Optimization of a LanthaScreen® Kinase assay for LCK

Overview

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

1. **Optimization of kinase concentration required to determine ATP $K_{m,app}$.**
   The assay is first performed at a high concentration of ATP (1 mM) against a dilution series of kinase in order to determine the amount of kinase required to elicit an approximately 80% change between the minimum and maximum TR-FRET emission ratios (the EC_{80} value).

2. **Determination of ATP $K_{m,app}$.**
   Using the concentration of enzyme determined in step 1, the assay is then performed against a dilution series of ATP in order to determine the amount of ATP required to elicit a 50% change between the minimum and maximum TR-FRET emission ratios (the EC_{50} value). This concentration of ATP is referred to as the "apparent" K_m value for ATP, or the ATP $K_{m,app}$.

3. **Optimization of kinase concentration required for assay at ATP $K_{m,app}$.**
   Using the ATP $K_{m,app}$ concentration of ATP determined in step 2, 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 experiments described in this document can be performed over two days, with steps one and two being performed on the first day, and step three and the inhibitor IC_{50} determination(s) being performed on the second day.

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 1 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 A	PV3189 (4 mL of 5X)	(1)
	Additives: *None*	*n.a.*	
Kinase	LCK	P3043 (10 µg)	
Substrate	Alexa Fluor® 647 – Poly GT	PV5693 (1 nmol) PV5836 (10 nmol)	(2)
10 mM ATP	10 mM ATP	PV3227 (500 µL)	
Antibody	Eu-PY20 Antibody	PV5692 (25 µg) PV5696 (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)	(5)
	Dasatinib	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 substrate is supplied at a concentration of ~30 µM, see the tube for the exact concentration.
(3) The Eu-PY20 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 3.3 µM, or 3300 nM.
(4) The antibody dilution buffer does not contain EDTA. EDTA is added separately, prior to addition of antibody.
(5) Staurosporine = *9S,10R,11R,13R*)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-(methylamino)-9,13-epoxy-1*H*,9*H*-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one.
(6) Dasatinib = *N*-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazole carboxamide monohydrate. CAS#371935-74-9.

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.

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 100 nM, and the 1x kinase reaction buffer consists of 50 mM HEPES pH 7.5, 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 Eu-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 HTRF® 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® Europium assays on your particular instrument.

Filter Selection

In general, excitation filters that work with other europium-based TR-FRET systems will perform well with LanthaScreen® Eu reagents. Typically, the europium donor is excited using a 340 nM excitation filter with a 30 nm bandwidth. However, the exact specifications of the excitation filter are not critical, and filters with similar specifications will work well. Emission from the FRET acceptor (Alexa Fluor® 647) is divided by (or ratioed) to the emission of the FRET donor (europium chelate) using filters at 665 nm and 620 nm, respectively, each with a 10 nm bandpass.

LanthaScreen® Europium:	
Excitation:	340 nm filter (30 nm bandwidth)
Alexa Fluor® 647 Emission:	665 nm filter (10 nm bandwidth)
Europium Emission:	620 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® Europium 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 1 mM ATP

(1.1) In an appropriate tube or vial, prepare 100 µL of kinase in 1x kinase reaction buffer at 2 times the highest concentration of kinase to be tested. In this example, 10 µg/mL was the highest concentration of kinase to be tested, and the stock concentration of kinase was 410 µg/mL.

Calculations:

Kinase: Stock = 410 µg/mL 1x = 10 µg/mL 2x = 20 µg/mL

			[Initial]				[Final 2x]
Kinase:	4.9 µL	*	410 µg/mL	=	100 µL	*	20 µg/mL
Buffer:	95.1 µL kinase reaction buffer						

Procedure:

Add 4.9 µL of 410 µg/mL kinase to 95.1 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 2–16 (B through P) with 5 µL kinase reaction buffer. Place 10 µL of the kinase solution prepared in step 1.1 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 substrate and ATP in kinase reaction buffer at 2 times the final concentration of each that is desired in the assay. If a 1 mL solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed using a multichannel pipette.

