T2079

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

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1 Abstract

Purpose: 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 (also known as "synucleinopathies") are also prevalent among patients suffering from dementia with LBs and multiple-system atrophy. Alpha-synuclein has been detected in several biological matrices, such as 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.

Methods: The assay was developed using MSD's MULTI-ARRAY® technology. Antibodies targeting the human alpha-synuclein protein were previously 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.

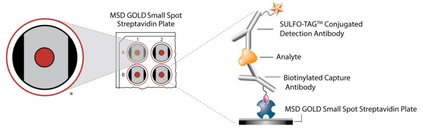
Results: The MSD® human alpha-synuclein assay demonstrated excellent sensitivity, precision, and inter-lot reproducibility. Precision and accuracy were determined from artificial and human CSF control samples with typical intra-plate CVs of less than 10%. Measured levels of alpha-synuclein in all five tested 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 with all five matrices. Calculated concentrations of serially-diluted and spike samples recovered within 80-120% of expected values. The assay exhibited less than 0.1% cross-reactivity to closely related beta- and gamma-synuclein proteins.

Conclusions: 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 Principle of the Assay

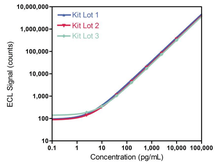
The U-PLEX Human Alpha-Synuclein Assay is a sandwich immunoassay (Figure 1). MSD provides a pre-coated MSD GOLD™ Small Spot Streptavidin Plate. Biotin-conjugated anti-human alpha-synuclein capture antibody is added to the plate, followed by the addition of sample, and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™). Analyte in the sample binds to capture antibodies immobilized on the working electrode surface, then recruitment of the detection antibodies by the bound analytes completes the sandwich. Detection is enabled with the addition of MSD Read Buffer, a solution containing reactants required for electrochemiluminescence. The plate is loaded into an MSD instrument where a voltage applied to the plate electrodes causes the captured labels to emit light. The intensity of emitted light is measured, providing a quantitative measure of analyte in the sample.



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 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

	Human Alpha-Synuclein			
	Median LLOD (pg/mL)	LLOD Range (pg/mL)	LLOQ (pg/mL)	ULOQ (pg/mL)
Kit Lot 1	0.70	0.46 - 1.22	3.99	6,800
Kit Lot 2	0.91	0.71 - 1.29	3.14	6,800
Kit Lot 3	1.14	0.74 - 1.68	2.59	6,800
Cross-lot	1.06	0.46 - 1.68	8.00	6,800

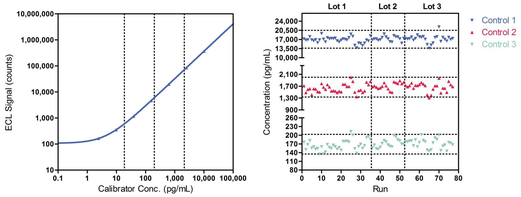
The U-PLEX Human Alpha-Synuclein Assay is sensitive and measures alpha-synuclein over a wide dynamic range. Assay sensitivity, as reported by lower limit of detection (LLOD), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), was determined for each of the three independent kit lots. The LLOD is a calculated concentration based on a signal 2.5 standard deviations above the blank (zero calibrator). The range of LLODs measured across three kit lots (n=123 plates) is presented. The ULOQ and LLOQ samples were created by spiking nominal quantities of alpha-synuclein calibrator into assay diluent. LLOQ and ULOQ are, respectively, the lowest and highest concentration of calibrator tested which has a %CV of 20% or less, with recovered concentration within 80-120%. The quantitative range is 8.00-6,800 pg/mL, which accounts for variation in the LLOD and Top-of-Curve. Testing for each kit involved a minimum of five runs. In-well concentrations are reported.

5 Cross-reactivity

The U-PLEX Human Alpha-Synuclein Assay specifically recognizes the target protein, and has no detectable cross-reactivity with closely-related proteins in the synuclein family, beta- and gamma-synuclein. The tested levels are above expected physiological concentrations. 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
2,700	101,208	2,500	66	2,500	95
675	20,650	625	62	625	71
169	5,283	156	66	156	64
42	1,247	39	60	39	75
11	393	9.8	65	9.8	74
2.6	159	2.4	61	2.4	65
0	72	0	66	0	69

6 Precision and Accuracy



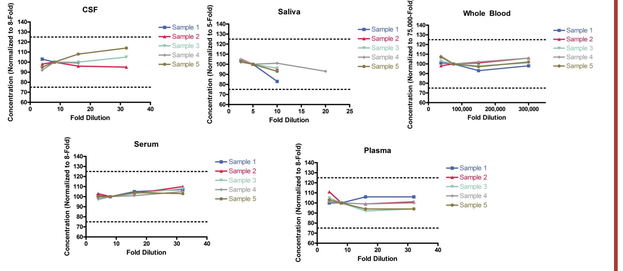
Human Alpha-Synuclein Diluent-based Controls				
	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %CV	Inter-lot %CV
Control 1	17079	3.2	7.3	1.5
Control 2	1671	3.2	8.7	2.7
Control 3	169	3.6	10.0	2.1

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. Results for the three controls, tested on kit lots 1–3, are presented. 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.

