LRRK2 Inhibitor Evaluation and Profiling in Both Biochemical and Cellular HTS Formats by SelectScreen® Screening Services

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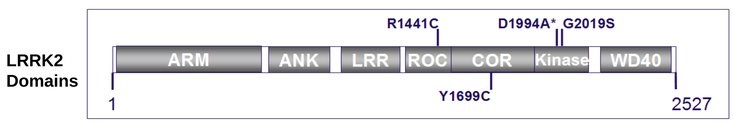
ABSTRACT

Parkinson's Disease (PD) is a progressive neurological disorder that results from degeneration of neurons in a region of the brain that controls movement, impacting an estimated 6.3 million people worldwide. To date, mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are the most common genetic cause of PD. LRRK2 is a protein kinase now being aggressively pursued as a target for PD therapeutics. However, studies of LRRK2 function and inhibition have been hampered by a lack of tools to express LRRK2 in diverse cell types as well as high-throughput biochemical and cell based assays. To address these needs, we have developed BacMam particles to express both untagged and green fluorescent protein (GFP) tagged full-length LRRK2 (WT and disease relevant mutants). BacMam technology has enabled higher levels of expression of LRRK2 in both workhorse cell lines (e.g. U-2 OS, HEK293T) and cells more physiologically relevant to neuroscience such as neuroblastoma SH-SY5Y and primary astrocytes compared to standard lipid transfection. Furthermore, the ability to express high levels of LRRK2 in these systems has enabled modular high-throughput assays for full-length LRRK2. Utilizing BacMam expression and LanthaScreen® technology, biochemical assays to measure inhibitor binding and cellular assays to measure inhibitor effects on kinase activity were developed and implemented by SelectScreen® Screening Services. Together these technologies will advance the field of PD research and therapeutics by addressing the currently unmet need for modular over expression, inhibitor binding assays and cellular kinase assays for full-length LRRK2.

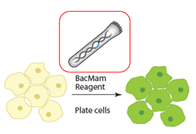
Figure 1. The Role of LRRK2 in Parkinson's Disease



The normal function of LRRK2 is unknown but it is expressed in many tissues and organs including the brain. LRRK2 is reported to be associated with microtubules, cytoskeleton rearrangements, synaptic vesicle trafficking, neurite outgrowth and translational control. LRRK2 is a large protein (2527 amino acid) belonging to the ROCO family of proteins defined by the GTP-binding Ras of complex protein (ROC) and carboxy-terminal of Roc (COR) domains that confer weak GTPase activity. A serine/threonine protein kinase domain is positioned next to the ROC and COR domains completing the catalytic core of LRRK2. The catalytic core is flanked by leucine-rich repeats (LRR) on one side and a WD40 domain on the opposite side, both domains are thought to function as protein-protein interaction domains. Over 40 mutations of LRRK2 have been reported but only mutations in the catalytic core of LRRK2 segregate with PD in a Mendelian fashion suggesting the enzymatic functions of LRRK2 play a key role in disease pathogenesis. Mutations in the ROC GTPase domain (R1441C, R1441G, R1441H) and the COR domain (Y1699C) are reported to reduce GTPase activity in in-vitro studies. The most frequent PD associated LRRK2 mutation G2019S is located in the activation loop of the kinase domain resulting in increased kinase activity and reported to cause neurotoxicity in cultured cells.