Calculations:

Substrate:	Stock = 30 µM	1x = 0.1 µM	2x = 0.2 µM
ATP:	Stock = 10 mM	1x = 1 mM	2x = 2 mM

			[Initial]				[Final 2x]
Substrate:	6.7 µL	*	30 µM	=	1000 µL	*	0.2 µM
ATP:	200 µL	*	10 mM	=	1000 µL	*	2 mM
Buffer:	793.3 µL kinase reaction buffer						

Procedure:

Add 6.7 µL of 30 µM substrate and 200 µL of 10 mM ATP to 793.3 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 the 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 Eu-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 = 3300 nM	1x = 2 nM	2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	40 µL	*	500 mM	=	1000 µL	*	20 mM
Antibody:	1.2 µL	*	3300 nM	=	1000 µL	*	4 nM
Buffer:	958.8 µL TR-FRET Dilution Buffer						

Procedure:

Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer.

(1.7) Add 10 µL of the Eu-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 kinase, 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))
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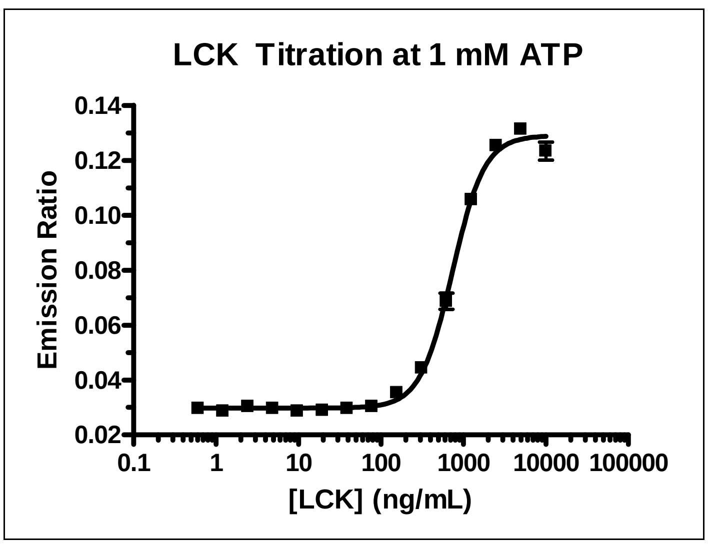
Alternatively, the amount of kinase needed to elicit an 80% change in TR-FRET response may be estimated from a visual inspection of the curve. It is important that the reactions in the next step of this protocol be performed at or below the EC_{80} concentration of kinase.

Figure 1: Example of Kinase Titration at 1 mM ATP



The EC_{80} value determined from the example data was 1400 ng/mL kinase. Based on this result, 1.4 µg/mL kinase was used for the following step of this protocol.

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: Titration of ATP at the Initial EC_{80} Concentration of Kinase to determine ATP $K_{m,app}$.

(2.1) In a small tube or vial, prepare 50 µL of a 1 mM ATP solution by adding 5 µL of 10 mM ATP to 45 µL of kinase reaction buffer.

(2.2) In a low-volume 384-well plate, fill each well in columns 1–3, rows 2 through 16 (B through P) with 5 µL kinase reaction buffer. Place 10 µL of the 1 mM ATP solution prepared in step 2.1 in the top well of each column, and then perform a 2-fold serial dilution down the plate by removing 5 µL of ATP 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 ATP solution in kinase reaction buffer.

(2.3) In an appropriate container, prepare 1 mL of a solution of substrate and kinase in kinase reaction buffer at 2 times the final concentration of each that is 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 = 30 µM	1x = 0.1 µM	2x = 0.2 µM
Kinase:	Initial conc. = 410 µg/mL	1x = 1.4 µg/mL	2x = 2.8 µg/mL

			[Initial]				[Final 2x]
Substrate:	6.7 µL	*	30 µM	=	1000 µL	*	0.2 µM
Kinase:	6.8 µL	*	410 µg/mL	=	1000 µL	*	2.8 µg/mL
Buffer:	986.5 µL kinase reaction buffer						

Procedure:

Add 6.7 µL of 30 µM substrate and 6.8 µL of 410 µg/mL kinase to 986.5 µL kinase reaction buffer.

(2.4) Start the kinase reaction by adding 5 µL of the substrate + kinase solution prepared in step 2.3 to each well of the assay plate.

