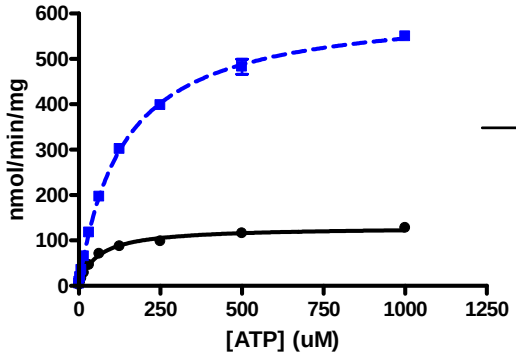
Optimization of LanthaScreen™ Kinase Assays for LRRK2 and LRRK2 G2019S

Overview

This protocol describes how to develop a LanthaScreen™ kinase assay designed to detect and characterize kinase inhibitors.

1. **Determination of ATP $K_{m,app}$.**
   ATP $K_{m,app}$ is often determined in LanthaScreen™ format. However, for LRRK2 ATP $K_{m,app}$ values were determined using a radiometric, phospho-cellulose filter-binding assay using 400 µM LRRKtide (PV5093) substrate in Kinase Buffer S supplemented with DTT (50 mM Tris pH 8.5, 10 mM MgCl2, 0.01% Brij-35, 1 mM EGTA, 2 mM DTT). The enzyme concentrations used were 2.1 µg/mL and 1.0 µg/mL for wt and G2019S, respectively. The ATP $K_{m,app}$ values were 57 µM for wt LRRK2 and 134 µM for the G2019S mutant, and ATP was used at these concentrations for the following LanthaScreen™ experiments.



	LRRK2	LRRK2 G2019S
VM	129.4	618.2
KM	56.88	134.1

2. **Optimization of kinase concentration required for assay at ATP $K_{m,app}$.**
   Using the ATP $K_{m,app}$ concentration of ATP determined in step 1, a kinase titration is performed in order to determine the concentration of kinase required to elicit an approximately 80% change between the minimum and maximum TR-FRET emission ratios at the ATP $K_{m,app}$ concentration of ATP (the EC_{80} value). This is the concentration of kinase that is typically used in an assay to determine an IC_{50} value for an inhibitor, though the exact concentration may be adjusted on a case-by-case basis.

Using the ATP and kinase concentrations determined above, the reaction is then performed in the presence of a dilution series of inhibitor, and the amount of inhibitor required to elicit a 50% change in TR-FRET ratio (the IC_{50}) is determined.

The optimization presented here is designed to maximize sensitivity of the assay towards both ATP-competitive as well as non-ATP competitive inhibitors. If desired, the assay can be performed at higher concentrations of ATP in order to be less sensitive towards ATP-competitive compounds. If such an assay is desired, step 2 is the only step that needs to be performed (at the chosen concentration of ATP) prior to performing the assay in the presence of inhibitors.

The data presented in this document are example data that were generated at Invitrogen. Specific results may vary based upon a variety of factors including the specific activity of the kinase, or the particular plate reader being used. In particular, the Emission Ratio measured can vary greatly between instruments. However, the quality of the data generated should be comparable to the data presented here. If you are reproducing the work presented in this document you should move between the various steps using the values determined in *your* experiments. If you are having trouble reproducing the data presented here, please do not hesitate to contact Invitrogen Technical Services or your Invitrogen representative.

Materials Required

Description	Part Name	Catalog #	Notes
Kinase Reaction Buffer	5X Kinase Buffer S (250 mM Tris pH 8.5, 50 mM MgCl2, 0.05% Brij-35, 5 mM EGTA)	PV5213	(1)
	Additives: DTT (2 mM)	P2325 (1 M)	
Kinase	LRRK2	PV4873	
	LRRK2 G2019S	PV4881	
Antibody	LanthaScreen™ Tb-anti-pERM (pLRRKtide)	PV4899 (25 µg) PV4900 (1 mg)	
Substrate	Fluorescein-ERM (LRRKtide)	PV4901 (1 mg)	
Antibody Dilution Buffer	TR-FRET Dilution Buffer	PV3574 (100 mL)	(2)
500 mM EDTA	Kinase Quench Buffer	P2825 (1 mL)	
10 mM ATP	10 mM ATP	PV3227 (500 µL)	

(1) The kinase reaction buffer is supplied as a 5x concentrated stock without DTT. Prepare a 1x solution from this stock as described below with 2 mM fresh DTT. The 1x kinase reaction buffer is stable at room temperature.