RESULTS

Figure 2. BacMam Enabled Expression of Parkinson's Disease Drug Target LRRK2

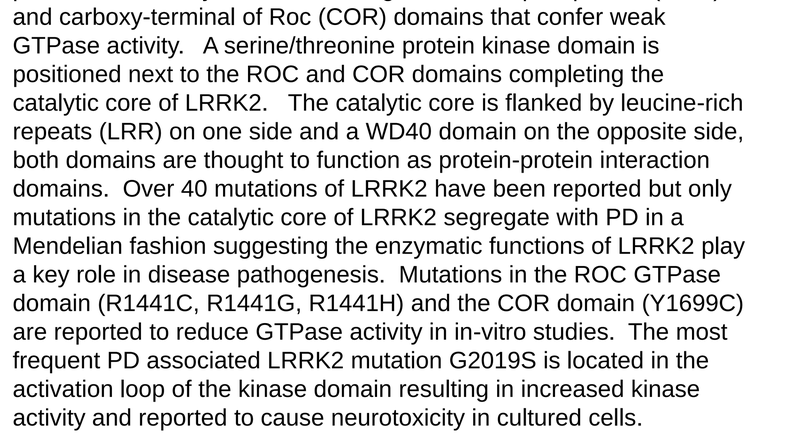


BacMam is the use of modified baculovirus to deliver and transiently express genes in mammalian cells

BacMam Gene Delivery

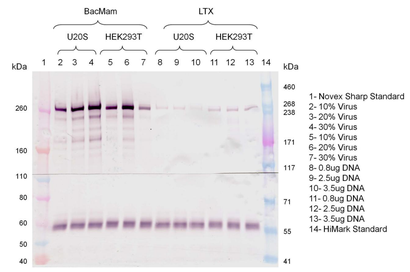
- **Baculovirus is an insect cell virus**
- **Non-replicating in mammalian cells**
- **High, CMV-driven expression**
- **Transient expression**
- **Tolerates large inserts up to 38 kb**
- **Convenient and Fast** – just add virus to cells
- **Efficient** – high expression level
- **Titratable** – control level of expression
- **Safe** – non-toxic to cells
- **Portable** – works efficiently with most cell types *(including primary and stem cells)*

U-2 OS | **SH-SY5Y** | **Human Astrocytes**



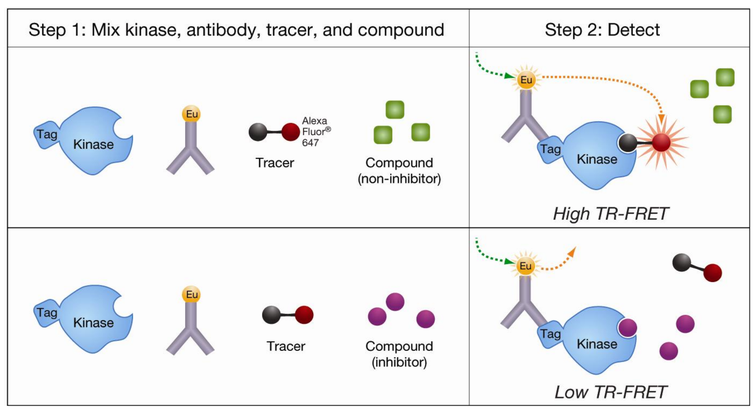
U-2 OS cells, SH-SY5Y Cells and human astrocytes (N7805100) were transduced with BacMam viruses of GFP tagged LRRK2 (A14170) and mutants. Cells were exposed to 20% virus for 24 hours, media was exchanged and cells were allowed to incubate an additional 24 hours before imaging by fluorescent microscopy.

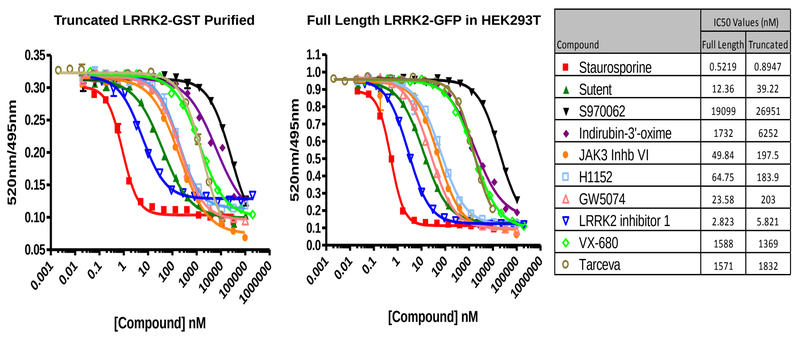
Figure 3. Expression Level Comparison of BacMam VS Lipid Transfection



