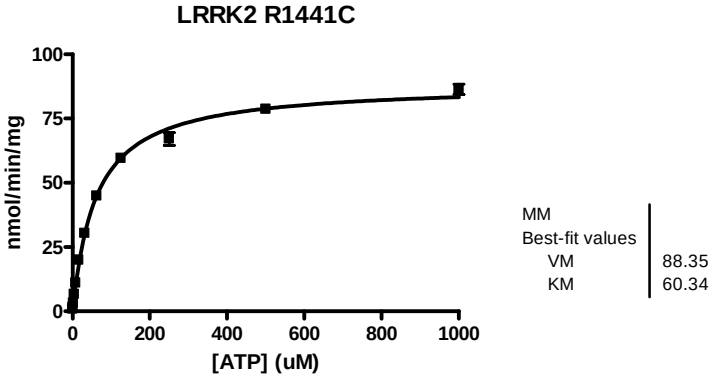
# Optimization of a LanthaScreen® Kinase assay for LRRK2 R1441C

## Overview

This protocol describes how to develop a LanthaScreen® kinase assay designed to detect and characterize kinase inhibitors. The development is performed in 2 steps:

1. **Determination of ATP $K_{m,app}$.**
   ATP $K_{m,app}$ is often determined in LanthaScreen® format. However, for LRRK2 R1441C mutant, ATP $K_{m,app}$ value was determined using a radiometric, phosphor-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). 2.1 µg/mL of LRRK2 R1441C was used and the ATP $K_{m,app}$ value was 60.3 µM. Therefore 60.3 µM of ATP was used for the following LanthaScreen® experiments.




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, the kinase titration is repeated 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 will be used in an assay to determine an $IC_{50}$ value for an inhibitor.

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 is example data that was 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 | PV5213 (4 mL of 5X) | (1) |
| | Additives: *DTT (2 mM)* | P2325 (1M) | |
| Kinase | LRRK2 R1441C | PV5858 (10 µg) | |
| Substrate | Fluorescein-ERM (LRRKtide) | PV4901 (1 mg) | (2) |
| 10 mM ATP | 10 mM ATP | PV3227 (500 µL) | |
| Antibody | LanthaScreen® Tb-anti-pERM (pLRRKtide) | PV4899 (25 µg)<br>PV4900 (1 mg) | (3) |
| Antibody Dilution Buffer | TR-FRET Dilution Buffer | PV3574 (100 mL) | (4) |
| 500 mM EDTA | Kinase Quench Buffer | P2825 (1 mL) | |
| Inhibitors (optional) | Staurosporine | PHZ1271 (100 µg) | |
| | K252a | PHZ1131 (100 µg) | |
| | JAK3 Inhibitor VI | N/A | (5) |
| | Sunitinib (Su-11248) | N/A | (6) |

(1) The kinase reaction buffer is supplied as a 5x concentrated stock. Prepare a 1x solution from this stock as described below. The 1x kinase reaction buffer is stable at room temperature.

(2) The molecular weight of Fluorescein-ERM (LRRKtide) substrate is 2475.8 g/mol. Therefore, a 1 mg/mL solution of this substrate would be ~404 µM.

(3) The LanthaScreen® Tb-anti-pERM (pLRRKtide) antibody is supplied at approximately 0.5 mg/mL. The molecular weight of the antibody is 150 kD. Thus, the stock concentration of the antibody is 3.6 µM, or 3600 nM.

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

(5) JAK3 Inhibitor VI, Adams, C; Aldous, D.J. and etc. Mapping the Kinase Domain of Janus Kinase 3. Bioorganic & Medicinal Chemistry Letters 13 (2003) 3105–3110. JAK3 Inhibitor VI can be purchased from Calbiochem (Cat #: 420126). CAS# 856436-16-3

(6) Sunitinib = *N*-[2-(diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-1,2-dihydro-2-oxo-3*H*-indol-3-ylidine)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide. CAS# 557795-19-4

## 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 module 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/instrumentsetup 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® Terbium assays on your particular instrument.

