Development and Validation of U-PLEX® Human Alpha-Synuclein Assay

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1 Abstract

Introduction: The human alpha-synuclein protein is well-studied in Parkinson's disease (PD); it aggregates to form toxic soluble oligomers (i.e., protofibrils) and insoluble fibrils known as Lewy bodies (LBs). Alpha-synuclein pathologies are also prevalent among patients suffering from dementia. Alpha-synuclein has been detected in cerebrospinal fluid (CSF), serum, plasma, and whole blood, which highlights its potential as a biomarker for disease. A biomarker that can be detected during the pre-symptomatic or early active stages and/or distinguish PD from other neurodegenerative conditions could significantly impact the selection of patients for clinical trials, and ultimately inform treatment options. In this study, we describe the development and validation of an immunoassay for detection of alpha-synuclein in human CSF, saliva, serum, plasma, and whole blood.

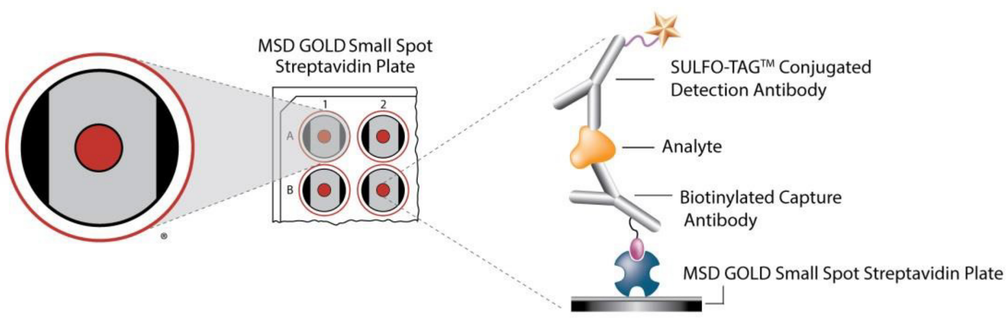
Materials and Methods: The assay was developed using MSD's MULTI-ARRAY® technology. Antibodies targeting the human alpha-synuclein protein were selected to maximize assay sensitivity and specificity. Analytical validation was performed across three independent kit lots to confirm consistency, accuracy, and precision. We optimized the assay to minimize matrix interferences and verify compatibility with human CSF, saliva, serum, plasma, and whole blood.

Results and Discussion: The MSD® human alpha-synuclein assay demonstrated excellent sensitivity, precision, and inter-lot reproducibility. Precision and accuracy were determined from artificial and human CSF control samples with typical intra-plate CVs of < 10%. Measured levels of alpha-synuclein in all matrices fell within the quantitative range of the assay and were consistent with literature reports. Dilution linearity and spike recovery testing demonstrated minimal matrix effects and accurate quantitation. Calculated concentrations of serially-diluted and spike samples recovered within 80-120% of expected values. The assay exhibited < 0.1% cross-reactivity to beta- and gamma-synuclein proteins.

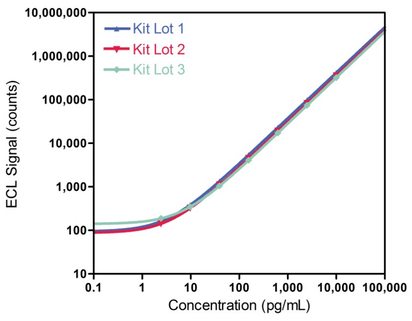
Conclusion: MSD has developed and validated an assay to measure alpha-synuclein in human CSF, serum, plasma, saliva, and whole blood. The assay provides accurate, precise measurements, as well as consistent performance across lots. This assay will support ongoing efforts to evaluate alpha-synuclein as a biomarker for characterization of PD cohorts. The reported research was funded by the Michael J. Fox Foundation for Parkinson's Research.

2 Methods

The U-PLEX human alpha-synuclein assay is a sandwich immunoassay (Figure 1). MSD provides a pre-coated MSD GOLD™ Small Spot Streptavidin Plate. MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the microplate. The intensity of emitted light is measured, providing a quantitative measure of analyte in the sample.



3 Standard Curve and Assay Protocol



	Human Alpha-Synuclein	
Conc. (pg/mL)	Average Signal	%CV
10,800	410,627	4.3
2700	86,788	4.3
675	19,405	3.6
169	4,689	2.7
42.2	1,242	2.5
10.55	364	2.9
2.64	143	3.7
0	68	7.0

Protocol

1. Add capture antibody solution (25 µL per well). Incubate 1 hour at RT.
2. Wash with PBS-T. Add detection antibody solution (25 µL per well). Add 25 µL of standard or diluted sample. Incubate 2 hours at RT.
3. Wash and add Read Buffer (150 µL per well). Analyze with MSD instrument.

Left: Standard curves from three independently built kit lots are presented, illustrating the wide dynamic range of the assay and the highly reproducible standard curve signals across manufactured kit lots. Each curve represents the average signals from a multi-run, multi-analyst, multi-day data set. **Middle:** Representative data of three kit lots. **Right:** The assay protocol is described.

