

# Optimization of a LanthaScreen™ Kinase assay for JAK2 JH1 JH2 V617F

## 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 50% change between the minimum and maximum TR-FRET emission ratios at the ATP  $K_{m,app}$  concentration of ATP (the  $EC_{50}$  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

<u>Description</u>	<u>Part Name</u>	<u>Catalog #</u>	<u>Notes</u>
Kinase Reaction Buffer	5X Kinase Buffer	PV3189 (4 mL of 5X)	(1)
	Additives: <i>None</i>	<i>n.a.</i>	
Kinase Antibody	JAK2 JH1 JH2 V617F LanthaScreen™ Tb-anti-pSTAT1 (pTyr701)	PV4336 (10 µg) PV4844 (25 µg) PV4845 (1 mg)	(2)
Substrate	GFP-STAT1	PV5211 (10 nmol)	(3)
Antibody Dilution Buffer	TR-FRET Dilution Buffer	PV3574 (100 mL)	(4)
500 mM EDTA	Kinase Quench Buffer	P2825 (1 mL)	
10 mM ATP	10 mM ATP	PV3227 (500 µL)	
Inhibitors	Staurosporine	PHZ1271 (100 µg)	
	JAK inhibitor I (Pyridone 6)		(5)
	JAK3 inhibitor VI		(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 STAT1 (pTyr701) 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. The antibody concentration may vary slightly from lot-to-lot. Perform all calculations based on the actual stock concentration of antibody.
- (3) The substrate is supplied at a concentration of ~5.0 µM; see the tube for the exact concentration.
- (4) The antibody dilution buffer does not contain EDTA. EDTA is added separately, prior to addition of antibody.
- (5) JAK inhibitor I is available from Calbiochem (catalog # 420099). JAK inhibitor I references: Pedranzini, L., et al. 2006. *Cancer Res.* **66**, 9714. Lucet, I.S., et al. 2005. *Blood* **107**, 176. Thompson, J.E., et al. 2002. *Bioorg. Med. Chem. Lett.* **12**, 1219.
- (6) JAK3 inhibitor VI is available from Calbiochem (catalog # 420126). JAK3 inhibitor VI references: Chen, J., et al. 2006. *Bioorg. Med. Chem. Lett.* **16**, 5633. Adams, C., et al. 2003. *Bioorg. Med. Chem. Lett.* **13**, 3105.

## 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<sub>2</sub>O 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<sub>2</sub>, 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 Tecan Infinite F500 plate reader using the LanthaScreen™ filters. The assay can be performed on a variety of plate readers including those from Tecan (Ultra, Safire<sup>2</sup>), Molecular Devices (Analyst and M5), BMG LABTECH PHERAstar and Perkin Elmer (EnVision, Victor, and ViewLux). Visit [www.invitrogen.com/Lanthascreen](http://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](mailto: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 1 mM ATP

- (1.1) In an appropriate tube or vial, prepare 100  $\mu\text{L}$  of kinase in 1x kinase reaction buffer at 2 times the highest concentration of kinase to be tested. In this example, 10  $\mu\text{g/mL}$  was the highest concentration of kinase to be tested, and the stock concentration of kinase was 310  $\mu\text{g/mL}$ .

### Calculations:

Kinase: Stock = 310  $\mu\text{g/mL}$       1x = 10  $\mu\text{g/mL}$       2x = 20  $\mu\text{g/mL}$