Not shown: Similar results were obtained from measurements of CSF-based controls across numerous runs and kit lots. CSF-based controls were comprised of unique, de-identified patient CSF samples, which were selected to span the expected physiological range (100-1000 pg/mL). Measured concentrations were typically within ±25% of the assigned, expected values. Intra-run CVs were less than or equal to 3.0%. Inter-lot CVs were less than 5.0%.

The U-PLEX Human Alpha-Synuclein Assay produces consistent measurements of control samples (diluent-based and matrix-based) both within and across runs and kit lots.

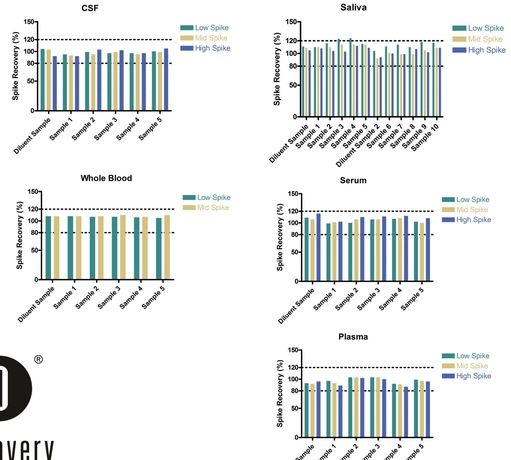
7 Dilution Linearity



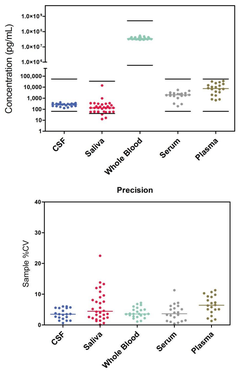
The U-PLEX Human Alpha-Synuclein Assay is robust to matrix interferences, and provides consistent quantification of human samples at the minimum required dilution and beyond. Samples of human CSF (n=5), saliva (n=5), whole blood (n=5), serum (n=5), and plasma (n=5) were acquired following Parkinson's Progression Markers Initiative (PPMI) guidelines and tested on the U-PLEX Human Alpha-Synuclein Assay. Samples 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. Dotted lines represent guard bands of 25% below and above the expected concentration. Some CSF and saliva samples were undetectable at higher dilutions, e.g., 32-fold and 20-fold (respectively). Results are representative data from two kit lots.

8 Spike Recovery

The U-PLEX Human Alpha-Synuclein Assay is robust to matrix interferences, and provides accurate quantification in five human matrices. Samples of human CSF (n=5), saliva (n=10), whole blood (n=5), serum (n=5), and plasma (n=5) were acquired following 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. Dotted lines represent guard bands of 20% below and above the expected concentration. Results are representative data from two kit lots.



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 U-PLEX Human Alpha-Synuclein Assay quantifies the target protein in five human matrices, demonstrating its utility and versatility. Samples of human CSF (n=20), saliva (n=32), whole blood (n=23), serum (n=20), and plasma (n=20) were acquired following PPMI guidelines, and tested on the U-PLEX Human Alpha-Synuclein Assay. **Top Left:** Representative data from three kit lots. 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:** Representative data from three kit lots. Intra-plate %CVs of calculated concentrations from testing of each sample. **Right:** Summary of testing averaged across three kit lots, showing typical values and % of samples falling within the detectable range of the assay.

10 Conclusion

The MSD U-PLEX Human Alpha-Synuclein Assay has been analytically validated for measurement of alpha-synuclein in human CSF, saliva, whole blood, serum, and plasma. The kit was built using rigorously characterized critical reagents. The assay provides adequate sensitivity to detect alpha-synuclein across a wide range of concentrations. Precision has been demonstrated across runs and kit lots using both artificial and human CSF. 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 Assay is a promising tool to support ongoing efforts to evaluate alpha-synuclein as a biomarker for characterization of PD cohorts.

Acknowledgements

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