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Novel Assays for LRRK2 and pSer935 LRRK2

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❶ Abstract

Background: Leucine-rich repeat kinase 2 (LRRK2) is the strongest known genetic contributor to Parkinson's disease (PD). LRRK2 protein is a kinase and the subject of numerous drug development programs throughout the pharmaceutical industry. As such, assays that quantify LRRK2 and LRRK2 specifically phosphorylated at serine 935 (pSer935 LRRK2) are desirable to screen for inhibitors and to quantify pharmacodynamic markers. The development of such assays has proven challenging since there is a paucity of antibodies available against these targets.

Methods: Using MSD's U-PLEX® platform, we have developed sandwich immunoassays for both analytes and shown their suitability for use with human peripheral blood mononuclear cells (PBMCs). Antibody pairs were selected and their concentrations optimized to create the most robust assays possible from the available antibodies. As test samples, human PBMCs were prepared and treated with or without a LRRK2 kinase inhibitor. Cell lysates were prepared from the PBMCs and analyzed with the new assays.

Results: Cells treated with the inhibitor showed a time-dependent decrease in the abundance of pSer935 LRRK2. A modest decrease in "total" LRRK2 was also noted. The results confirm that the pSer935 LRRK2 assay is specific for quantifying reversible phosphorylation of LRRK2.

Conclusion: Immunoassays for LRRK2 and pSer935 LRRK2 were successfully developed. The assays are capable of high throughput and are compatible with PBMCs, a sample type common to clinical research. The assays should prove useful for LRRK2 drug development programs both during pre-clinical research and to provide ancillary data in support of clinical studies.

❷ Principle of the Assay

MSD's electrochemiluminescence (ECL) 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.

❸ Methods

The total and pSer935 LRRK2 assays are singleplex sandwich immunoassays on the MSD® U-PLEX platform. The protocol below describes the PBMC preparation and the U-PLEX assay procedure.

Human PBMC Cell Model

1. Prepare PBMCs at 2.5 x 10^6 cells/mL.
2. For the control cells, treat cells with DMSO for 1 hour.
3. For the LRRK2 pSer935 phosphorylation inhibited cells, treat cells with 1 µM LRRK2-IN-1 inhibitor for 30 min, 1 hour, and 2 hours.
4. Pellet cells in the presence of phosphatase inhibitors.
5. Prepare cell lysates using protease and phosphatase inhibitors.

Assay Protocol

1. Prepare a solution containing capture antibodies coupled with U-PLEX linkers. Coat the wells (50 µL per well) and incubate 1 hour at room temperature (RT) with shaking.
2. Wash and add 25 µL of prepared sample or calibrator standard to each well. Incubate 1 hour at RT with shaking.
3. Wash and add detection antibody solution (25 µL per well). Incubate 1 hour at RT with shaking.
4. Wash and add read buffer (150 µL per well). Analyze with MSD instrument.

❹ Primary Screening of Antibodies

An unbiased primary screen of antibodies was performed using seven anti-total LRRK2 and three anti-pSer935 specific antibodies as capture and detection antibodies with 50,000 pg/mL wild-type LRRK2 calibrator in pairwise combinations in both orientations. ECL signal intensity and background were used to select promising antibody pairs.

ECL Signal Counts		TOTAL LRRK2 DETECTION ANTIBODIES						
		Antibody 1	Antibody 2	Antibody 3	Antibody 4	Antibody 5	Antibody 6	Antibody 7
TOTAL LRRK2 CAPTURE ANTIBODIES	Antibody 1	1,087	15,827	2,834	74	47	14,407	8,231
	Antibody 2	11,960	4,307	13,715	196	70	70,417	38,433
	Antibody 3	1,410	2,241	71	27	41	2,353	1,523
	Antibody 4	66	120	71	42	89	139	149
	Antibody 5	106	559	127	-7	49	463	279
	Antibody 6	3,828	22,058	4,576	38	31	740	596
	Antibody 7	5,319	22,194	4,217	35	55	1,583	1,740