4 Sensitivity

Assay sensitivity, as reported by lower limit of detection (LLOD), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), was determined from testing of three independent kit lots.

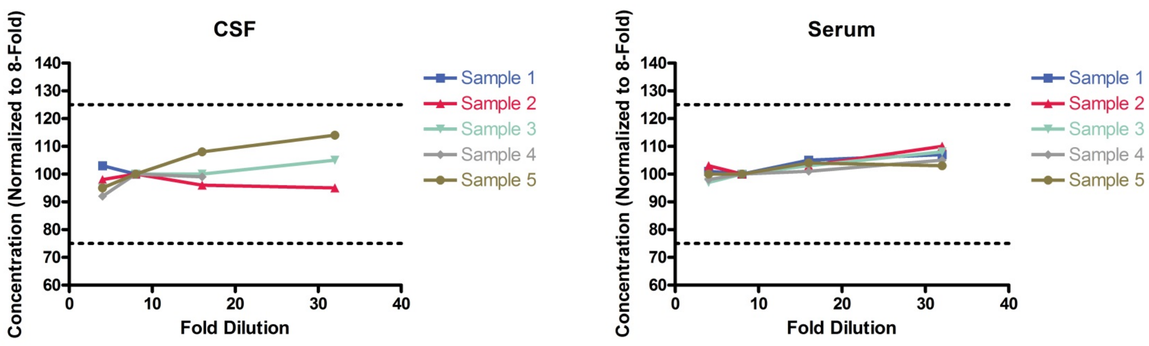
Human Alpha-Synuclein			
Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
0.900	0.464 - 1.68	8.00	6,800

5 Cross-reactivity

The assay specifically recognizes the target protein and has no detectable cross-reactivity with beta- and gamma-synuclein. Results are representative data from three kit lots.

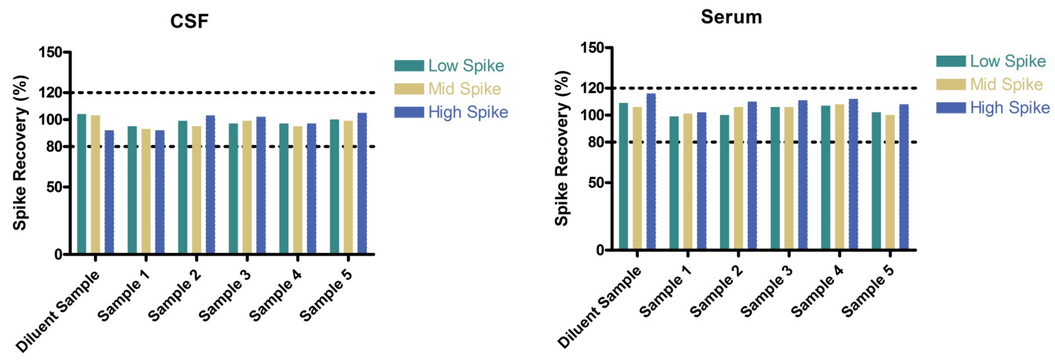
Alpha-Synuclein		Beta-Synuclein		Gamma-Synuclein	
Conc. (pg/mL)	Signal	Conc. (pg/mL)	Signal	Conc. (pg/mL)	Signal
10,800	550,365	10,000	62	10,000	196
0	72	0	66	0	69

6 Dilution Linearity



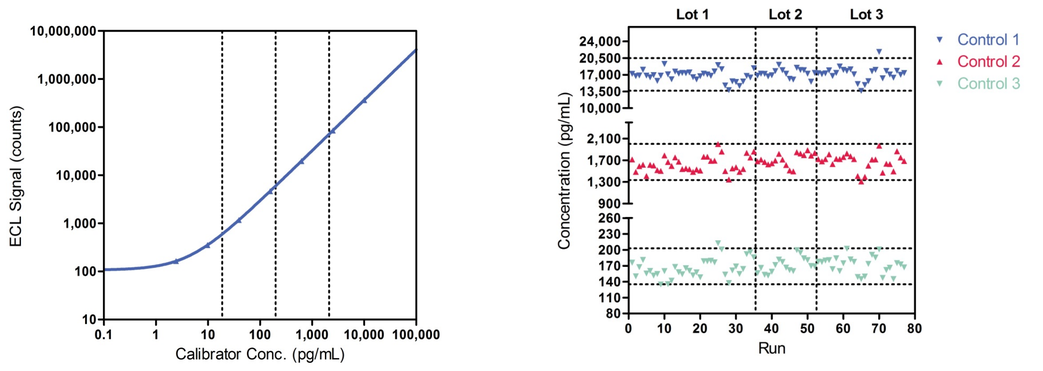
Samples of human CSF (n=5), saliva (n=5), whole blood (n=5), serum (n=5), and plasma (n=5) were serially diluted in assay diluent. Measured concentrations were corrected for dilution factor. Recovery at each dilution was calculated relative to the target sample dilution. Results for CSF and serum are shown above. Dotted lines represent guard bands of 25% below and above the expected concentration. Results are representative data from two kit lots.