(2) The antibody dilution buffer does not contain EDTA. EDTA is added separately, prior to addition of antibody.

Preparing the 1x Kinase Reaction Buffer

Prepare a 1x solution of kinase reaction buffer from the 5x Kinase Buffer stock (listed above) by adding 4 mL of 5x stock to 16 mL H_2O to make 20 mL of 1x kinase reaction buffer. Add 40 µL of 1M DTT for a final concentration of 2 mM on the day of use.

General Assay Conditions

Kinase reactions are performed in a 10 µL volume in low-volume 384-well plates. Typically, Corning model 3676 (black) or 3673 (white) plates are used. The concentration of substrate in the assay is 400 nM, and the 1x kinase reaction buffer consists of 50 mM Tris pH 8.5, 0.01% BRIJ-35, 10 mM $MgCl_2$, and 1 mM EGTA, plus any additional additives that may be required for a specific kinase. Kinase reactions are allowed to proceed for 1 hour at room temperature before a 10 µL preparation of EDTA (20 mM) and Tb-labeled antibody (4 nM) in TR-FRET dilution buffer are added. The final concentration of antibody in the assay well is 2 nM, and the final concentration of EDTA is 10 mM. The plate is allowed to incubate at room temperature for at least 30 minutes before being read on a plate reader configured for LanthaScreen™ TR-FRET.

Plate Readers

The data presented in this document were generated using a BMG Pherastar plate reader using the LanthaScreen™ filter block available from BMG. The assay can be performed on a variety of plate readers including those from Tecan (Ultra, Safire^2, and InfiniTE F500), Molecular Devices (Analyst and M5), and Perkin Elmer (EnVision, Victor, and ViewLux). Visit www.invitrogen.com/Lanthascreen or contact Invitrogen Discovery Sciences technical support at 800-955-6288 (select option 3 and enter 40266), or email tech_support@invitrogen.com for more information on performing LanthaScreen™ assays on your particular instrument.

Example Protocols

The following example protocols describe the various steps using 16-point dilutions of the variable reagent (kinase or inhibitor) in triplicate.

Step 1: Titration of Kinase at ATP K_m

(1.1) In a low-volume 384-well plate, fill each well in columns 1–3, rows 2 through 16 (B through P) with 5 µL of kinase reaction buffer. Place 10 µL of the stock kinase in the top well of each column, and then perform a 2-fold serial dilution down the plate by removing 5 µL of kinase from the top well, adding this to the well below, mixing, and repeating with the next well below. Discard 5 µL of solution from the bottom well such that each well contains 5 µL of kinase solution.

(1.2) In an appropriate container, prepare 1 mL of a solution of substrate and ATP in kinase reaction buffer at 2 times the final concentration of each reagent desired in the assay.

If a 1000 µL solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed with a multichannel pipette.

Calculations:

Substrate:	Stock = 372 µM	1x = 0.4 µM	2x = 0.8 µM
ATP:	Stock = 10 mM	1x = 57 µM	2x = 114 µM **(for LRRK2 wt)**
ATP:	Stock = 10 mM	1x = 134 µM	2x = 268 µM **(for LRRK2 G2019S)**

LRRK2 wt			[Initial]				[Final 2x]
Substrate:	2.1 µL	*	372 µM	=	1000 µL	*	0.8 µM
ATP:	11.4 µL	*	10 mM	=	1000 µL	*	0.114 mM
Buffer:	987 µL kinase reaction buffer						

LRRK2 G2019S			[Initial]				[Final 2x]
Substrate:	2.1 µL	*	372 µM	=	1000 µL	*	0.8 µM
ATP:	26.8 µL	*	10 mM	=	1000 µL	*	0.268 mM
Buffer:	971 µL kinase reaction buffer						

Procedure:

For LRRK2 wt, add 2.1 µL of 372 µM substrate and 11.4 µL of 10 mM ATP to 987 µL kinase reaction buffer.