Expression level comparison of BacMam versus Lipofectamine LTX (with plus reagent) was tested in U-2 OS and HEK293T cells under optimized conditions. Expression levels of full length LRRK2 were assessed by western blot (rabbit anti-LRRK2 – Cell Signaling Technologies). Sample loading was normalized by Bradford assays and confirmed by anti-AKT (Cell Signaling Technologies) blot (lower bands).

Figure 4. Kinase Binding Assay Utilizing Lysates from BacMam LRRK2 Transduced HEK293T Cells

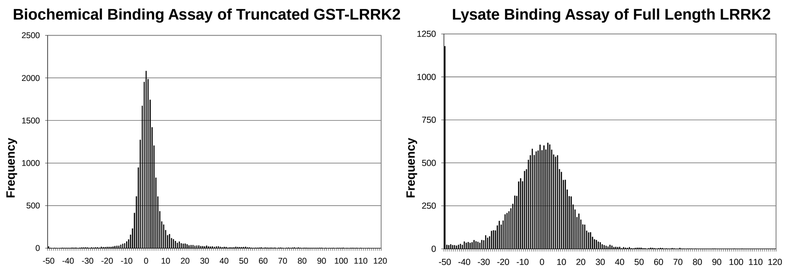




Compound	IC50 Values (nM) Full Length	Truncated
Staurosporine	0.5219	0.8947
Sutent	12.36	39.22
S970062	19099	26951
Indirubin-3'-oxime	1732	6252
JAK3 Inhb VI	49.84	197.5
H1152	64.75	183.9
GW5074	23.58	203
LRRK2 inhibitor 1	2.823	5.821
VX-680	1588	1369
Tarceva	1571	1832

BacMam LRRK2-GFP reagent was used to transduce HEK293T and generate a cellular lysate containing full length LRRK2. A lysate based Kinase Binding assay was developed using the full length LRRK2 lysate and compared to purified truncated LRRK2. Optimal lysate and tracer concentration were determined. 5nM truncated kinase (PV4873) or 3nM full length kinase (A14171), 2 nM Eu-anti GFP antibody (A14173) and 20 nM Tracer 236 (PV5592) were used to assay binding of several kinase inhibitors to LRRK2.

Figure 5. Enamine Library Screen Using Full Length LRRK2 Kinase Binding Assay



	Truncated LRRK2 (970-2527)	Full Length LRRK2
>70% Inhibition	93/20,155 (0.46%)	12/20,155 (0.06%)
>30% Inhibition	475/20,155 (2.36%)	270/20,155 (1.34%)
Average Z'-factor	0.80	0.55