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

(2.6) Prior to completion of the kinase reaction, prepare 1 mL of a solution of EDTA and Eu-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 = 3300 nM	1x = 2 nM	2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	40 µL	*	500 mM	=	1000 µL	*	20 mM
Antibody:	1.2 µL	*	3300 nM	=	1000 µL	*	4 nM
Buffer:	958.8 µL TR-FRET Dilution Buffer						

Procedure:

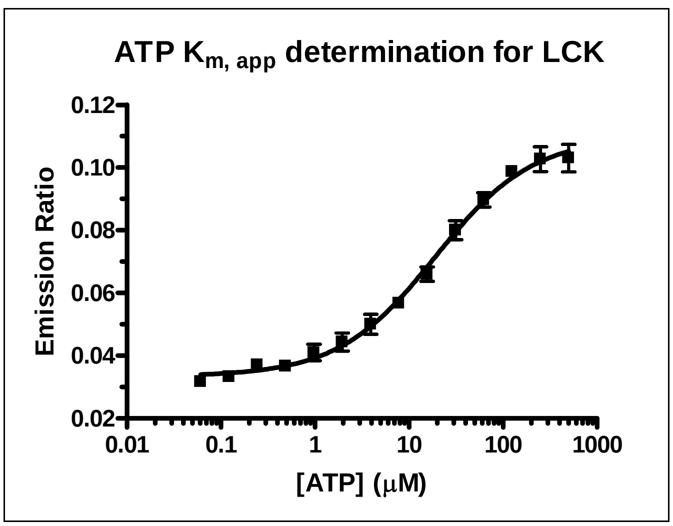
Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer.

(2.7) Add 10 µL of the Eu-antibody + EDTA solution prepared in step 2.6 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 incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

(2.9) 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_{50} concentration from the curve. This is the ATP $K_{m,app}$ for your kinase under these assay conditions.

Figure 2: Example of ATP $K_{m,app}$ Determination Curve



The EC_{50} value determined from the example data was 19 µM ATP. Based on this result, 19 µM ATP was used for the following step of this protocol.

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

Step 3: Titration of Kinase at ATP $K_{m,app}$.

(3.1) In an appropriate tube or vial, prepare 100 µL of kinase in 1x kinase reaction buffer at 2 times the highest concentration of kinase to be tested. In this example, 10 µg/mL was the highest concentration of kinase to be tested, and the stock concentration of kinase was 410 µg/mL.

Calculations:

Kinase: Stock = 410 µg/mL 1x = 10 µg/mL 2x = 20 µg/mL

			[Initial]				[Final 2x]
Kinase:	4.9 µL	*	410 µg/mL	=	100 µL	*	20 µg/mL
Buffer:	95.1 µL kinase reaction buffer						

Procedure:

Add 4.9 µL of 410 µg/mL kinase to 95.1 µL kinase reaction buffer.

Keep the diluted kinase on ice until needed.

(3.2) 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.

(3.3) 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 = 30 µM 1x = 0.1 µM 2x = 0.2 µM

ATP: Stock = 10 mM 1x = 0.019 mM 2x = 0.038 mM

			[Initial]				[Final 2x]
Substrate:	6.7 µL	*	30 µM	=	1000 µL	*	0.2 µM
ATP:	3.8 µL	*	10 mM	=	1000 µL	*	0.038 mM
Buffer:	989.5 µL kinase reaction buffer						

Procedure:

Add 6.7 µL of 30 µM substrate and 3.8 µL of 10 mM ATP to 989.5 µL kinase reaction buffer.

(3.4) Start the kinase reaction by adding 5 µL of the substrate + ATP solution prepared in step 3.3 to each well of the assay plate.

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

(3.6) Prior to completion of the kinase reaction, prepare 1 mL of a solution of EDTA and Eu-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 = 3300 nM	1x = 2 nM	2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	40 µL	*	500 mM	=	1000 µL	*	20 mM
Antibody:	1.2 µL	*	3300 nM	=	1000 µL	*	4 nM
Buffer:	958.8 µL TR-FRET Dilution Buffer						

Procedure:

Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer.

(3.7) Add 10µL of the Eu-antibody + EDTA solution prepared in step 3.6 to each well of the assay plate and mix briefly, either by pipette or on a plate shaker.