For LRRK2 G2019S, add 2.1 µL of 372 µM substrate and 26.8 µL of 10 mM ATP to 971 µL kinase reaction buffer.

(1.3) Start the kinase reaction by adding 5 µL of the substrate + ATP solution prepared in step 3.2 to each well of the assay plate.

(1.4) Cover the assay plate and allow reaction to proceed for 1 hour at room temperature.

(1.5) Prior to completion of the kinase reaction, prepare 1.5 mL of a solution of EDTA and Tb-labeled antibody at 2 times the desired final concentrations of each reagent in TR-FRET dilution buffer. The antibody is stable in EDTA for several hours, but because it is sensitive to high concentrations of EDTA we recommend first adding the concentrated EDTA to the dilution buffer, mixing the solution well, and then adding the antibody before mixing further.

Calculations:

EDTA:	Stock = 500 mM	1x = 10 mM	2x = 20 mM
Antibody:	Stock = 3600 nM	1x = 2 nM	2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	60 µL	*	500 mM	=	1500 µL	*	20 mM
Antibody:	1.7 µL	*	3600 nM	=	1500 µL	*	4 nM
Buffer:	1438 µL TR-FRET Dilution Buffer						

Procedure:

Add 60 µL of 500 mM EDTA and 1.7 µL of 3600 nM antibody to 1438 µL TR-FRET Dilution Buffer.

(1.6) Add 10 µL of the Tb-antibody + EDTA solution prepared in step 1.5 to each well of the assay plate and mix briefly, either by pipette or on a plate shaker.

(1.7) Cover the assay plate and incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

(1.8) Plot the resulting TR-FRET emission ratio against the concentration of ATP, and fit the data to a sigmoidal dose-response curve with a variable slope. Calculate the EC_{80} concentration from the curve. The following equation can be used with GraphPad™ Prism® software:

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F=80
logEC50=logECF-(1/HillSlope)*log(F/(100-F))
Y=Bottom+(Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))
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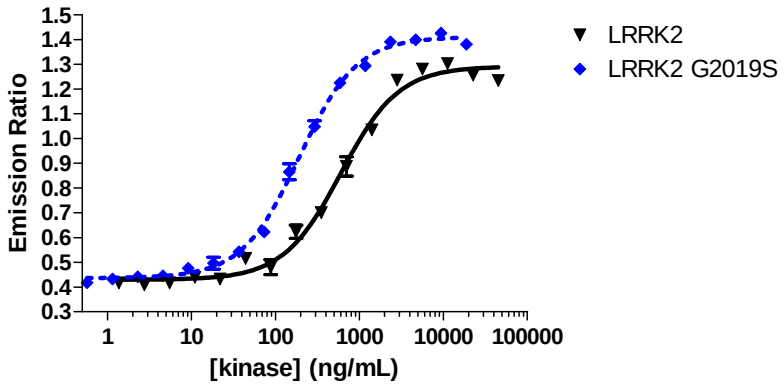
Alternatively, the amount of kinase needed to elicit an 80% change in TR-FRET response may be estimated from a visual inspection of the curve. It is important that the reactions performed to determine the IC_{50} value of an inhibitor be performed at or below the EC_{80} concentration of the kinase determined from this graph.

Figure 1: Example of Kinase Titration at ATP $K_{m,app}$



The EC_{80} values determined from the example data were 1800 ng/mL and 580 ng/mL kinase, for LRRK2 and LRRK2 G2019S, respectively. Based on this result, 1800 ng/mL and 580 ng/mL kinase, was used for the following step of this protocol.

Step 2: Determination of Inhibitor IC_{50} Value.