Top Library Hits For LRRK2 Followed-Up Using Multiple Assay Formats

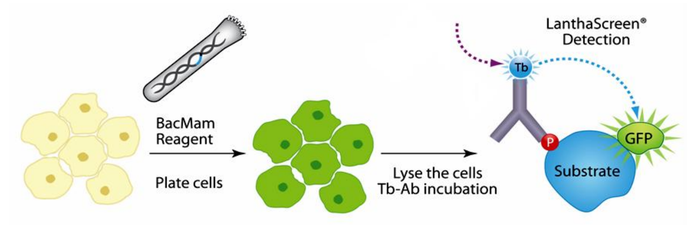
	Cellular				Biochemical Binding								Biochemical Activity	
IC_{50} (μM)	SHSY5Y cells		U-2 OS cells		FL Lysate-based binding assay			Truncated, purified biochemical assay					LRRKtide activity assay	
Compound Name	G2019S	WT	G2019S	WT	G2019S	WT	R1441C	G2019S	WT	R1441C	D1994A	I2020T	G2019S	WT
LRRK2-IN-I	0.094	0.22	0.055	0.11	0.005	0.006	0.005	0.003	0.002	0.003	0.002	0.004	0.02	0.02
JAK3 Inh. VI	0.73	1.2	0.21	1.4	0.23	0.2	nd	0.04	0.05	nd	nd	nd	nd	nd
Indirubin-3-oxine	3.8*	>10	3.9*	1.9*	9.3	6.2	nd	2.1	1.7	nd	nd	nd	nd	nd
Compd 2	>20	>20	>20	>20	0.13	0.15	1.9	3*	3*	5.4*	>20	>20	>20	>20
Compd 5	>20	>20	>20	>20	3.3	1.8	7.2	1.2	1	1.2	1.1	1.3	>20	>20
Compd 6	0.53	0.76	0.2	0.31	0.09	0.06	0.06	0.025	0.012	0.017	0.011	0.029	0.49	0.18
Compd 10	>10	>10	0.37*	0.59*	2	1.9	4.2	0.09	0.11	0.21	0.11	0.23	1.3*	1.1*
Compd 17	>20	>20	20.9*	>20	0.84	0.8	0.59	0.39	0.26	0.34	0.24	0.52	1.8	2
Compd 25	0.17	0.88	0.99	1.22	0.03	0.18	0.12	0.01	0.019	0.028	0.014	0.053	0.11	0.26
Compd 27	nd	nd	0.44	1.29	0.25	0.274	0.6	0.054	0.034	0.1	0.056	0.19	0.54	0.68
Compd 30	>20	>20	>20	>20	8.5	1.6	1.3	10.6*	18.3*	>20	>20	11.1*	>20	>20

Top Library Hits Profiled Against Kinase Panel To Determine Compound Specificity *Partial Inhibition

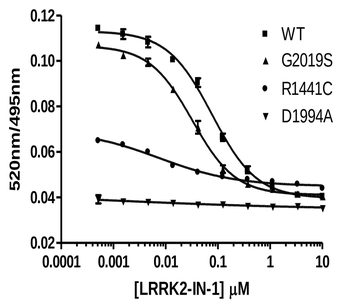
	LRRK2 IN-1	GW5074	Cmpd 2	Cmpd 5	Cmpd 6	Cmpd 10	Cmpd 17	Cmpd 25	Cmpd 27	Cmpd 30
>80% inhibition	7	126	0	216	52	8	115	14	221	5
20-80% inhibition	58	93	8	5	170	142	103	154	1	66

The lysate binding assay performed well in a high throughput screening (HTS) format when compared against the biochemical binding assay in a screen of the Enamine library - a drug like set of 20,155 compounds. Top hits from both screens were followed-up with IC_{50} curves in multiple assay formats including lysate and biochemical binding assay, cellular assay and biochemical activity assay. Selected compounds were also profiled by SelectScreen® Profiling Services against a panel of 223 kinases to assess compound specificity.

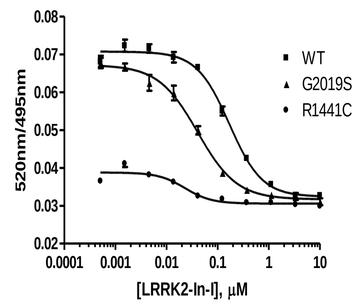
Figure 6. Cellular Assay for Monitoring the Phosphorylation of LRRK2 Ser935



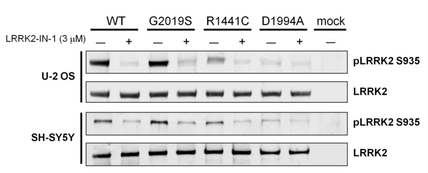
BacMam-enabled LanthaScreen® pLRRK2 [Ser935] Assay in U-2 OS cells