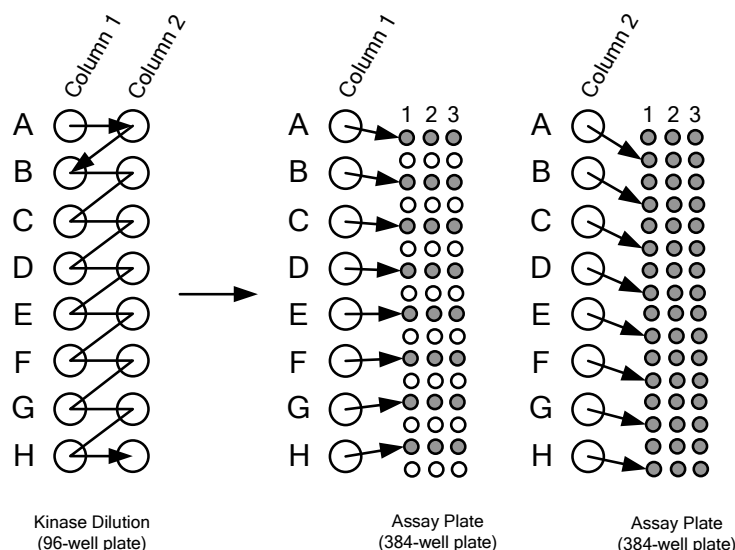
	[Initial]			[Final 2x]		
Kinase:	6.4 $\mu\text{L}$	*	310 $\mu\text{g/mL}$	=	100 $\mu\text{L}$	* 20 $\mu\text{g/mL}$
Buffer:	93.6 $\mu\text{L}$ kinase reaction buffer					

### Procedure:

Add 6.4  $\mu\text{L}$  of 310  $\mu\text{g/mL}$  kinase to 93.6  $\mu\text{L}$  kinase reaction buffer.

Keep the diluted kinase on ice until needed.

- (1.2) A dilution series of kinase is prepared in a 96-well plate and then transferred to the 384-well plate to be assayed. The dilutions are “staggered” between columns as shown in the left side of figure below. To prepare the kinase dilution series:
1. Add 50  $\mu\text{L}$  of kinase assay buffer to the wells in row B–H of column 1, and all wells of column 2.
  2. Add 100  $\mu\text{L}$  of kinase prepared in step 1.1 to well A1.
  3. Transfer 50  $\mu\text{L}$  of kinase solution from well A of column 1 to well A of column 2.
  4. After mixing, transfer 50  $\mu\text{L}$  from well A of column 2 to well B of column 1.
  5. This process is repeated for all wells (see left side of figure below).
  6. Using an multi-channel pipette, add 5  $\mu\text{L}$  of kinase solution from the 96-well plate to the 384-well assay plate as shown in figure below. Use column 1 of the 96-well plate 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.



- (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 = 5.0 $\mu$ M	1x = 0.1 $\mu$ M	2x = 0.2 $\mu$ M
ATP:	Stock = 10 mM	1x = 1 mM	2x = 2 mM

			[Initial]			[Final 2x]
Substrate:	40 $\mu$ L	*	5.0 $\mu$ M	=	1000 $\mu$ L	* 0.2 $\mu$ M
ATP:	200 $\mu$ L	*	10 mM	=	1000 $\mu$ L	* 2 mM
Buffer:	760 $\mu$ L kinase reaction buffer					

**Procedure:**

Add 40  $\mu$ L of 5.0  $\mu$ M substrate and 200  $\mu$ L of 10 mM ATP to 760  $\mu$ L kinase reaction buffer.

- (1.4) Start the kinase reaction by adding 5  $\mu$ L 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 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 = 3300 nM	1x = 2 nM	2x = 4 nM

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

**Procedure:**

Add 40  $\mu$ L of 500 mM EDTA and 1.2  $\mu$ L of 3300 nM antibody to 958.8  $\mu$ L TR-FRET Dilution Buffer.

- (1.7) Add 10  $\mu$ L 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 kinase, and fit the data to a sigmoidal dose-response curve with a variable slope. Calculate the EC<sub>80</sub> concentration from the curve. The following equation can be used with GraphPad<sup>TM</sup> Prism<sup>®</sup> software:

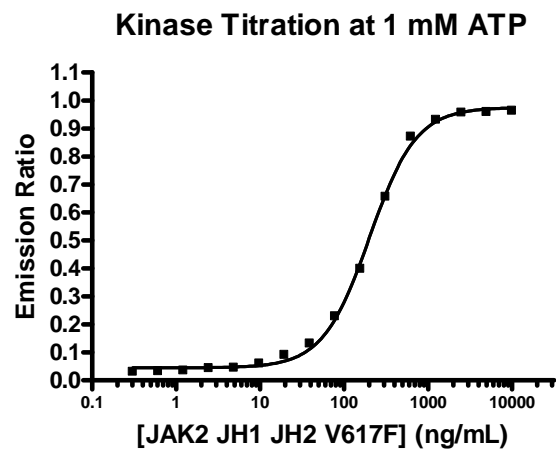
$$F=80$$

$$\log EC_{50} = \log ECF - (1/\text{HillSlope}) * \log(F/(100-F))$$

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\log EC_{50} - X) * \text{HillSlope})})$$

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 in the next step of this protocol be performed at or below the EC<sub>80</sub> concentration of kinase.