(2.1) The general procedure for determining an inhibitor IC_{50} value is as follows:

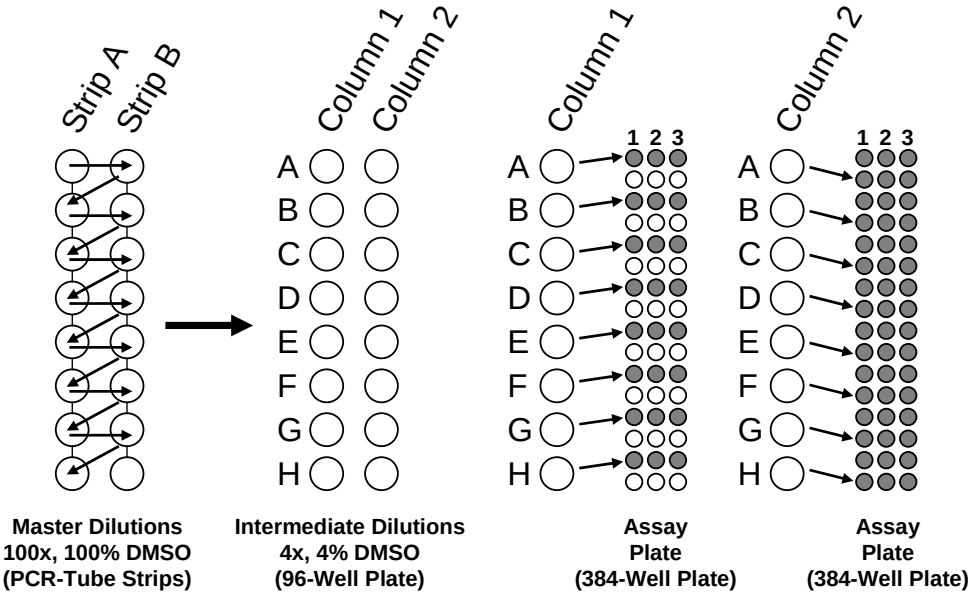
1. Add 2.5 µL of inhibitor in 4% DMSO at 4-fold the final assay concentration to triplicate assay wells.
2. To start the reaction, add 2.5 µL of kinase at 4-fold the final assay concentration, followed by 5 µL of substrate + ATP at 2-fold the final reaction concentrations.
3. The remainder of the protocol is similar to previous steps.

(2.2) A dilution series of inhibitor in 100% DMSO is first prepared at 100 times the concentrations to be assayed. By performing the initial dilutions in 100% DMSO, solubility problems associated with dilutions into aqueous buffer can be minimized.

This "master" dilution series of inhibitor can be prepared in two separate 8-tube PCR strips, and stored at -20˚ or -80˚ for use in future experiments. The dilutions are "staggered" between strips as shown in the left side of Figure 4:

1. Add 50 µL of DMSO to tubes 2–8 of strip A, and all tubes of strip B.
2. Add 100 µL of inhibitor in DMSO, at 100-fold the highest concentration to be tested in the experiment, to tube 1 of strip A.
3. Transfer 50 µL of inhibitor from tube 1 of strip A to tube 1 of strip B.
4. After mixing, transfer 50 µL from tube 1 of strip B to tube 2 of strip A.
5. This process is repeated for all but the final tube of strip B, which contains only DMSO (no inhibitor).

Figure 4: Preparing a Dilution Series of Inhibitor



(2.3) From the master dilutions of inhibitor in 100% DMSO, intermediate dilutions are then prepared in two columns of a 96-well plate. The 96-well plate is used only as a convenient vessel for preparing the intermediate dilutions.

1. First, place 96 µL of kinase reaction buffer into all wells of two columns of a 96-well plate.
2. Then, transfer 4 µL of the master inhibitor stock from strip A into column 1 of the 96 well plate, and 4 µL of the master inhibitor stock from strip B into column 2 of the 96-well plate.

