Use of BacMam technology to express full-length LRRK2 and to profile inhibitors using cell-based and biochemical assays

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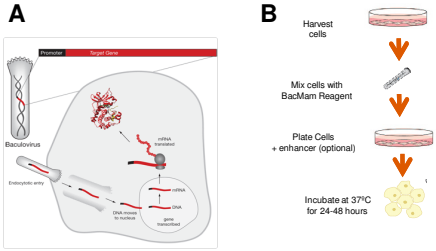
ABSTRACT

Studies of LRRK2 function and inhibition have been hampered by a lack of tools to express full-length 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 tagged and untagged LRRK2 (wild-type and mutants). Compared to standard lipid transfection, 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. Furthermore, the ability to express high levels of LRRK2 in these systems has enabled modular high-throughput assays for compounds that bind to 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. In addition, application of BacMam technology has enabled expression levels sufficient to produce highly purified, full-length LRRK2.

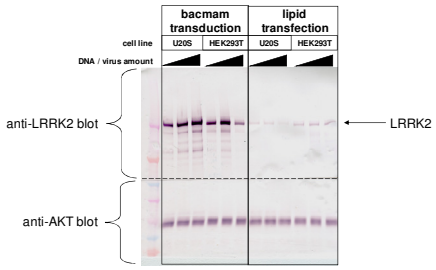
Figure 1. Introduction to BacMam Technology



The BacMam technology (A) is based on use of a modified baculovirus to efficiently deliver and express genes in mammalian cells. Baculoviruses are non-replicating in mammalian cells and thus have an excellent safety profile combined with being well-tolerated by cells. BacMam reagents have been used in cell based assays, live cell imaging, stem cell biology and many other applications.

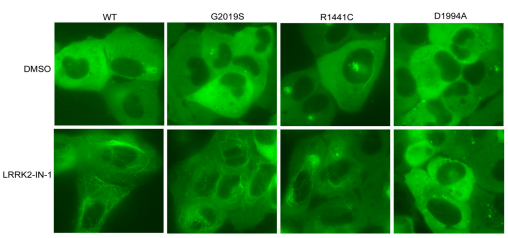
BacMam reagents are used in your normal workflow as any other reagent for cell-based research; take the reagent from the fridge, add it to cells, incubate and assay (B). Transduction is efficient and reproducible in most cell lines, including primary and stem cells. The BacMam platform enables easy transduction of large quantities of cells in batch mode; transduced cells can be stored frozen in aliquots for later use, providing assay-ready cells when you need them - with no loss of activity. Cells can be assayed within hours of thawing and plating.

Figure 2. LRRK2 expression levels are higher with the BacMam system than lipid transfection



Expression levels for LRRK2 were compared for the BacMam system and for a lipid transfect ion method. Much higher expression was observed for the BacMam system.

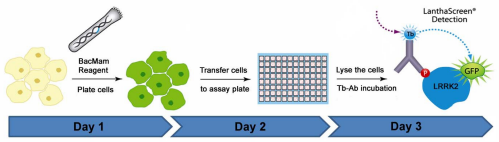
Figure 3. BacMam-expressed GFP-LRRK2 recapitulates inhibitor dependent relocalization



U-2 OS cells were transduced with 20% BacMam for WT and the indicated mutants. Cells were treated with DMSO only or LRRK2-IN-1 (3 µM) for 90 min. GFP images were captured and representative images are shown. LRRK2-IN-1 treatment resulted in the relocalization of the wild-type, G2019S and R1441C[1] to fibrillar-like structures

CELLULAR LRRK2 ASSAY

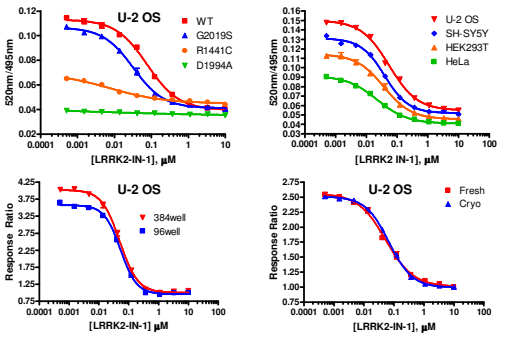
Figure 4. Principle of BacMam-enabled LanthaScreen® LRRK2 [pSer935] Celluar Assay



