Toward Standardization of Alpha-synuclein Measurement: A Multi-pronged/Multi-center Approach for Assay Verification and Validation

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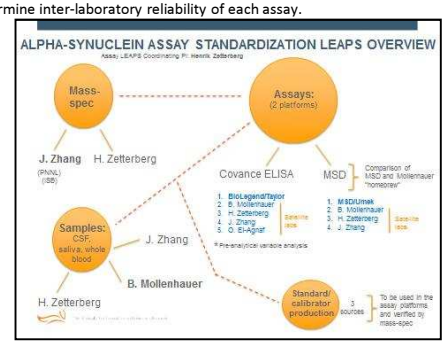
Background

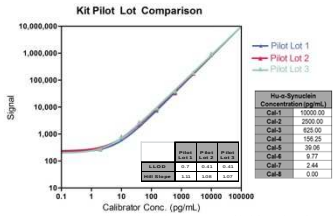
Alpha-synuclein has been implicated in the pathogenesis of Parkinson's disease (PD) based upon accumulation of the protein in Lewy bodies, a hallmark of PD, and the association of mutations in the synuclein gene with the onset of an inherited form of PD. Alpha-synuclein levels in various biological fluids have been studied as a potential biomarker that may enable discrimination of PD affected individuals from non-affected counterparts; and results suggest that, at least in the cerebrospinal fluid, levels of the protein may differ between these groups (decrease in PD and alpha-synuclein related disorders compared to controls). Establishing the diagnostic or prognostic value of alpha-synuclein as a biomarker requires robust assay(s) that can generate reliable measurement. The assays utilized to date detect various forms of alpha-synuclein, and have been developed primarily for basic research use not for use in controlled clinical trials. To accelerate the adoption of validated alpha-synuclein assays into observational and interventional clinical trials, The Michael J. Fox Foundation has assembled a consortium of investigators with a goal of standardizing alpha-synuclein measurements in cerebrospinal fluid, blood and saliva collected under controlled protocols from healthy and affected individuals. To achieve this goal the consortium is working collaboratively to compare performance of multiple assay platforms (conventional ELISA, ECL based ELISA and Mass Spectrometry), evaluating pre- and peri-analytical variables that may impact consistent and reliable measurements of alpha-synuclein, and preparing to conduct a 'round-robin' style assessment to determine inter-laboratory reliability of each assay.



Study Objectives

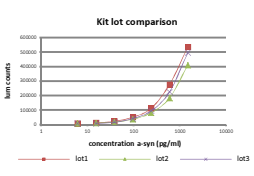
1) Demonstrate consistent lot to lot performance of critical immunoassay components (PT and RU)
2) Validate performance of the immunoassays in CSF, blood and saliva matrices (PT and RU)
3) Establish 'gold standard' reference method using MS-based approach (JZ and HZ)
4) Assess impact of multiple pre- and peri-analytical variables on alpha-synuclein measurement (PT and BM)
5) Confirm robustness of the assay via inter-laboratory analysis of a prospective cohort (all)

Reproducibility of Immunoassay Lots



	Pilot Lot 1		Pilot Lot 2		Pilot Lot 3	
	Average	CV%	Average	CV%	Average	CV%
Cal 1	843091	5.60%	963834	3.00%	946541	5.60%
Cal 2	170046	7.60%	180231	3.80%	192132	5.30%
Cal 3	31574	4.00%	35538	4.60%	38724	5.70%
Cal 4	7183	5.70%	8815	2.80%	9212	6.00%
Cal 5	2059	4.70%	2547	4.30%	2808	3.60%
Cal 6	628	4.20%	803	9.00%	768	3.40%
Cal 7	261	4.90%	304	10.20%	281	21.20%
Cal 8	120	6.90%	60	10.20%	76	5.80%

Figure 1. MSD Assay : Three pilot kit lots were assembled from three lots of raw materials including capture antibody, detection antibody and calibrator. The performance of the three lots were compared using the optimized assay diluent and protocol. The signals and %CV were averaged across all plate runs for each lot. The calculated LLODs and Hill slopes are derived from representative runs. The performance is highly reproducible across the three pilot kit lots.