## Filter Selection

The terbium donor is excited using a 340 nm excitation filter with a 30 nm bandpass. However, the exact specifications of the excitation filter are not as critical, and filters with similar specifications will work well. In general, excitation filters that work with europium-based TR-FRET systems will perform well with the LanthaScreen® terbium chelates. Because it is important to measure energy transfer to the acceptor (fluorescein or GFP) without interference from terbium, a filter centered at 520 nm with a 25 nm bandpass is used for this purpose. In general, **standard "fluorescein" filters may not be used**, because such filters also pass light associated with the terbium spectra. The emission of fluorescein due to FRET is referenced (or "ratioed") to the emission of the first terbium peak, using a filter that isolates this peak. This is typically accomplished with a filter centered at 490 or 495 nm, with a 10 nm bandpass. In general, a 490 nm filter will reduce the amount of fluorescein emission that "bleeds through" into this measurement, although instrument dichroic mirror choices (such as those on the Tecan Ultra Evolution instrument) may necessitate the use of a 495 nm filter. The effect on the quality of the resulting measurements is minimal in either case.

| LanthaScreen® Terbium: | |
|---|---|
| Excitation: | 340 nm filter (30 nm bandwidth) |
| Fluroescein Emission: | 520 nm filter (25 nm bandwidth) |
| Terbium Emission: | 490 nm filter (10 nm bandwidth) |

Visit www.invitrogen.com/instrumentsetup 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® Terbium assays on your particular instrument.

## Example Protocols

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

If you are reproducing the work presented in this document you should move between the various steps using the values determined in **your** experiments.

## Step 1: Titration of Kinase at ATP $K_{m,app}$.

**(1.1)** In an appropriate tube or vial, prepare 40 µL of kinase in 1x kinase reaction buffer at 2 times the highest concentration of kinase to be tested. In this example, 36.25 µg/mL was the highest concentration of kinase to be tested, and the stock concentration of kinase was 290 µg/mL.

**Calculations**:

Kinase: Stock = 290 µg/mL 1x = 36.25 µg/mL 2x = 72.5 µg/mL

| | | | [Initial] | | | | [Final 2x] |
|---|---|---|---|---|---|---|---|
| Kinase: | 10.0 µL | * | 290 µg/mL | = | 40 µL | * | 72.5 µg/mL |
| Buffer: | 30.0 µL kinase reaction buffer | | | | | | |

**Procedure:**

Add 10.0 µL of 290 µg/mL kinase to 30.0 µL kinase reaction buffer.

Keep the diluted kinase on ice until needed.

**(1.2)** 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 kinase solution as prepared above 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.

**(1.3)** 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 = 404 µM 1x = 0.4 µM 2x = 0.8 µM

ATP: Stock = 10,000 µM 1x = 60.3 µM 2x = 120.6 µM

| | | | [Initial] | | | | [Final 2x] |
|---|---|---|---|---|---|---|---|
| Substrate: | 2.0 µL | * | 404 µM | = | 1000 µL | * | 0.8 µM |
| ATP: | 12.1 µL | * | 10,000 µM | = | 1000 µL | * | 120.6 µM |
| Buffer: | 985.9 µL kinase reaction buffer | | | | | | |

**Procedure:**

Add 2.0 µL of 404 µM substrate and 12.1 µL of 10 mM ATP to 985.9 µL kinase reaction buffer.

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

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

**(1.6)** Prior to completion of the kinase reaction, 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: | 40 µL | * | 500 mM | = | 1000 µL | * | 20 mM |
| Antibody: | 1.1 µL | * | 3600 nM | = | 1000 µL | * | 4 nM |
| Buffer: | 958.9 µL TR-FRET Dilution Buffer | | | | | | |

**Procedure:**

Add 40 µL of 500 mM EDTA and 1.1 µL of 3600 nM antibody to 958.9 µL TR-FRET Dilution Buffer.