Signal/Background Ratio		TOTAL LRRK2 DETECTION ANTIBODIES						
		Antibody 1	Antibody 2	Antibody 3	Antibody 4	Antibody 5	Antibody 6	Antibody 7
TOTAL LRRK2 CAPTURE ANTIBODIES	Antibody 1	1	633	44	1	3	81	12
	Antibody 2	1,196	23	386	4	2	1,548	183
	Antibody 3	2	39	-12	1	1	17	7
	Antibody 4	1	2	35	1	1	4	1
	Antibody 5	10	37	6	0	2	12	5
	Antibody 6	166	455	166	1	1	51	7
	Antibody 7	4	347	256	1	3	8	2

ECL Signal Counts		pSer935 LRRK2 DETECTION ANTIBODIES		
		Antibody 1	Antibody 2	Antibody 3
TOTAL LRRK2 CAPTURE ANTIBODIES	Antibody 1	9,693	46	3,347
	Antibody 2	48,560	88	18,215
	Antibody 3	1,912	195	740
	Antibody 4	167	99	114
	Antibody 5	414	71	138
	Antibody 6	16,048	39	4,954
	Antibody 7	14,993	66	5,710

Signal/Background Ratio		pSer935 LRRK2 DETECTION ANTIBODIES		
		Antibody 1	Antibody 2	Antibody 3
TOTAL LRRK2 CAPTURE ANTIBODIES	Antibody 1	57	1	152
	Antibody 2	372	1	156
	Antibody 3	39	1	4
	Antibody 4	3	1	1
	Antibody 5	4	2	2
	Antibody 6	1,003	1	73
	Antibody 7	96	1	55

Signal intensities expressed as ECL counts and signal to background ratios for total LRRK2 (above left) and pSer935 LRRK2 assays (above right). **From the primary screen, six antibody pairs for the total LRRK2 assay and four antibody pairs for the pSer935 LRRK2 assay were selected.**

❺ Secondary Screening of Antibodies

Eight-point calibration curves were tested for the secondary screen. Signal intensities, Hill slope, and assay sensitivity were used as criteria for selecting the best pairs.

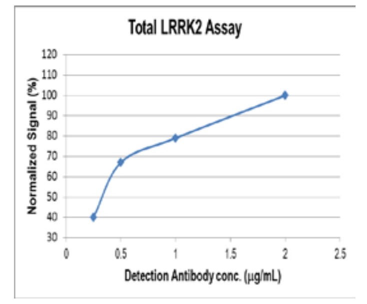
ECL Signal Counts		TOTAL LRRK2 ASSAY					
Conc. (pg/ml)	Antibody Pair	1	2	3	4	5	6
200,000	STD1	34,436	149,438	498,367	380,582	1,317	14,844
50,000	STD2	11,030	45,757	154,961	120,934	954	4,856
12,500	STD3	3,469	11,777	38,400	30,221	775	2,100
3,125	STD4	949	3,225	10,050	8,309	763	1,398
781	STD5	310	961	2,706	2,490	729	1,222
195	STD6	205	388	831	1,134	831	1,189
49	STD7	287	277	444	985	860	1,176
0	STD8	212	204	410	891	943	1,376
Hill Slope		1.13	1.00	1.06	1.07	16.22	1.10
LLOD (pg/mL)		908	103	81	111	49,449	1,360
S/B STD4		4	16	25	9	1	1

ECL Signal Counts		pSer935 LRRK2 ASSAY			
Conc. (pg/ml)	Antibody Pair	1	2	3	4
200,000	STD1	414,186	135,204	138,507	139,805
50,000	STD2	121,105	34,845	36,319	37,644
12,500	STD3	30,544	9,141	7,933	8,461
3,125	STD4	7,528	2,446	1,992	2,055
781	STD5	2,212	797	614	618
195	STD6	884	455	264	263
49	STD7	567	470	129	148
0	STD8	732	841	125	159
Hill Slope		1.09	1.15	1.02	1.07
LLOD		197	320	128	145
S/B STD4		3	3	16	13

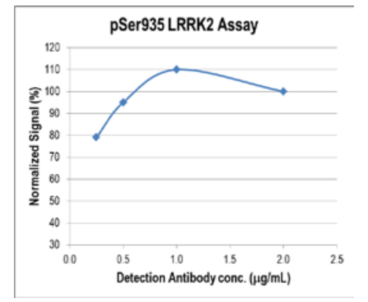
Antibody pairs highlighted in red yielded the best signal and assay sensitivity.

