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LanthaScreen® Eu Kinase Binding Assay for LRRK2-GFP Lysate

Catalog nos. A14171, A14194

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Overview

The following protocol describes how to perform a LanthaScreen® Eu Kinase Binding Assay designed to detect and characterize kinase inhibitors that bind to LRRK2 using a LRRK2-GFP lysate. This assay uses recombinant full-length human LRRK2 with a C-terminal GFP tag that is present in a lysate from HEK293T cells. LRRK2 was expressed in the HEK293T cells using BacMam technology, which uses a modified baculovirus to efficiently deliver and robustly express genes in mammalian cells.

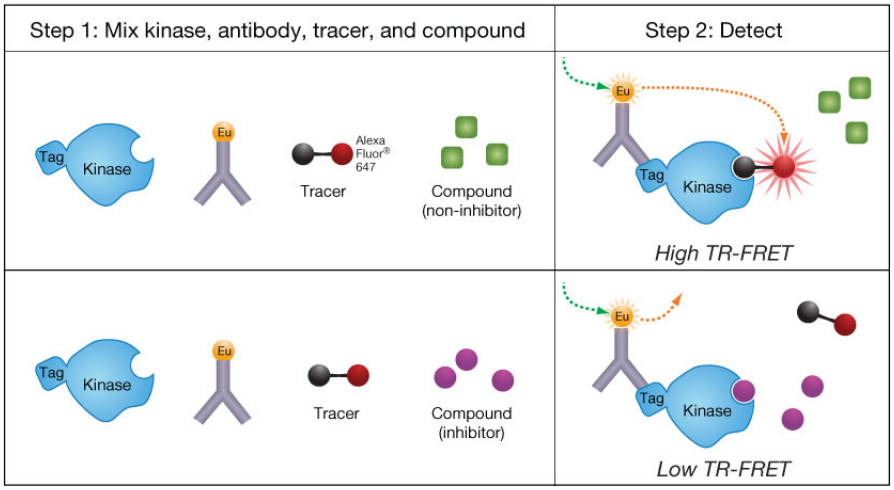
LanthaScreen® Eu Kinase Binding Assays are based on the binding and displacement of a proprietary, Alexa Fluor® 647-labeled, ATP-competitive kinase inhibitor scaffold (kinase tracer) to the kinase of interest (Figure 1). Tracers based on a variety of scaffolds have been developed to address a wide range of kinase targets. The binding of the tracer to the kinase is detected using a europium-labeled anti-tag antibody, which binds to the kinase of interest. Simultaneous binding of the tracer and antibody to the kinase results in a high degree of FRET (fluorescence resonance energy transfer) from the europium (Eu) donor fluorophore to the Alexa Fluor® 647 acceptor fluorophore on the kinase tracer. Binding of an inhibitor to the kinase competes for binding with the tracer, resulting in a loss of FRET. Note that the instrinsic fluorescence of the GFP tag is used for quantifying the target in the lysate product, but is not utilized in the assay itself.

Invitrogen's Kinase Tracers are based on ATP-competitive kinase inhibitors, making them suitable for detection of any compound that binds to the ATP site or to an allosteric site altering the conformation of the ATP site that results in the displacement of the tracer. Inhibitors that bind the ATP site include both Type I kinase inhibitors, which bind solely to the ATP site, and Type II inhibitors (e.g., Gleevec®/Imatinib, Sorafenib, BIRB-796), which bind to both the ATP site and a hydrophobic site exposed in the DFG-out (non-active) conformation. Compounds that do not compete with ATP (Type III inhibitors) have also been shown to displace the tracer from the kinase. A study of 15 diverse Type III inhibitors demonstrated that all but one compound was detected in the binding assay with equivalent potency to activity assays. The sole exception was a substrate-competitive compound that did not disrupt the phophorylation of an alternate substrate, demonstrating that the active site remained catalytically competent.