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

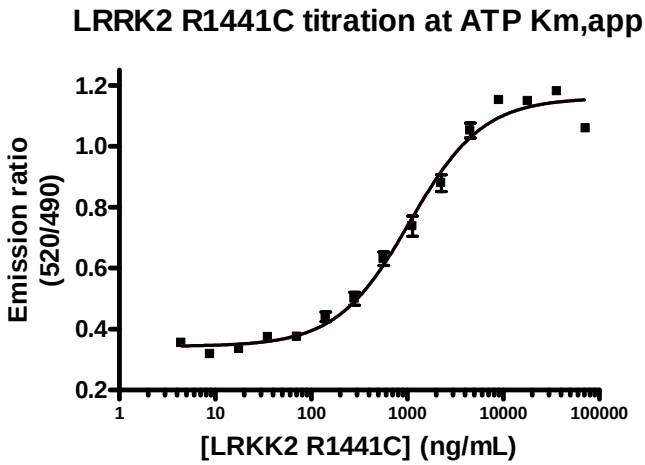
**(1.8)** Cover the assay plate and incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

**(1.9)** 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:

```
F=80
logEC50=logECF-(1/HillSlope)*log(F/(100-F))
Y=Bottom+(Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))
```

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 $EC_{80}$ value determined from the example data was 3600 ng/mL kinase. Based on this result, 3600 ng/mL kinase was used to determine inhibitor $IC_{50}$ values when performing the assay at 60.3 µM ATP.

If you are reproducing the work presented in this document you should move between the various steps using the values determined in **your** experiments.

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

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

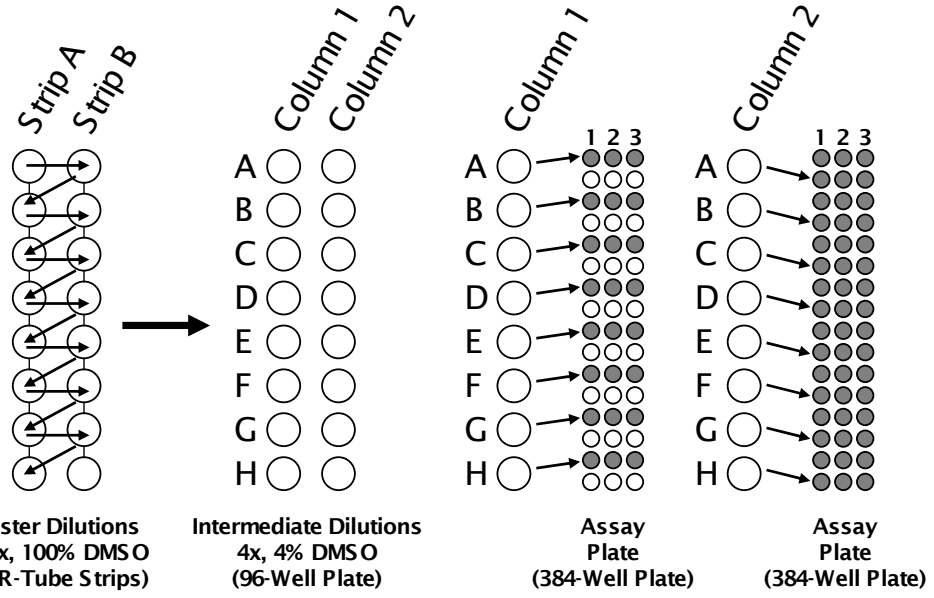
1. Add 2.5 µL of inhibitor in 4% DMSO at 4-fold the final assay concentration to triplicate assay wells.
2. Add 5 µL of 2-fold kinase + substrate, followed by 2.5 µL of ATP at 4-fold the final reaction concentrations to start the reaction.
3. The remainder of the protocol is similar to previous steps.