❻ Detection Antibody Titration

A detection antibody titration was performed for both assays to maximize performance.



Calibrator Conc.	Signal / Background ratio			
	Detection Antibody Conc. (µg/mL)			
	2	1	0.5	0.25
50,000	755	922	670	586
3,125	55	64	45	41
195	4	4	4	2



Conc. (pg/mL)	Signal / Background ratio			
	Detection Antibody Conc. (µg/mL)			
	2	1	0.5	0.25
50,000	104	186	334	343
3,125	8	14	24	25
195	1	2	3	3

The graphs show representative data obtained from the analysis of three calibrator levels (STD2, STD4, and STD6) as a function of detection antibody concentration. The signal at each calibrator level is normalized to the signal at the highest detection antibody concentration (2 µg/mL).The tables to the right of the graphs report the signal to background ratios, which were the deciding factors for selecting the optimal detection antibody concentration.

Based on the signal to background ratios, detection antibody concentrations of 1 µg/mL for the total LRRK2 assay and 0.5 µg/mL for the pSer935 LRRK2 assay were selected.

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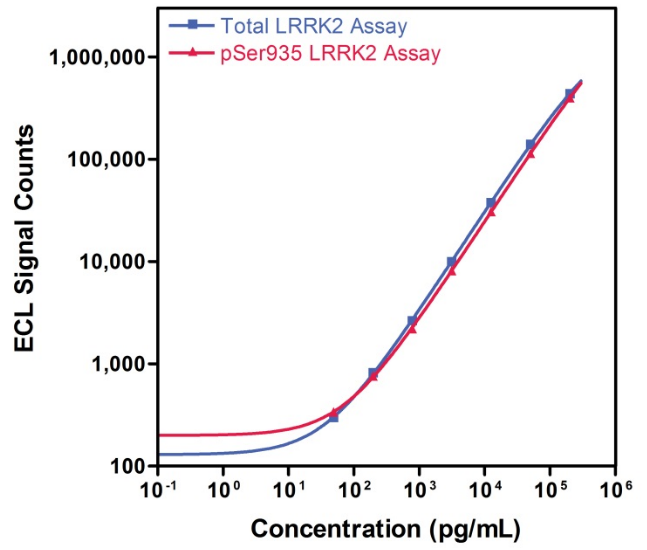
❼ Calibration Curves and Protocol

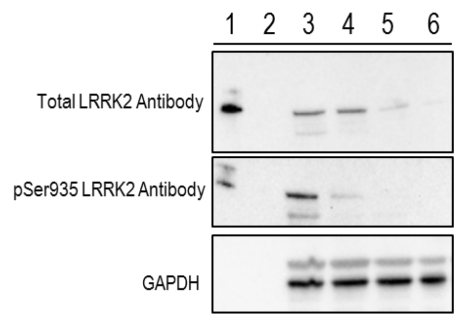


		Total LRRK2 Assay		pSer935 LRRK2 Assay	
Conc. (pg/ml)	Antibody Pair	Avg. ECL Signal (Counts)	%CV	Avg. ECL Signal (Counts)	%CV
200,000	STD1	438,338	4.8	397,379	5.4
50,000	STD2	140,153	3.9	114,480	3.5
12,500	STD3	37,772	3.4	30,711	2.8
3,125	STD4	10,028	2.5	8,148	3.5
781	STD5	2,658	4.2	2,215	6.0
195	STD6	821	3.1	760	4.1
49	STD7	300	8.7	340	8.6
0	STD8	130	10.3	199	17.9

	Median	Range	Median	Range
Hill Slope	0.954	0.934-0.964	0.960	0.928-0.972
LLOD (pg/ml)	31	27-36	40	36-71

Left: Average ECL signal counts for the points along the calibration curves are reported from six independent experiments. Both assays exhibit a broad dynamic range. **Right:** Average ECL signal counts along with %CV are reported from six independent experiments and indicate reproducibility across multiple runs. The tight range of Hill slopes indicate the reproducibility of the linear ranges for the total and pSer935 LRRK2 assays. The sensitivities of both assays are reported as the LLOD, including the means and ranges observed.