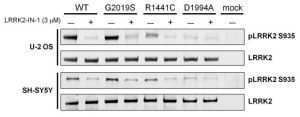
Using Time-Resolved Förster Resonance Energy Transfer (TR-FRET) technology, we developed a high-throughput compatible homogenous cellular assay for monitoring the LRRK2 phosphorylation at Ser935. LRRK2-GFP fusion proteins are transiently expressed in a variety of cell backgrounds via BacMam. Cells are plated in 384-well assay plates. Phosphorylation at Ser935 in these cells is detected using a terbium labeled pSer935 antibody that generates TR-FRET signals between terbium and GFP.

Figure 5. Validation of BacMam-enabled LanthaScreen® LRRK2 [pSer935] Cellular Assay

A. LRRK2-IN-1 profiles in multiple cell types and formats



B. Confirmation by Western blotting

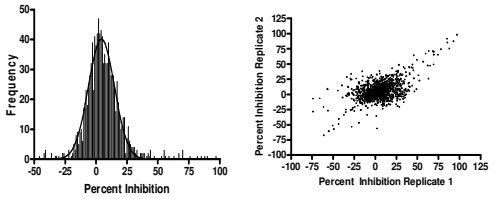


C. IC_{50} values for known Inhibitors

IC50 (µM)	U-2 OS		SHSY5Y		HEK293T	
Compound Name	WT	G2019S	WT	G2019S	WT	G2019S
LRRK2-IN1	0.08	0.06	0.23	0.094	0.03	0.01
JAK3 Inh. VI	0.24	0.18	1.2	0.73	1.17	0.31
Indirubin-3-oxime	5.12	2.54	>10	3.8*	5.95	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
Staurosporin	0.001	0.001	nd	nd	0.001	0.002

* Partial Inhibition

D. Test screen of 1120 compound set



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

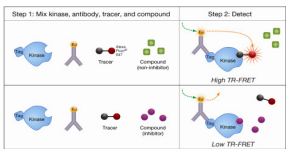
% Inhibition (at 20 µM)	Compound Name	Known Compound Activity	Cellular TR-FRET assay LRRK2 G2019S		Cytotoxicity Assay	Biochemical LRRK2 assay
			Max % Inhibition	IC_{50} (µM)	IC_{50} (µM)	IC_{50} (µM)
97.5	Bay 11-7085	Irreversible inhibitor of TNFa-induced IkB phosphorylation	96.6	8.3	>20	>100
90.1	JTC 801	ORL1 antagonist	84.0	15.6	10	>100
86.3	SP 600125	JNK and other kinase inhibitor	103.1	0.9	>20	0.40
85.5	Bay 11-7821	Irreversible inhibitor of TNFa-induced IkB phosphorylation	94.2	5.2	20	>100
81.7	IKK 16	Inhibitor of IKK	82.5	2.1	>20	0.78
76.1	NSC 95397	Selective Cdc25 dual specificity phosphatase inhibitor	95.9	2.2	7.8	1.9
69.8	Ro 106-9920	Inhibitor of NFkB activation	76.4	7.6	20	>100
69.7	GW 441756	TrkA inhibitor	72.2	2.3	>20	0.65
69.7	BNTX maleate	Standard d1 selective antagonist	88.4	5.0	>20	>100
67.3	PD 407824	Inhibitor of Chk1 and Wee1	65.3	0.6	15	0.68
67.3	Y-27632 dihydrochloride	p160ROCK inhibitor	68.8	14.5	>20	0.88
63.5	Indirubin-3-oxime	GSK-3b inhibitor. Also inhibits other protein kinases	ND	ND	ND	10
59.1	NSC 663284	Cdc25 phosphatase inhibitor	45.6	4.9	2	0.13
56.2	Aminopurvalanol A	Cyclin-dependent kinase inhibitor	62.9	4.5	>20	ND
55.3	SB 218078	Inhibitor of checkpoint kinase 1 (Chk1)	58.9	0.1	>20	6.0
55.0	CV 1808	Adenosine A2 receptor agonist	ND	ND	ND	ND
54.8	TPCA-1	Inhibitor of IKK-2	46.2	9.2	>20	ND
53.1	3'-Fluorobenzylspiperone maleate	D2-like receptor ligand	ND	ND	ND	ND
46.6	Anisomycin	Protein synthesis inhibitor	49.3	0.1	>20	ND
100.0	LRRK2 IN-1	Positive Control Compound	100.2	0.1	>20	0.008