In contrast to most fluorescence-based kinase activity assays, LanthaScreen® Eu Kinase Binding Assays can be read continuously, which facilitates evaluation of compounds with slow binding kinetics. Also, unlike most activity assays, binding assays can be performed using activated or non-activated kinase preparations, which enables the characterization of compounds that preferentially bind to non-activated kinases, such as Gleevec®/imatinib and some allosteric inhibitors.

We have optimized the concentration of Kinase Tracer for use with the LRRK2-GFP lysate. The following protocol describes how to perform kinase inhibitor affinity (IC_{50}) measurements using this optimized tracer concentration. If you wish to perform tracer titration experiments to determine optimal tracer concentration under different assay conditions, refer to the general LanthaScreen® Eu Kinase Binding Assay user guide on our website at **www.invitrogen.com/bindingassay**. For additional information on the BacMam technology, visit **www.invitrogen.com/bacmam**.

Figure 1 Schematic of the LanthaScreen® Eu Kinase Binding Assay



Materials required

Product	Part no.	Concentration	Amount	Notes
LRRK2-GFP lysate	A14171 A14194	Lot specific; refer to CoA	25 pmol 250 pmol	1
5X Kinase Buffer A	PV3189 PV6135	5X	4 mL 100 mL	2
Kinase Tracer 236	PV5592	50 µM in DMSO	25 µL	
LanthaScreen® Eu-anti-GFP Antibody	A14173 A14190 A14193	0.1 mg/mL (0.67 µM)	2.5 µg 25 µg 250 µg	3
Staurosporine (optional)	N/A	N/A	N/A	4

(1) LRRK2-GFP lysate is supplied at a concentration of greater than 50 nM LRRK-GFP in the cellular lysate. The concentration is determined by GFP fluorescence intensity using a GFP-fusion protein as a standard.

(2) Kinase Buffer A is supplied as a 5X concentrated stock. Prepare a 1X solution by adding 4 mL of the 5X solution to 16 mL of distilled H_2O. The 1X kinase reaction buffer is stable at room temperature. 1X Kinase Buffer A consists of 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35.

(3) Prior to use, centrifuge the antibody tube at approximately 10,000 × *g* for 5 minutes, and aspirate the amount needed for the assay from the top of the solution. This centrifugation step eliminates spurious data points that can arise on occasion due to any particulates in the product.

(4) Staurosporine can be used as a control compound for this assay because it competes with Kinase Tracer 236 for binding to LRRK2-GFP.

Guidelines for inhibitor studies

Final assay conditions for inhibitor studies

3 nM kinase[1]

2 nM Eu-Anti-GFP Antibody

20 nM Kinase Tracer 236[2]

1X Kinase Buffer A

[1] We recommend a kinase concentration of 3 nM as a starting point for assay development because this typically results in a robust signal. Decreasing the kinase concentration may be necessary for accurate measurement of very tight-binding inhibitors, similar to kinase activity assays.

[2] We suggest a tracer concentration of 20 nM. However, the tracer concentration could also be experimentally determined or optimized, if other assay conditions are varied.

Plates

Assays are typically performed in white low-volume 384-well plates (Corning, part no. 3673, or Greiner, part no. 784207) or black, low-volume 384-well plates (Corning, part no. 3676). White plates are generally recommended because they yield higher quality data for many assays, especially those with a relatively low assay window (1.5 to 2 fold) or those that are being measured on monochromater-based instruments or some filter-based instruments (e.g. Perkin Elmer EnVision®). In other cases, black and white plates yield comparable data.