❽ Cell Model Validation using Western Blotting



Sample 1	LRRK2 Calibrator (1 ng)
Sample 2	Empty
Sample 3	PBMC untreated
Sample 4	PBMC treated with LRRK2-IN-1 30 min
Sample 5	PBMC treated with LRRK2-IN-1 1h
Sample 6	PBMC treated with LRRK2-IN-1 2h

Western blot analysis with total and pSer935 LRRK2 antibodies was used to validate the PBMC cell model.

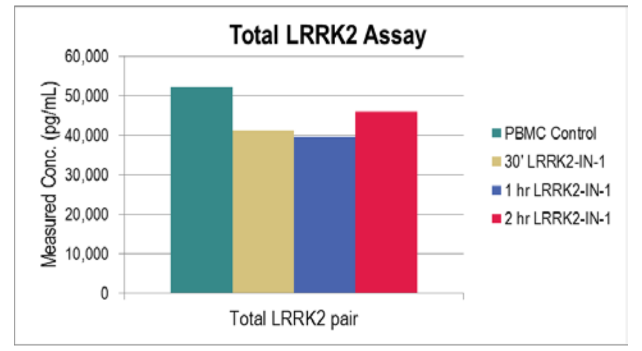
Top Blot: Total LRRK2 levels show reduction with longer exposures to LRRK2 inhibitor.

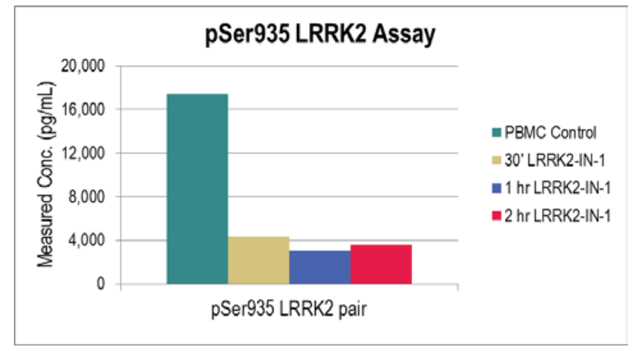
Middle Blot: pSer935 specific antibody shows reduction in LRRK2 phosphorylation, correlating with the duration of exposure to the inhibitor.

Lower Blot: Shows GAPDH house keeping gene as loading control.

Western blots establish the properties of the PBMC cell model. At 30 minutes there is significant reduction in LRRK2 phosphorylation with little or no reduction in total LRRK2.