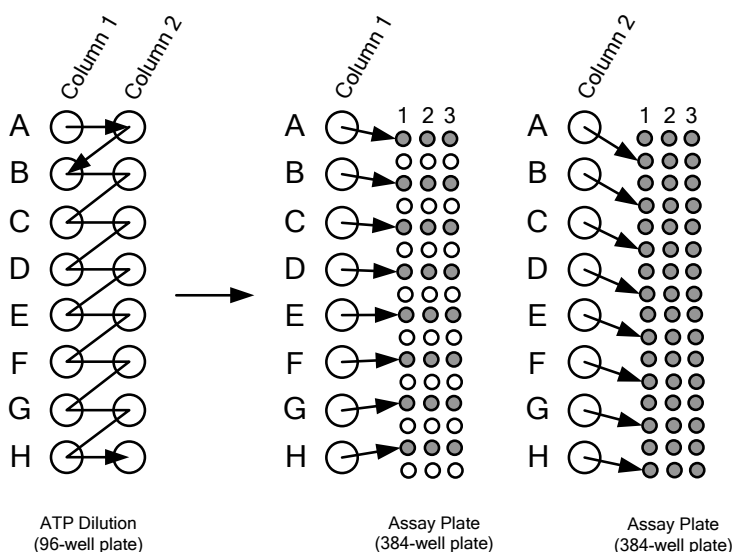
Figure 1: Example of Kinase Titration at 1 mM ATP



The  $EC_{80}$  value determined from the example data was 488 ng/mL kinase. Based on this result, 488 ng/mL kinase was used for the following step of this protocol.

## Step 2: Titration of ATP at the Initial EC<sub>80</sub> Concentration of Kinase to determine ATP K<sub>m,app</sub>

- (2.1) In a small tube or vial, prepare 100  $\mu\text{L}$  of ATP in 1x kinase reaction buffer at 2 times the highest concentration of ATP to be tested. In this example, 1 mM was the highest concentration of ATP to be tested. Therefore, prepare a 2 mM stock of ATP by adding 20  $\mu\text{L}$  of 10 mM ATP to 80  $\mu\text{L}$  of kinase reaction buffer.
- (2.2) A dilution series of ATP is prepared in a 96-well plate and then transferred to the 384-well plate to be assayed. The dilutions are “staggered” between columns as shown in the left side of figure below. To prepare the ATP dilution series:
1. Add 50  $\mu\text{L}$  of kinase assay buffer to the wells in row B–H of column 1, and all wells of column 2.
  2. Add 100  $\mu\text{L}$  of 2 mM ATP prepared in step 2.1 to well A1.
  3. Transfer 50  $\mu\text{L}$  of ATP solution from well A of column 1 to well A of column 2.
  4. After mixing, transfer 50  $\mu\text{L}$  from well A of column 2 to well B of column 1.
  5. This process is repeated for all wells (see left side of figure below).
  6. Using an multi-channel pipette, add 5  $\mu\text{L}$  of ATP solution from the 96-well plate to the 384-well assay plate as shown in figure below. Use column 1 of the 96-well plate 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.



- (2.3) 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  $\mu\text{L}$  solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed using a multichannel pipette.

### Calculations:

Substrate: Stock = 5.0  $\mu\text{M}$       1x = 0.1  $\mu\text{M}$       2x = 0.2  $\mu\text{M}$   
 Kinase: Initial conc. = 310  $\mu\text{g/mL}$       1x = 488 ng/mL      2x = 976 ng/mL

			[Initial]			[Final 2x]
Substrate:	40 $\mu\text{L}$	*	5.0 $\mu\text{M}$	=	1000 $\mu\text{L}$	* 0.2 $\mu\text{M}$
Kinase:	3.15 $\mu\text{L}$	*	310 $\mu\text{g/mL}$	=	1000 $\mu\text{L}$	* 0.976 $\mu\text{g/mL}$
Buffer:	956.9 $\mu\text{L}$ kinase reaction buffer					

**Procedure:**

Add 40  $\mu\text{L}$  of 5.0  $\mu\text{M}$  substrate and 3.15  $\mu\text{L}$  of 310  $\mu\text{g/mL}$  kinase to 956.9  $\mu\text{L}$  kinase reaction buffer.