Plate readers

The data presented in this document were generated using a BMG LABTECH (PHERAstar*Plus*) plate reader using the appropriate filters and instrument settings for europium-based LanthaScreen® assays. The assay can be performed on a variety of plate readers including those from Tecan (Ultra, Infinite F-500, Safire²), Molecular Devices (Analyst and M5) and Perkin Elmer (EnVision®, Victor, and ViewLux) or any other plate reader configured for LANCE® or HTRF® assays. General instrument settings are listed in the table below:

Excitation	340 nm (30 nm bandpass)
Kinase Tracer Emission	665 nm (10 nm bandpass)
LanthaScreen® Eu-anti-Tag Antibody Emission	615 nm (10 nm bandpass)
Dichroic Mirror	Instrument dependent
Delay Time	100 µs
Integration Time	200 µs

For additional assistance, ask your Invitrogen representative for instrument-specific setup guidelines, or contact Invitrogen Discovery Sciences Technical Support at 800-955-6288 (select option 3 and enter 40266), or email **drugdiscoverytech@invitrogen.com** for more information on performing these assays on your particular instrument or for a control to test an instrument.

Basic protocol for inhibitor studies

LanthaScreen® Kinase Binding Assays for evaluating inhibitors are typically performed by the addition of 3 components each at 3X the final desired concentration prepared in 1X Kinase buffer A as follows:

1. Add 5 µL of **test compound**.
2. Add 5 µL of **9 nM LRRK2-GFP kinase/6 nM LanthaScreen® Eu-anti GFP antibody** mixture.
3. Add 5 µL of **60 nM Kinase Tracer 236.**
4. Incubate for 1 hour at room temperature and read the plate.

IC_{50} determination

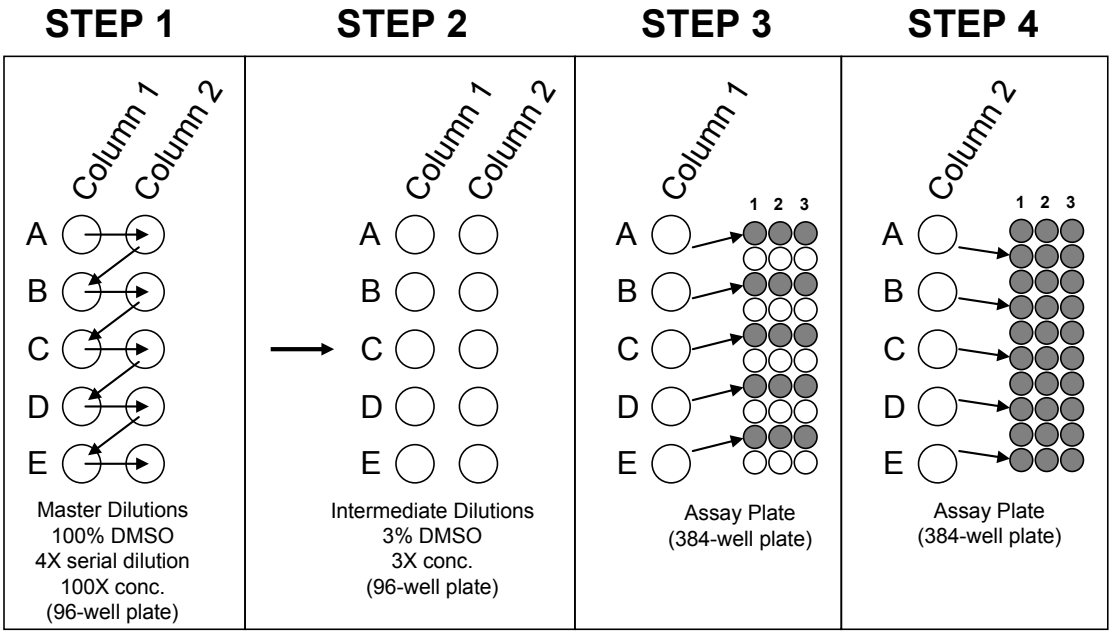
This procedure describes how to determine inhibitor potencies by generating a 10-point IC_{50} curve from a 4-fold dilution series of test compound.

We recommend using 20 nM of Kinase Tracer 236 for inhibitor studies. Under these conditions a high Z′ value (> 0.7) was obtained while keeping the tracer concentration close to the average K_d value of 23 nM).