BacMam-enabled LanthaScreen® pLRRK2 [Ser935] Assay in SH-SY5Y cells



Assay Results Confirmed by Western Blot

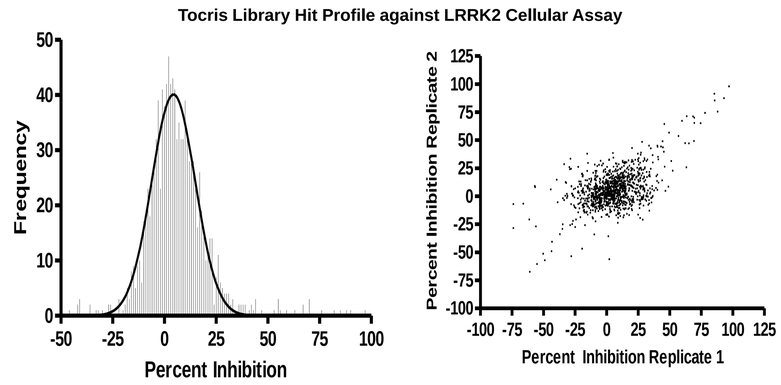


IC_{50} Value Summary Table

IC50 (μM)	U-2 OS		SHSY5Y		HEK293T	
Compound Name	WT	G2019S	WT	G2019S	WT	G2019S
LRRK2-IN-I	0.08	0.06	0.22	0.094	0.03	0.01
JAK3 Inh. VI	0.24	0.18	1.2	0.73	1.17	0.31
Indirubin-3-oxine	5.12	2.54	>10	3.8*	5.96	3.55
Sunitinib	0.27	0.15	0.23	0.11	0.60	0.48
GW5074	>20	0.03*	>20	>20	>20	0.28*
H-1152	2.80	0.85	1.7	0.62	3.88	1.43
H-89	>20	>20	>20	>20	nd	nd
Staurosporine	0.001	0.001	nd	nd	0.001	0.002

Using Time-Resolved Förster Resonance Energy Transfer (TR-FRET) technology, we developed a high-throughput compatible homogenous cellular assay for monitoring the LRRK2 phosphorylation at Ser935. LRRK2-GFP fusion proteins were transiently expressed in a variety of cell backgrounds via BacMam gene delivery system. Cells were plated in 384-well assay plates. The phosphorylation at Ser935 in these cells was detected using a terbium labeled anti-Ser935 phosphorylation specific antibody that generates TR-FRET signals between terbium and GFP. Consistent with previous reports and our western blot results, wild type and G2019S LRRK2 are constitutively phosphorylated at Ser935 in cells measured by TR-FRET. The phosphorylation level was reduced for R1441C mutant and little could be detected for the kinase activity-dead mutant D1994A. The TR-FRET cellular assay was further validated with reported LRRK2 inhibitors including LRRK2-IN-I and further confirmed that inhibition of LRRK2 kinase activity can reduce the phosphorylation level at Ser935.

Figure 7. Tocris Mini Library Screen Using LRRK2 pSer935 Cellular Assay



>70% Inhibition	>30% Inhibition	Average Z'-factor
6/1120 (0.54%)	38/1120 (3.39%)	0.75

To demonstrate the utility of the LRRK2 cellular assay in HTS applications a compound screen was performed using the Tocris mini library – a collection of 1120 biologically active compounds.

CONCLUSIONS

An extensive set of tools have been developed to enable the discovery of kinase inhibitors for PD target LRRK2. These tools allow for HTS compatible screens in multiple formats that provide detailed mechanistic data on how compounds are binding LRRK2 and inhibiting kinase activity of LRRK2 and its mutants in biochemical and cellular environments. Additionally, all assays described are available through SelectScreen ® Screening Services.

Useful Weblinks

www.lifetechnologies.com/bindingassay

www.lifetechnologies.com/lanthascreencellular

www.lifetechnologies.com/kinaseprofiling