- (2.4) Start the kinase reaction by adding 5  $\mu\text{L}$  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 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 = 3300 nM            1x = 2 nM            2x = 4 nM

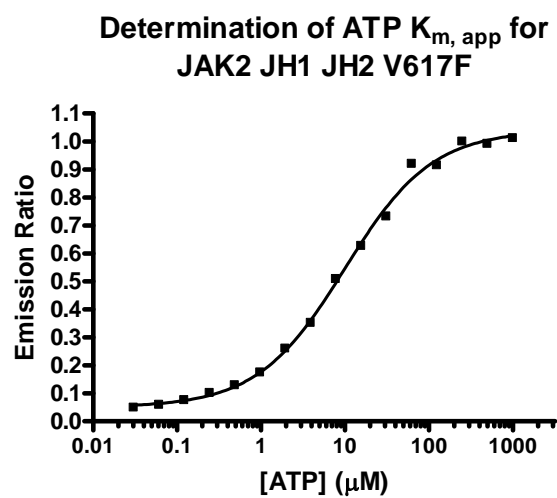
			<u>[Initial]</u>			<u>[Final 2x]</u>
EDTA:	40 $\mu\text{L}$	*	500 mM	=	1000 $\mu\text{L}$	* 20 mM
Antibody:	1.2 $\mu\text{L}$	*	3300 nM	=	1000 $\mu\text{L}$	* 4 nM
Buffer:	958.8 $\mu\text{L}$ TR-FRET Dilution Buffer					

**Procedure:**

Add 40  $\mu\text{L}$  of 500 mM EDTA and 1.2  $\mu\text{L}$  of 3300 nM antibody to 958.8  $\mu\text{L}$  TR-FRET Dilution Buffer.

- (2.7) Add 10  $\mu\text{L}$  of the Tb-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  $\text{EC}_{50}$  concentration from the curve. This is the ATP  $K_{m,\text{app}}$  for your kinase under these assay conditions.

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



The EC<sub>50</sub> value determined from the example data was 9.9 μM ATP. Based on this result, 9.9 μM ATP was used for the following step of this protocol.



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

- (3.1) In an appropriate tube or vial, prepare 100  $\mu\text{L}$  of kinase in 1x kinase reaction buffer at 2 times the highest concentration of kinase to be tested. In this example, 20  $\mu\text{g/mL}$  was the highest concentration of kinase to be tested, and the stock concentration of kinase was 310  $\mu\text{g/mL}$ .

#### Calculations:

Kinase: Stock = 310  $\mu\text{g/mL}$       1x = 20  $\mu\text{g/mL}$       2x = 40  $\mu\text{g/mL}$

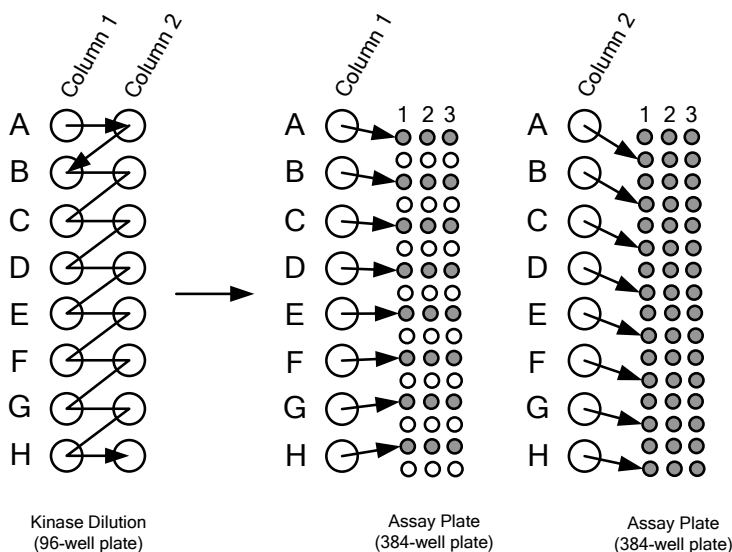
	[Initial]				[Final 2x]			
Kinase:	12.9 $\mu\text{L}$	*	310 $\mu\text{g/mL}$	=	100 $\mu\text{L}$	*	40 $\mu\text{g/mL}$	
Buffer:	87.1 $\mu\text{L}$ kinase reaction buffer							