Preparing reagents

1. Prepare an intermediate dilution series of each test compound by 4-fold serial dilution in DMSO such that the top concentration is 1 mM (suggested starting point) (Figure 2, Step 1).
    a. Prepare 4 mM test compound in DMSO.
    b. Add 60 µL of DMSO to 5 wells in each of 2 columns of a 96-well plate (wells A1 to E2).
    c. Add 20 µL of 4 mM compound to well A1 and mix.
    d. Remove 20 µL from well A1, transfer to well A2 and mix.
    e. Remove 20 µL from well A2, transfer to well B1 and mix.
    f. Continue process as depicted in Step 1 of Figure 2.

Figure 2 Compound serial dilution



2. Dilute the "Master Dilution" series 33.3-fold into Kinase Buffer A. Remove 5 µL of each concentration of the diluted compound, transfer to another 96-well plate, add 162 µL of Kinase Buffer A and mix (Figure 2, Step 2 on page 4).
3. Prepare tracer solution in Kinase Buffer A at 60 nM tracer (3X the desired final assay concentration). Add the volumes of reagents calculated below to calculated volume of Kinase Buffer A.

   Calculations (for a 1000 µL solution):

   $$\textbf{Tracer volume needed (µL)} = \frac{\text{(final volume of solution)} \times \text{(desired 3X tracer concentration)}}{\text{(1,000 nM/µM)} \times \text{(stock tracer concentration)}}$$

   $$= \frac{1{,}000\ \mu L \times 60\ nM}{(1{,}000\ nM/\mu M) \times 50\ \mu M}$$

   Kinase Buffer A needed (µL) = 1,000 µL – tracer volume needed (µL)

4. Prepare kinase/antibody solution at a concentration of 9 nM for the kinase and 6 nM for the antibody (i.e., 3X the desired final assay concentration). Centrifuge the antibody tube at approximately 10,000 × *g* for 5 minutes and aspirate the desired volume from the top of the solution. Add the volumes of reagents calculated as shown below to the calculated volume of Kinase Buffer A.

   Calculations (for a 1000 µL solution):

   Stock kinase concentartion (nM) = reported on tube and on COA

   $$\textbf{Kinase volume needed (µL)} = \frac{1{,}000\ \mu L \times 9\ nM}{\text{stock kinase concentration (nM)}}$$

   $$\textbf{Antibody volume needed (µL)} = \frac{1{,}000\ \mu L \times 6\ nM}{\text{stock antibody concentration (µM)} \times 1{,}000\ (nmol/\mu mol)}$$

   Kinase Buffer A needed (µL) = 1000 µL – kinase volume needed (µL) – antibody volume needed (µL)

Experimental procedure

1. Add 5 µL of each concentration of serially diluted compound to triplicate assay wells in a 384-well plate (columns 1–3) as depicted in Steps 2 and 3 of Figure 2 on page 4.
2. Add 5 µL of kinase/antibody solution to all wells.
3. Add 5 µL of tracer solution to all wells.
4. Incubate the plate at room temperature for 60 min and read.

   Note: 60 minutes is a general guideline for incubation. However, in some cases multiple read times or continuous measurements may be used to examine the kinetics of binding reactions as might be of interest for studies on slow-binding compounds.

Data analysis

1. Divide the acceptor/tracer emission (665 nM) by the antibody/donor emission (615 nM) to calculate the "emission ratio".
2. Plot the concentration of test compound versus the emission ratio. The sigmoidal dose-response curve with a variable slope can be fit to the data. The following equation can be used with GraphPad Prism software:

$$F = 50$$

$$\log EC_{50} = \log EC_F - \left(\frac{1}{HillSlope}\right) \times \log\left(\frac{F}{100 - F}\right)$$

$$Y = Bottom + \frac{(Top - Bottom)}{\left(1 + 10^{\log(EC_{50} - X) \times HillSlope}\right)}$$