7 Spike Recovery



Samples of human CSF (n=5), saliva (n=10), whole blood (n=5), serum (n=5), and plasma (n=5) were acquired following Parkinson's Progression Markers Initiative (PPMI) guidelines and tested on the U-PLEX human alpha-synuclein assay. Recombinant alpha-synuclein protein was spiked into each sample at various concentrations. Expected concentrations were based on the endogenous values plus the spiked amounts. Recovery at each spike level was calculated relative to the expected concentration. Results for CSF and serum samples are shown. Dotted lines represent guard bands of 20% below and above the expected concentration. Results are representative data from two kit lots.

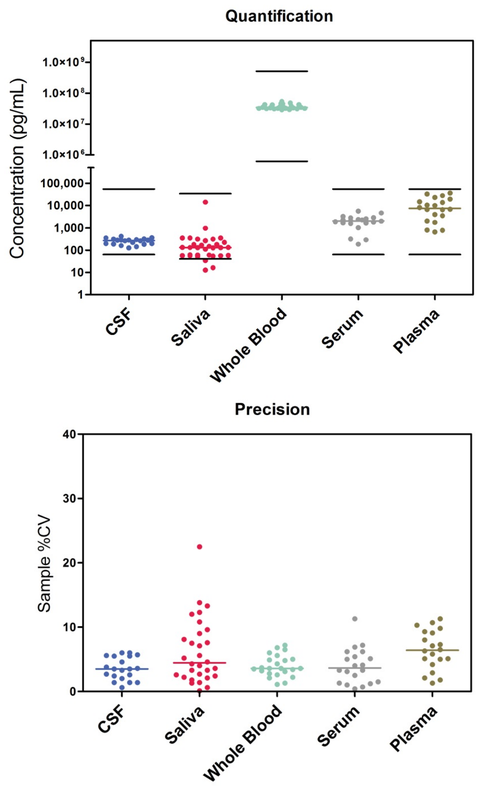
8 Precision and Accuracy



Human Alpha-Synuclein Diluent-based Controls				
	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
Control 1	17079	3.2	7.3	1.5
Control 2	1671	3.2	8.7	2.7
Control 3	169	3.6	10.0	2.1

Alpha-synuclein levels were measured in diluent-based controls (n=3). Controls were produced by spiking nominal quantities of alpha-synuclein calibrator into a diluent designed to serve as artificial CSF. **Left:** Diagram showing the target concentrations (8-fold, dilution-adjusted, dotted lines) of the controls in relation to the typical calibration curve. The control levels were selected to span the quantitative range of the assay. **Right:** Measurements were made across three kit lots using multiple analysts and plates during 77 runs over a five-month period. Horizontal lines represent guard bands of 20% above and below the assigned concentration. Vertical lines separate runs between the different kit lots. **Bottom:** The precision results for the controls are presented in a tabular format. Reported concentrations are adjusted for sample dilution.

9 Measurement of Human Samples



Human Alpha-Synuclein		
Sample Type	Statistic	Concentration
CSF (n=20) (pg/mL)	Median	266
	Range	137 - 383
	% Detected	100
Saliva (n=32) (pg/mL)	Median	137
	Range	33 - 15,424
	% Detected	91
Whole Blood (n=23) (µg/mL)	Median	29
	Range	24 - 45
	% Detected	100
Serum (n=20) (pg/mL)	Median	2,090
	Range	204 - 5,155
	% Detected	100
Plasma (n=20) (pg/mL)	Median	6,314
	Range	627 - 31,435
	% Detected	100

The target protein was quantified in five human matrices. Samples of human CSF (n=20), saliva (n=32), whole blood (n=23), serum (n=20), and plasma (n=20) were tested. Representative data are from three kit lots. **Top Left:** Calculated concentrations from testing of each sample. Black lines represent LOQs (dilution-adjusted). Dilutions were as follows: CSF (8-fold), saliva (5-fold), whole blood (75,000-fold), serum (8-fold), and plasma (8-fold). **Bottom Left:** Intra-plate %CVs of calculated concentrations from testing of each sample. **Top Right:** Summary of testing averaged across three kit lots, showing % of samples falling within the detectable range of the assay.

10 Conclusion

The U-PLEX Human Alpha-Synuclein Kit has been analytically validated for measurement of alpha-synuclein in human CSF, saliva, whole blood, serum, and plasma across a wide range of concentrations. Precision has been demonstrated across runs and kit lots. The kit can accurately measure alpha-synuclein in all tested matrices and is robust to matrix effects. Finally, the assay shows no significant cross-reactivity with closely related proteins. Collectively, these results demonstrate that the U-PLEX Human Alpha-Synuclein Kit is a promising tool to support ongoing efforts to evaluate alpha-synuclein as a biomarker for characterization of PD cohorts.

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Acknowledgements: The Michael J. Fox Foundation for Parkinson's Research, Grand Central Station, P.O. Box 4777, New York, NY 10163-4777