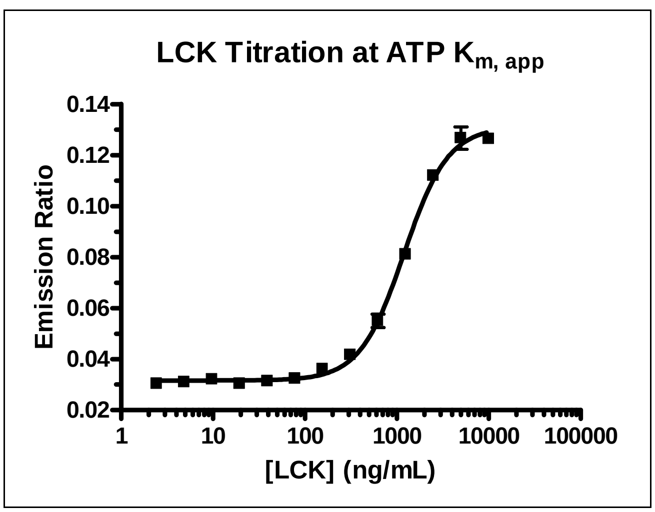
(3.8) Cover the assay plate and incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

(3.9) 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 3: Example of Kinase Titration at ATP $K_{m,app}$



The EC_{80} value determined from the example data was 2.6 µg/mL kinase. Based on this result, 2.6 µg/mL kinase was used to determine inhibitor IC_{50} values when performing the assay at 19 µM ATP.

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

Step 4: Determination of Inhibitor IC_{50} Value.

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

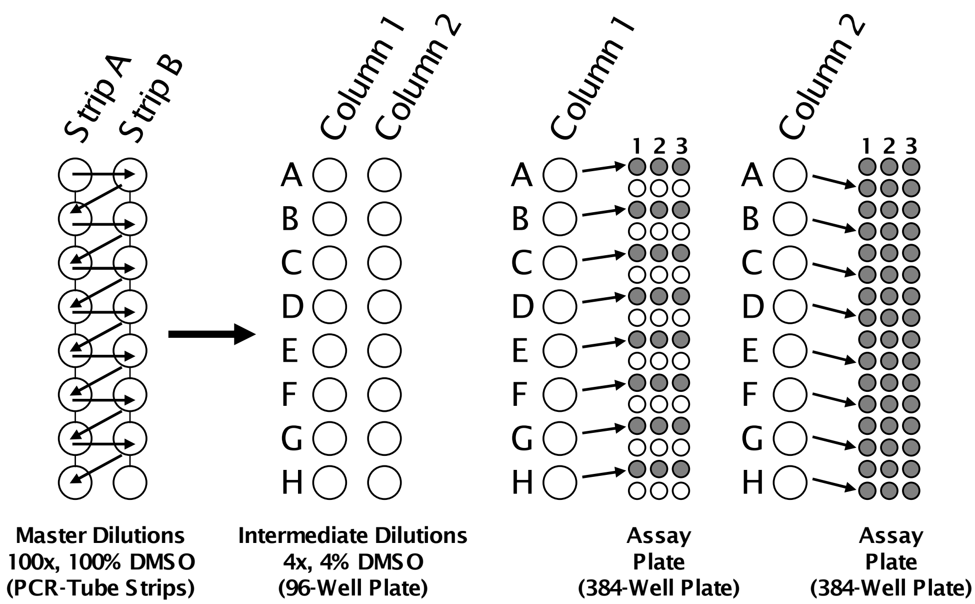
1. Add 2.5 µL of inhibitor in 4% DMSO at 4-fold the final assay concentration to triplicate assay wells.
2. Add 5 µL of 2-fold kinase + substrate, followed by 2.5 µL of ATP at 4-fold the final reaction concentrations to start the reaction.
3. The remainder of the protocol is similar to previous steps.

(4.2) 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



(4.3) 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.

(4.4) In an appropriate tube or vial, prepare 1000 µL of kinase + substrate in kinase reaction buffer at 2 times the highest concentration to be tested.

Calculation:

Kinase:	Initial conc. = 410 µg/mL	1x = 2.6 µg/mL	2x = 5.2 µg/mL
Substrate:	Stock = 30 µM	1x = 0.1 µM	2x = 0.2 µM

			[Initial]				[Final 2x]
Kinase:	12.7 µL	*	410 µg/mL	=	1000 µL	*	5.2 µg/mL
Substrate:	6.7 µL	*	30 µM	=	1000 µL	*	0.2 µM
Buffer:	980.6 µL kinase reaction buffer						

Procedure:

Add 12.7 µL of 410 µg/mL kinase and 6.7 µL of 30 µM substrate to 980.6 µL kinase reaction buffer

(4.5) Add 5.0 µL of the kinase + substrate solution prepared in step 4.4 to each well of the assay plate.