3. Mix the solutions well, either with a plate shaker or by mixing with a 20 µL multichannel pipette.
4. Using an 8-channel pipette, add 2.5 µL of inhibitor from the intermediate dilution in the 96-well plate to the 384-well assay plate as shown in figure 4. Use column 1 of the intermediate stock to fill rows A, C, E, etc. of the 384-well assay plate, and column 2 to fill the alternating rows B, D, F, etc.

(2.4) Prepare a 1 mL solution of kinase in kinase reaction buffer at 4x the final desired reaction concentration of the kinase. From Step 3.8, 1800 ng/mL and 580 ng/mL kinase was determined to be the concentration required for the assay for LRRK2 wt and G2019S, respectively.

Calculation:

LRRK2: Initial conc. = 180,000 ng/mL 1x = 1,800 ng/mL 4x =7,200 ng/mL

LRRK2 G2019S: Initial conc. = 150,000 ng/mL 1x = 580 ng/mL 4x =2,320 ng/mL

LRRK2 wt			[Initial]				[Final 4x]
Kinase:	40 µL	*	180,000 ng/mL	=	1000 µL	*	7,200 ng/mL
Buffer:	960 µL kinase reaction buffer						

LRRK2 G2019S			[Initial]				[Final 4x]
Kinase:	15.5 µL	*	150,000 ng/mL	=	1000 µL	*	2320 ng/mL
Buffer:	984.5 µL kinase reaction buffer						

Procedure:

For LRRK2, add 40 µL of 180,000 ng/mL kinase to 960 µL kinase reaction buffer.

For LRRK2 G2019S, add 15.5 µL of 150,000 ng/mL kinase to 984.5 µL kinase reaction buffer.

(2.5) Add 2.5 µL of the kinase solution prepared in step 2.4 to each well of the assay plate.

(2.6) In an appropriate container, prepare 1 mL of a solution of substrate and ATP in kinase reaction buffer at 2 times the final concentration of each reagent desired in the assay.

If a 1000 µL solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed using a multichannel pipette.

Calculations:

Substrate: Stock = 372 µM 1x = 0.4 µM 2x = 0.8 µM

LRRK2 ATP: Stock = 10 mM 1x = 57 µM 2x = 114 µM

LRRK2 G2019S ATP: Stock = 10 mM 1x = 134 µM 2x = 268 µM

LRRK2 wt			[Initial]				[Final 2x]
Substrate:	5.4 µL	*	372 µM	=	2500 µL	*	0.8 µM
ATP:	28.5 µL	*	10 mM	=	2500 µL	*	0.114 µM
Buffer:	2466 µL kinase reaction buffer						

LRRK2 G2019S			[Initial]				[Final 2x]
Substrate:	5.4 µL	*	372 µM	=	2500 µL	*	0.8 µM
ATP:	67.0 µL	*	10 mM	=	2500 µL	*	0.268 mM
Buffer:	2428 µL kinase reaction buffer						

Procedure:

For LRRK2 wt, add 5.4 µL of 372 µM substrate and 28.5 µL of 10 mM ATP to 2466 µL kinase reaction buffer.

For LRRK2 wt, add 5.4 µL of 372 µM substrate and 67.0 µL of 10 mM ATP to 2428 µL kinase reaction buffer.

(2.7) Start the kinase reaction by adding 5 µL of the substrate + ATP solution prepared in step 4.6 to each well of the assay plate and mix briefly, either by pipette or on a plate shaker.

(2.8) Cover the assay plate and allow reaction to proceed for 1 hour at room temperature.

(2.9) Prior to completion of the assay, prepare 1 mL of a solution of EDTA and Tb-labeled antibody at 2 times the desired final concentrations of each reagent in TR-FRET dilution buffer. The antibody is stable in EDTA for several hours, but because it is sensitive to high concentrations of EDTA, we recommend first adding the concentrated EDTA to the dilution buffer, mixing the solution well, and then adding the antibody before mixing further.