LRRK2-IN-1 IC_{50} values (A,C) for cells transduced with WT and G2019S were similar to reported values and also recapitulate a slightly lower IC_{50} for G2019S relative to WT. Assays with the kinase dead mutant of LRRK2 (D1994A[1]) display no significant TR-FRET signal in the absence of inhibitor, consistent with negligible phosphorylation at Ser935. The TR-FRET signal was greatly reduced for R1441C[1], relative to WT and G2019S. TR-FRET results were consistent with phospho-Western results (B). IC_{50} values were performed for a number of known LRRK2 inhibitors (C). In addition, a small set of known bioactive compounds (Tocris Mini Library) were screened to test the assay's utility in an HTS environment (D).

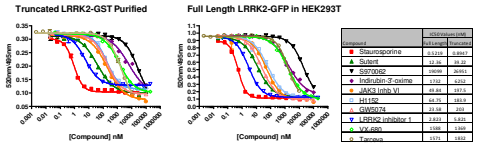
FULL-LENGTH LRRK2 BIOCHEMICAL ASSAYS

In order to develop more physiologically relevant biochemical assays for LRRK2 inhibition, formats have been established using full-length LRRK2.

Figure 6. Kinase Binding Assay in lysates with full-length LRRK2

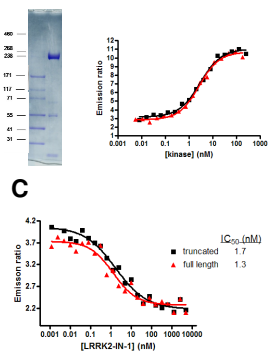


Lysate-based Kinase Binding Assays are based on the binding of a fluorescent "tracer" to the kinase active site, resulting in a TR-FRET signal from a Eu-labeled antibody bound to the kinase tracer complex. Displacement of the tracer by an inhibitor results in a loss of signal. In this configuration, the kinase is full-length LRRK2 present in a 293T lysate and the tag is GFP.



BacMam LRRK2-GFP reagent was used to transduce HEK293T cells and generate a lysate containing full-length LRRK2. Kinase Binding Assays were then performed comparing the full length LRRK2 lysate and 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.While many inhibitors appear to binding with similar affinity to truncated and full-length LRRK2, others (notably GW5074) appear to have significantly altered affinity.

Figure 7. Production of purified and active full-length LRRK2 [1,2]



Affinity tagged LRRK2 was expressed with the BacMam system, purified, and analyzed by Coomassie-stained SDS-PAGE (A). Purified, full length LRRK2 was active in a LanthaScreen® activity assay using LRRKtide as the substrate (B). The activity assay using full-length LRRK2 was validated with the relatively specific inhibitor LRRK2-IN-1 (C). We have successfully purified full-length WT, D1994A, G2019S and R1441C.

CONCLUSIONS

An extensive set of tools have been developed to enable the discovery and characterization of inhibitors of LRRK2. These tools allow for HTS compatible screens in multiple formats for full-lengthLRRK2 and its mutants in biochemical and cellular environments.

References

Hermanson, S.B.; Carlson, C.B.; Zhao, J.; Riddle, S. M.; Vogel, K; Nichols, R. J; Bi, K., Screening for Novel LRRK2 Inhibitors Using a High-Throughput TR-FRET Cellular Assay for LRRK2 Ser935 Phosphorylation. PLoS One. 2012;7(8):e43580.

Reichling LJ, Riddle SM (2009) Leucine-rich repeat kinase 2 mutants I2020T and G2019S exhibit altered kinase inhibitor sensitivity. Biochem Biophys Res Commun 384: 255–258.

Useful Weblinks

www.lifetechnologies.com/bindingassay
www.lifetechnologies.com/lanthascreencellular

[1]Full-length LRRK2 proteins mentioned in this publication are currently available for sale, please contact discoveryservices@lifetech.com to learn more.
[2]Funded by The Michael J. Fox Foundation for Parkinson's Research