3. Representative date generated for a set of kinase inhibitors is presented in Figure 3 (page 7). A comparison of IC_{50} values to the purified, truncated version of LRRK2 is provided as a reference in Table 1 (page 7).
4. In some cases, the Cheng-Prusoff equation[1] below can be used to convert the IC_{50} values to K_i based on the concentration of Tracer and an accurate Tracer K_d.

$$K_i = \frac{(IC_{50})}{\left(1 + \left(\frac{[Tracer]}{K_d}\right)\right)}$$

This relationship holds true when the following criteria are met:

1. [kinase] << [tracer] and [kinase] << IC_{50}
2. [kinase] < Tracer K_d
3. There is a single class of binding sites

To determine K_i values for very tight-binding compounds, it may be necessary to perform assays at lower kinase concentrations.

[1]Cheng, Y.C., Prusoff, W.H. *Biochem Pharmacol.* (22) 3099-3108 (1973).

Figure 3 Representative data generated for a set of kinase inhibitors using the LRRK2-GFP lysate.

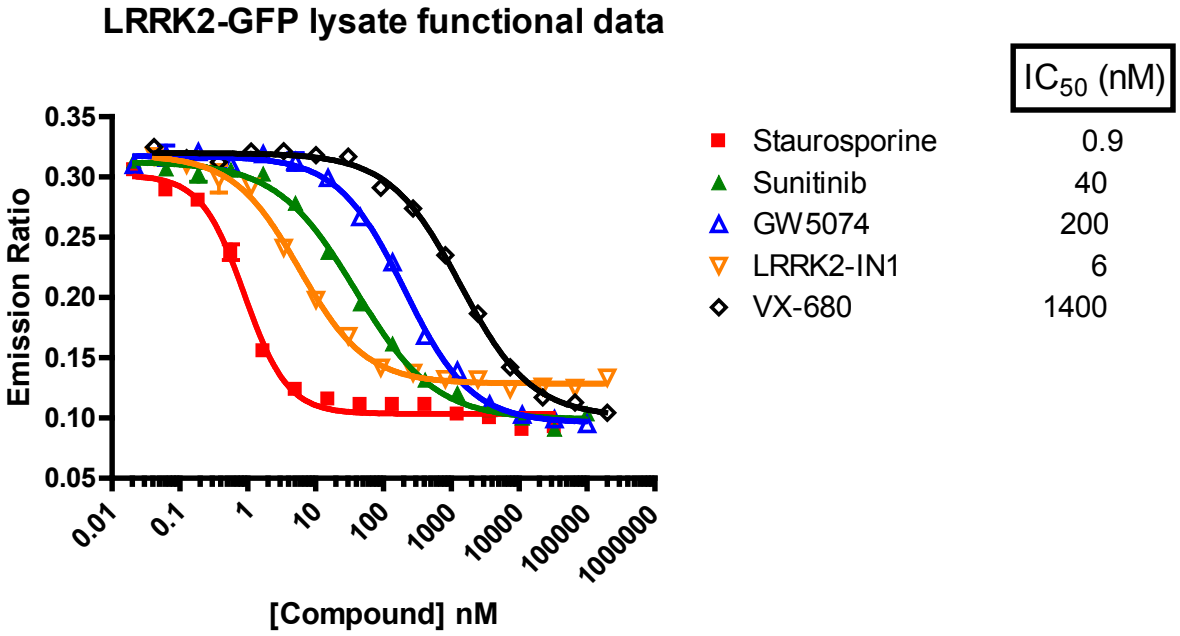


Table 1 IC_{50} values (nM) and their comparison to data generated with the truncated version of LRRK2-GFP in LanthaScreen® Eu Kinase Binding Assay.

Compound	LanthaScreen® Eu Kinase Binding Assay	
	Full-length LRRK2-GFP	LRRK2 (aa 970–2527)
Staurosporine	0.9	0.5
Sunitinib	40	12
VX680	1400	1600
GW5074	200	24
LRRK2-IN1	6	3

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