Calculations:

EDTA:	Stock = 500 mM	1x = 10 mM	2x = 20 mM
Antibody:	Stock = 3600 nM	1x = 2 nM	2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	120 µL	*	500 mM	=	3000 µL	*	20 mM
Antibody:	3.3 µL	*	3600 nM	=	3000 µL	*	4 nM
Buffer:	2877 µL TR-FRET Dilution Buffer						

Procedure:

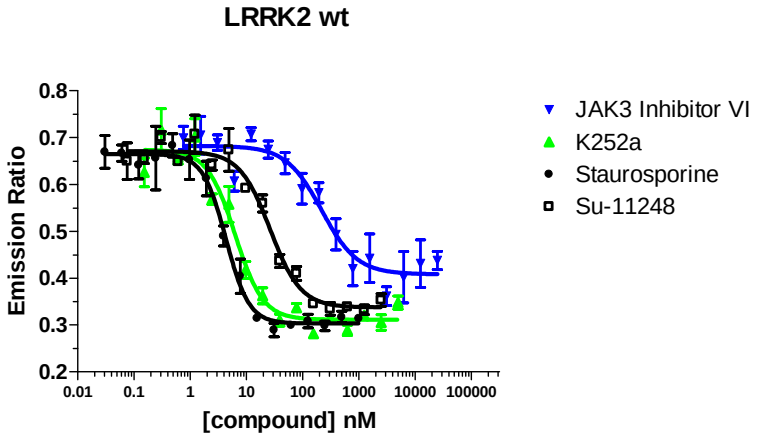
Add 120 µL of 500 mM EDTA and 3.3 µL of 3600 nM antibody to 2877 µL TR-FRET Dilution Buffer.

(2.10) Add 10 µL of the Tb-antibody + EDTA solution prepared in step 2.9 to each well of the assay plate.

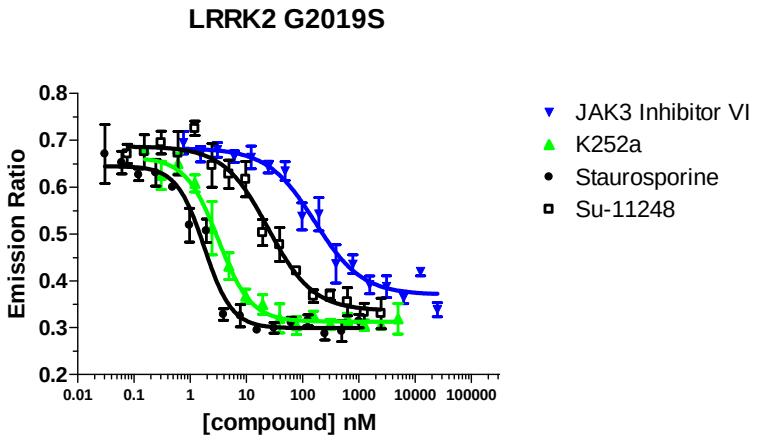
(2.11) Cover the assay plate and incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

(2.12) Plot the resulting TR-FRET emission ratio against the concentration of inhibitor, and fit the data to a sigmoidal dose-response curve with a variable slope. Calculate the EC_{50} concentration from the curve. This is equal to the IC_{50} value for the inhibitor.

Figure 2: Example of an IC_{50} Curves



	JAK3 Inhibitor VI	K252a	Staurosporine	Su-11248
EC50	217.5	6.318	4.333	26.47



	JAK3 Inhibitor VI	K252a	Staurosporine	Su-11248
EC50	163.9	3.167	1.782	24.52

Appendix:

Assay Validation Using a "Kinase Dead" Mutant of LRRK2 and Comparison of Disease-Associate Mutants