**(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˚C or -80˚C for use in future experiments. The dilutions are "staggered" between strips as shown in the left side of Figure 2:

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 2: 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)** In an appropriate tube or vial, prepare 1500 µL of kinase + substrate in kinase reaction buffer at 2 times the highest concentration to be tested.

**Calculation:**

Kinase: Initial conc. = 290 µg/mL 1x = 3.6 µg/mL 2x = 7.2 µg/mL

Substrate: Stock = 404 µM 1x = 0.4 µM 2x = 0.8 µM

| | | | [Initial] | | | | [Final 2x] |
|---|---|---|---|---|---|---|---|
| Kinase: | 37.3 µL | * | 290 µg/mL | = | 1500 µL | * | 7.2 µg/mL |
| Substrate: | 3.0 µL | * | 404 µM | = | 1500 µL | * | 0.8 µM |
| Buffer: | 1459.7 µL kinase reaction buffer | | | | | | |

**Procedure:**

Add 37.3 µL of 290 µg/mL kinase and 3.0 µL of 404 µM substrate to 1459.7 µL kinase reaction buffer

**(2.5)** Add 5.0 µL of the kinase + substrate 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 4 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:**

ATP: Stock = 10 mM 1x = 0.0603 mM 4x = 0.241 mM

| | | | [Initial] | | | | [Final 4x] |
|---|---|---|---|---|---|---|---|
| ATP: | 24.1 µL | * | 10 mM | = | 1000 µL | * | 0.241 mM |
| Buffer: | 975.9 µL kinase reaction buffer | | | | | | |

**Procedure:**

Add 24.1 µL of 10 mM ATP to 975.9 µL kinase reaction buffer.

**(2.7)** Start the kinase reaction by adding 2.5 µL of the ATP solution prepared in step 2.6 to each well of the assay plate .

**(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 2.5 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: | 100 µL | * | 500 mM | = | 2500 µL | * | 20 mM |
| Antibody: | 2.8 µL | * | 3600 nM | = | 2500 µL | * | 4 nM |
| Buffer: | 2397.2 µL TR-FRET Dilution Buffer | | | | | | |

**Procedure:**

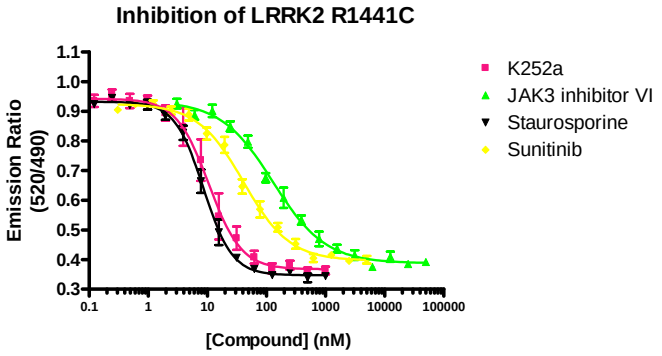
Add 100 µL of 500 mM EDTA and 2.8 µL of 3600 nM antibody to 2397.2 µL TR-FRET Dilution Buffer.

**(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 3: Example of an $IC_{50}$ Calculation Curve**




The inhibition of LRRK2 R1441C with the inhibitors Staurosporine, K252a, JAK3 inhibitor VI and Sunitinib are displayed above.

The $IC_{50}$ value for inhibition of LRRK2 R1441C with the above inhibitor is shown in the table below.

| | | $IC_{50}$ (nM) | |
|---|---|---|---|
| **Kinase** | **Inhibitor** | **Literature** | **Observed** |
| LRRK2 R1441C | K252a | NR | 10.6 |
| | JAK3 inhibitor VI | NR | 129.6 |
| | Staurosporine | NR | 8.6 |
| | Sunitinib | NR | 41.4 |

NR = No value reported

For convenience, titration curves can be normalized by dividing all values in the curve by the ratio obtained at the bottom of the curve. This will normalize the titration curves making data comparison between various instruments and gain settings easier. Normalizing the data will have no effect on the $IC_{50}$ values or Z prime.