Calibrator Conc (pg/ml)	Lot 1	Lot 2	Lot 3
1500	534964	409280	495446
600	275126	181956	224126
240	111621	80770	93515
96	50549	35941	40530
38.4	22057	16748	15386
15.4	11020	10057	7584
6.1	6276	7604	3965
0	3353	5593	2034

Figure 3. BioLegend Assay : Three pilot kit lots were assembled from three lots of raw materials including capture antibody, detection antibody and calibrator. The performance was reproducible across the three pilot kit lots.

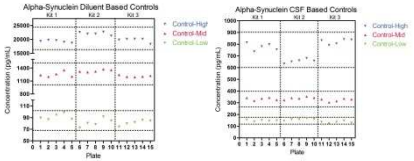
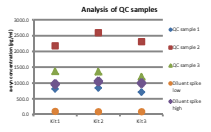


Figure 2. Analysis of Controls. The nominal levels of alpha-synuclein in control samples were determined with three pilot kit lots of the MSD assay. Controls were either recombinant, full-length alpha-synuclein spiked into diluent (diluent-based) or endogenous levels from three well-characterized human CSF samples (CSF-based). Each point is a "run". Vertical lines separate the three pilot kit lots and dashed lines indicate ±20% of the nominal values.



	Expected concentration (pg/ml)	Reported concentration as % of expected		
		Kit 1	Kit 2	Kit 3
QC sample 1	823.5	- 3%	3%	- 12%
QC sample 2	2212.2	- 3%	18%	5%
QC sample 3	1292.3	7%	6%	- 6%
Diluent spike high	1000.0	- 3%	6%	0%
Diluent spike low	100.0	9%	- 1%	1%

Figure 4. Analysis of Controls. The levels of alpha-synuclein in control samples were determined with three kit lots of the BioLegend assay. Controls were either recombinant, full-length alpha-synuclein spiked into diluent or endogenous levels in three well-characterized human CSF samples. Tabular data shows the values from each kit lot as a percentage of the expected concentration.

Assessment of Pre-analytical Variables

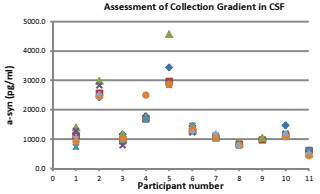


Figure 5: CSF was obtained from 11 study participants. To determine the potential effect of a concentration gradient during the collection of high volumes of CSF (up to 35 ml), up to seven separate samples were prepared per participant over the course of the tap. CSF a-synuclein levels were determined in each sample aliquot . Shown at right is a plot of a-synuclein levels by participant. The tight accordance of values in 10/11 participants indicates that CSF levels of a-synuclein do not vary widely over the course of sample collection.

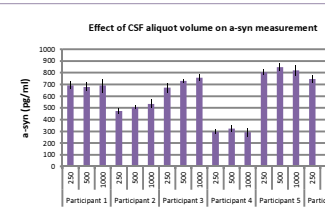


Figure 6: CSF was obtained from 6 study participants. To determine the potential effect of storage aliquot volume multiple aliquots were prepared at volumes of 1000 ul, 500 ul and 250 ul. CSF a-synuclein levels were determined in five independent aliquots at each volume from six subjects . Shown at right is a bar graph depicting the mean a-synuclein levels by volume by participant. The error bars shown represent one standard deviation. The results indicate that aliquot volume is not a critical pre-analytical variable affecting reproducibility of a-synuclein measurement.

MRM-Mass Spectrometry Assay Development

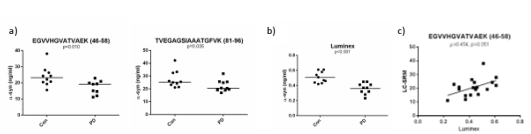


Figure 7. MRM mass spectrometry results: a) MRM data showing decreased α-syn levels in PD samples compared with controls. b) Luminex quantitation of α-syn levels of the same batch of CSF samples . c) The correlation between Luminex result and MRM result of peptide EGVVHGVATVAEK.

Next steps

1) Continue efforts to validate the immunoassays in CSF, blood and saliva
2) Validate the Mass Spectrometry method that will serve as the gold standard
3) Complete the assessment of pre-analytical and peri-analytical variables, including:
   - Collection tubes/ collection temperature
   - Centrifugation
   - Effect of non-ionic detergents
   - Time to freezing
4) Complete validation and verification of the MSD assay using manufactured lots
5) Complete the round-robin style analysis to determine inter-laboratory variability