❾ Testing Selected Antibody Pairs on the PBMC Cell Model



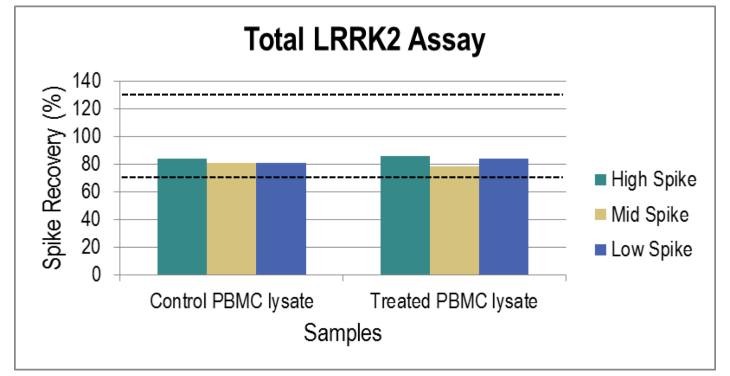


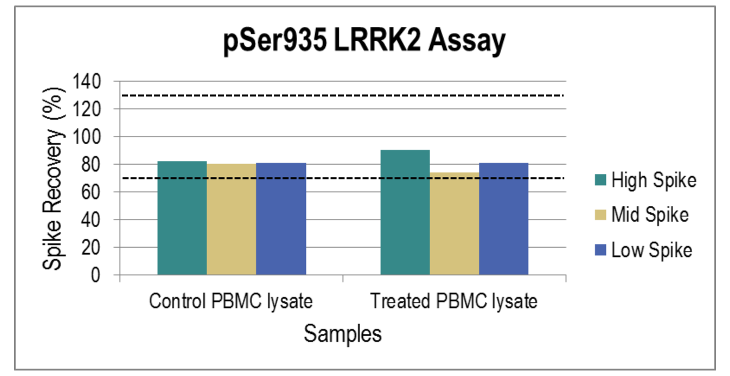
The PBMC cell model was tested with the selected total and pSer935 LRRK2 assays. Five micrograms of PBMC cell lysate was analyzed per well. Total and pSer935 LRRK2 concentrations in the cell lysates were determined by back fitting the signal to the calibration curve.

The MSD assays agree with the Western blot data inasmuch as pSer935 shows a significant reduction in phosphorylation (>75%) after LRRK2-IN-1 treatment compared to a modest reduction (20%) in total LRRK2 levels.

❿ Spike and Recovery

Recombinant, phosphorylated LRRK2 protein was spiked into PBMC cell lysate at three different concentrations. Expected concentrations were the sum of the endogenous and the spiked LRRK2 concentrations. Recovery at each spike level was calculated relative to the expected concentration. Dotted lines represent the guard bands of ± 30% of the expected concentration.

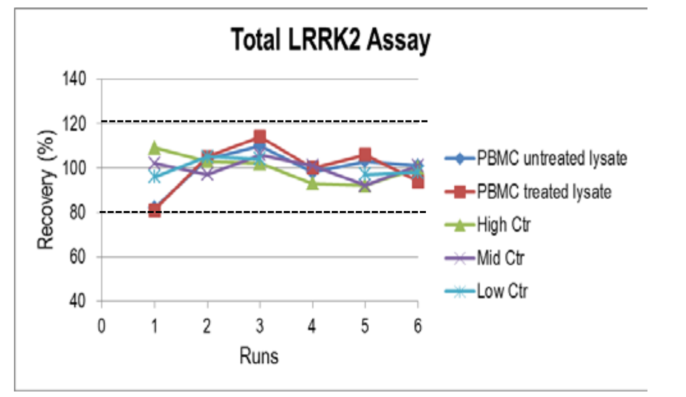


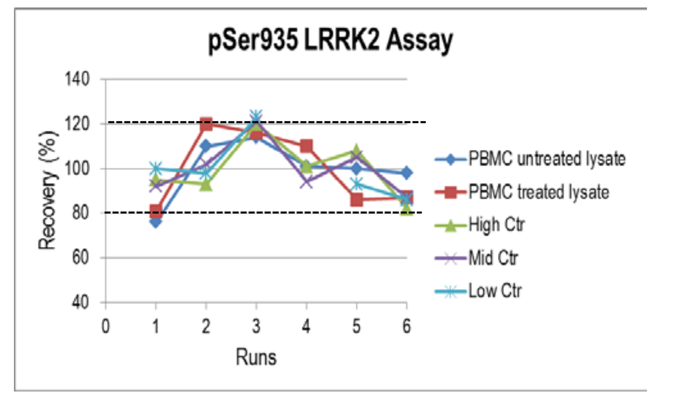


The total and pSer935 LRRK2 assays do not show matrix interference and afford accurate quantitation in Human PBMC cell lysates.

⓫ Assay Reproducibility

Total and pSer935 LRRK2 were measured in PBMC lysates treated or not with LRRK2-IN-1 for 30 minutes. Controls produced by spiking specific quantities of recombinant, phosphorylated calibrator into a diluent (Ctr) were also quantified. Dotted lines represent the guard bands of ± 20% of the expected concentration.





The experiment was repeated six times and showed reproducibility with percent recoveries within ± 20%.

⓬ Conclusion

MSD Total and pSer935 LRRK2 assays were robust and reproducible with excellent sensitivity and broad dynamic ranges. Using these assays, total and pSer935 LRRK2 levels were quantified in human PBMCs. Given the current paucity of total and phosphorylation specific assays for LRRK2, these assays should prove useful not only for understanding the role of LRRK2 and its phosphorylation in Parkinson's disease, but will also be instrumental in LRRK2 targeted drug development.

Acknowledgments

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