(4.6) 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 = 0.019 mM 4x = 0.076 mM

		[Initial]		[Final 4x]
ATP:	7.6 µL	10 mM	1000 µL	0.076 mM
Buffer:	992.4 µL kinase reaction buffer			

Procedure:

Add 7.6 µL of 10 mM ATP to 992.4 µL kinase reaction buffer.

(4.7) Start the kinase reaction by adding 2.5 µL of the ATP solution prepared in step 4.6 to each well of the assay plate .

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

(4.9) Prior to completion of the assay, prepare 1 mL of a solution of EDTA and Eu-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 = 3300 nM 1x = 2 nM 2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	40 µL	*	500 mM	=	1000 µL	*	20 mM
Antibody:	1.2 µL	*	3300 nM	=	1000 µL	*	4 nM
Buffer:	958.8 µL TR-FRET Dilution Buffer						

Procedure:

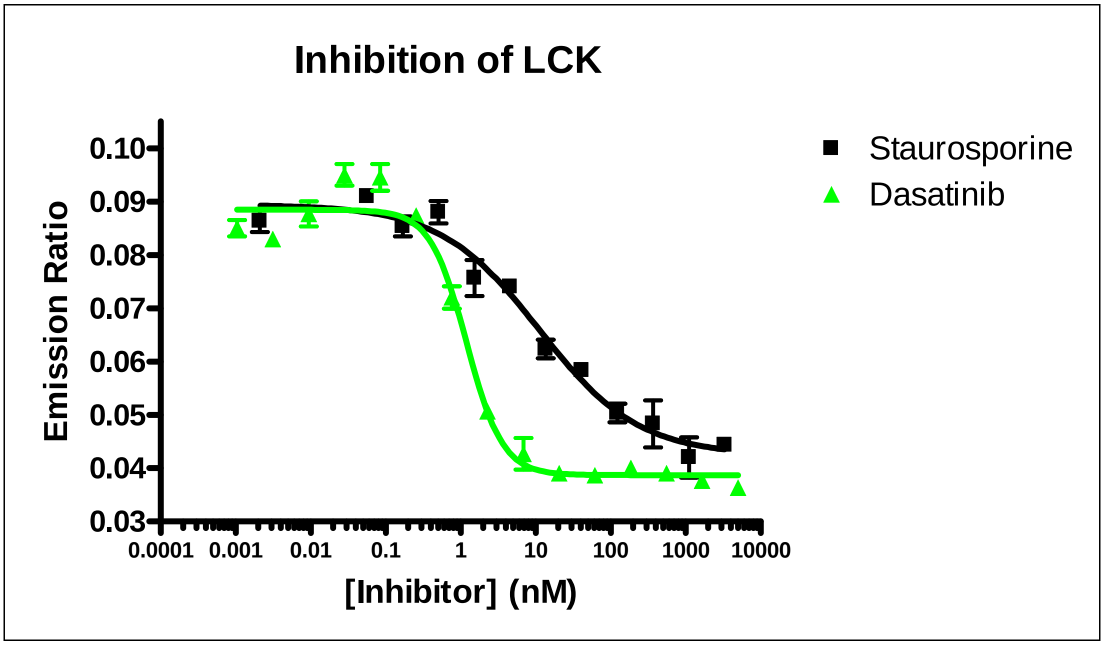
Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer.

(4.10) Add 10 µL of the Eu-antibody + EDTA solution prepared in step 4.9 to each well of the assay plate.

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

(4.12) 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 5: Example of an IC_{50} Calculation Curve



The inhibition of LCK with the inhibitors Staurosporine and Dasatinib are displayed above.

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

		IC_{50} (nM)	
Kinase	**Inhibitor**	**Literature**	**Observed**
LCK	Staurosporine	30[1]	11.2
	Dasatinib	0.2[1]	1.2

[1] Karaman MW. Et al **(2008)** *Nature Biotech.* **26**:127-132.

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.