Because of a lack of literature data regarding small-molecule inhibition of LRRK2 at the time of these experiments, a "kinase dead" mutant of LRRK2 was prepared by mutating the active site aspartate (D1994) to alanine. Additionally, we prepared a series of disease relevant LRRK2 mutants which were all purified in parallel by identical procedures. SDS-PAGE analysis of 2 μg of each of the kinases prepared demonstrates they were all produced with comparable purity (Figure A1). The activities of the mutants (at 2.1 μg/mL) were compared by radiometric assays using 400 μM unlabeled LRRKtide (PV5093) in Kinase Buffer S with 2 mM DTT (Figure A2) and by LanthaScreen™ assays (Figure A3) as performed as in Figure 1, except at 1 mM ATP. In both assay formats, the catalytic activity of the D1994A mutant is severely attenuated relative to the wildtype or disease-relevant mutants. Likewise, in both formats, G2019S has moderately higher activity, and I2020T has moderately lower activity. The robustness of the assay for HTS was assessed by determining the Z' value (Figure A4) by running assays as in Figure 2, in the absence of inhibitors, and with ½ of a 384 well plate with LRRK2 and ½ of the plate without enzyme.

Figure A1: SDS-PAGE Analysis of LRRK2 Proteins

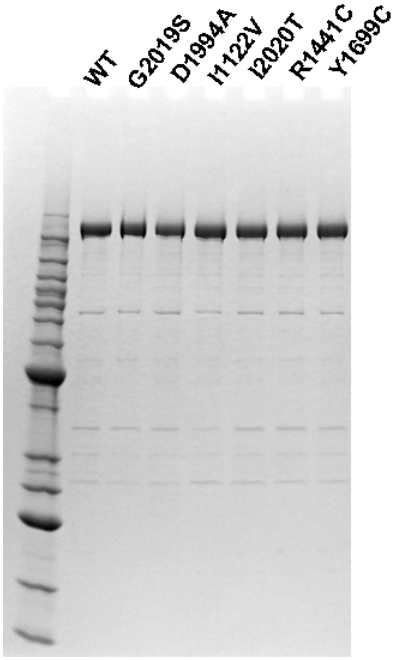


Figure A2: Comparison of LRRK2 mutants by radiometric assay

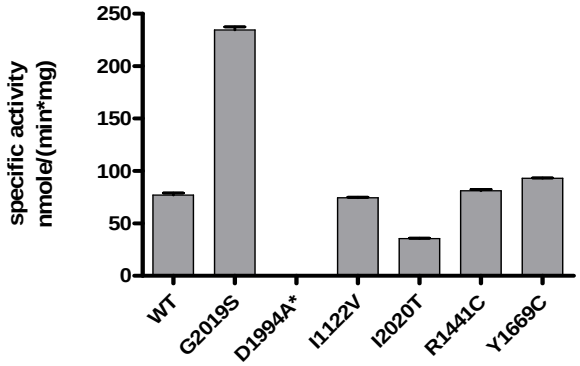


Figure A3: Comparison of LRRK2 mutants by LanthaScreen™ assay

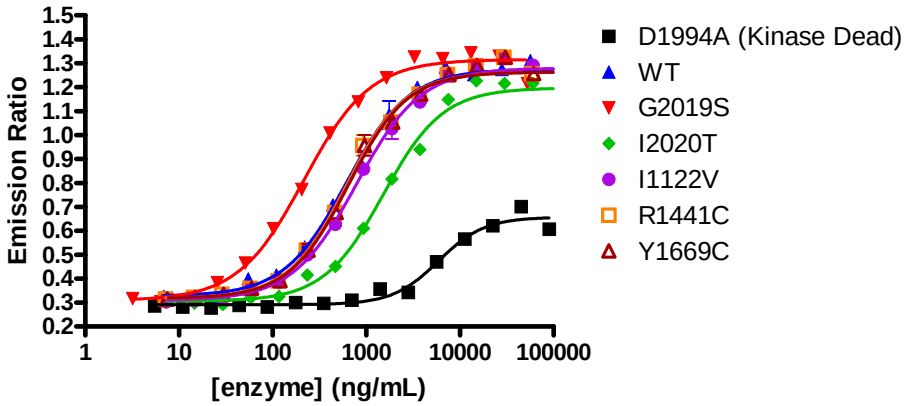


Figure A4: Determination of Z' for the LRRK2 LanthaScreen™ assay