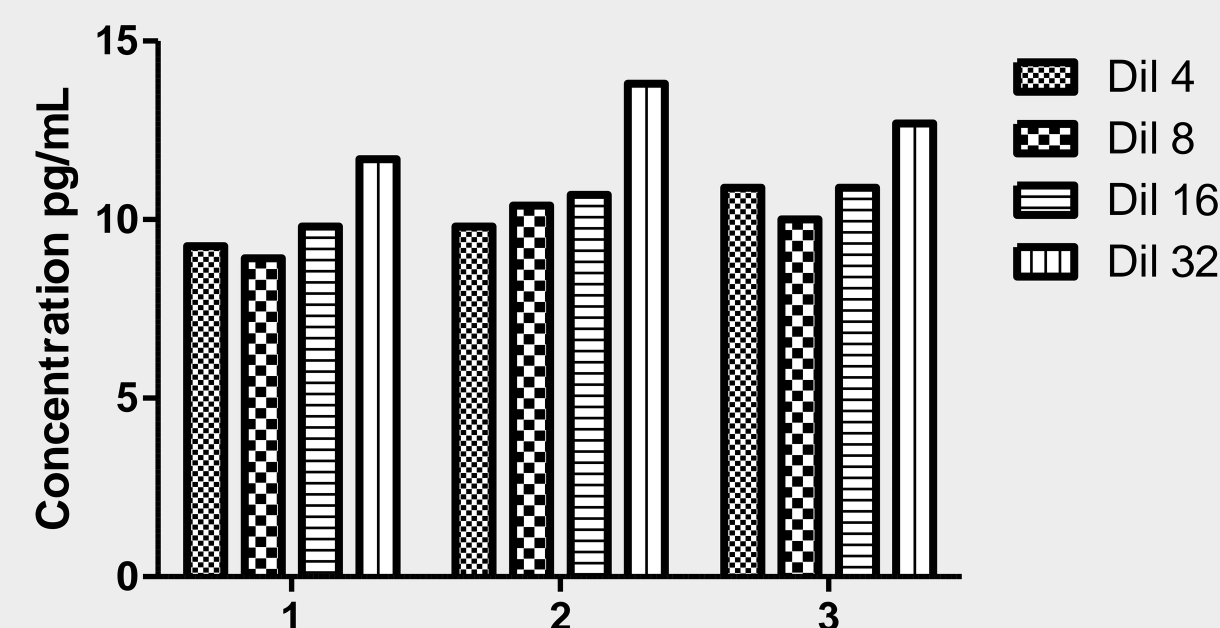

Figure 2: Evaluation of parallelism in K_2-EDTA plasma samples

To determine the stability of the analyte, two endogenous matrix controls whose concentration was established during the A&P runs were used as stability samples. Analyte stability at Room Temperature (RT) and Thawed Matrix (TM) and Freeze-Thaw (FT) stability was assessed in one analytical run. NfL was determined to be stable up-to three freeze-thaw cycles (FT 1 and FT 2) and for 24 hours at room temperature (RT1 and RT2) and for 72 hours at 2-8°C (TM1 and TM2). Data in Table 2 represents the precision expressed as percent coefficient of variation and accuracy expressed as percent difference from theoretical obtained for three replicates analyzed at each level.

Run ID	FT 1 Dil 4 (pg/mL)	FT 2 Dil 4 (pg/mL)	RT 1 Dil 4 (pg/mL)	RT 2 Dil 4 (pg/mL)	TM 1 Dil 4 (pg/mL)	TM 2 Dil 4 (pg/mL)
3RNBS	9.04	6.30	8.28	6.93	8.53	7.25
	8.79	6.34	8.76	6.23	8.59	5.73
	8.64	6.51	8.21	6.25	8.11	6.25
N	3	3	3	3	3	3
Theoretical Concentration	8.97	6.62	8.97	6.62	8.97	6.62
Mean	8.82	6.38	8.42	6.47	8.41	6.41
%C.V.	2.33	1.73	3.52	6.18	3.14	12.0
%DFT	-1.64	-3.62	-6.18	-2.31	-6.25	-3.13

Table 2: Neurofilament Light freeze-thaw and thawed matrix stability

CONCLUSION(S)

Neuronal damage and death occurs well before the onset of structural changes in brain and the associated clinical symptoms. Therefore, there is an urgent and unmet need to accurately detect and instill therapeutics early-on to reverse the injury and restore normal physiology. While probing for NfL in CSF allows accurate quantification of neurodegenerative biomarkers, repeated CSF drawing through lumbar puncture is complicated.

Hence, NfL detection in blood is preferable and the data presented here provides unequivocal evidence that NfL can be accurately quantified from plasma samples. Specifically, the error within inter-assay precision runs is below 10%, acceptable recoveries were obtained at dilutions beyond the MRD in parallelism samples, indicating the specificity of the assay and the analytical measurement range is computed to be from 16000 to 0.667 pg/mL. Data presented here should entice pharmaceutical companies and neurologists to probe for neurodegenerative biomarkers in peripheral blood more routinely to prevent unforeseen complications of the disease.

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