#### Procedure:

Add 12.9  $\mu\text{L}$  of 310  $\mu\text{g/mL}$  kinase to 87.1  $\mu\text{L}$  kinase reaction buffer.

Keep the diluted kinase on ice until needed.

- (3.2) A dilution series of kinase is prepared in a 96-well plate and then transferred to the 384-well plate to be assayed. The dilutions are “staggered” between columns as shown in the left side of figure below. To prepare the kinase dilution series:
1. Add 50  $\mu\text{L}$  of kinase assay buffer to the wells in row B–H of column 1, and all wells of column 2.
  2. Add 100  $\mu\text{L}$  of kinase prepared in step 3.1 to well A1.
  3. Transfer 50  $\mu\text{L}$  of kinase solution from well A of column 1 to well A of column 2.
  4. After mixing, transfer 50  $\mu\text{L}$  from well A of column 2 to well B of column 1.
  5. This process is repeated for all wells (see left side of figure below).
  6. Using an multi-channel pipette, add 5  $\mu\text{L}$  of kinase solution from the 96-well plate to the 384-well assay plate as shown in figure below. Use column 1 of the 96-well plate 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.



- (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.

Since you will be assaying your kinase at  $< 100 \mu\text{M}$  ATP, first prepare  $200 \mu\text{L}$  of an intermediate dilution of ATP consisting of  $1 \text{ mM}$  of ATP in kinase reaction buffer by adding  $20 \mu\text{L}$  of  $10 \text{ mM}$  ATP to  $180 \mu\text{L}$  of kinase reaction buffer, and use this intermediate dilution of ATP to prepare the solution.

If a  $1000 \mu\text{L}$  solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed with a multichannel pipette.

#### Calculations:

Substrate: Stock =  $5.0 \mu\text{M}$        $1x = 0.1 \mu\text{M}$        $2x = 0.2 \mu\text{M}$   
 ATP:      Stock =  $1000 \mu\text{M}$        $1x = 9.9 \mu\text{M}$        $2x = 19.8 \mu\text{M}$

			[Initial]			[Final 2x]
Substrate:	$40 \mu\text{L}$	*	$5.0 \mu\text{M}$	=	$1000 \mu\text{L}$	* $0.2 \mu\text{M}$
ATP:	$19.8 \mu\text{L}$	*	$1000 \mu\text{M}$	=	$1000 \mu\text{L}$	* $19.8 \mu\text{M}$
Buffer:	$940.2 \mu\text{L}$ kinase reaction buffer					

#### Procedure:

Add  $40 \mu\text{L}$  of  $5.0 \mu\text{M}$  substrate and  $19.8 \mu\text{L}$  of  $1000 \mu\text{M}$  ATP to  $940.2 \mu\text{L}$  kinase reaction buffer.

- (3.4) Start the kinase reaction by adding  $5 \mu\text{L}$  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  $1 \text{ mL}$  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 =  $500 \text{ mM}$        $1x = 10 \text{ mM}$        $2x = 20 \text{ mM}$   
 Antibody:      Stock =  $3300 \text{ nM}$        $1x = 2 \text{ nM}$        $2x = 4 \text{ nM}$

			[Initial]			[Final 2x]
EDTA:	$40 \mu\text{L}$	*	$500 \text{ mM}$	=	$1000 \mu\text{L}$	* $20 \text{ mM}$
Antibody:	$1.2 \mu\text{L}$	*	$3300 \text{ nM}$	=	$1000 \mu\text{L}$	* $4 \text{ nM}$
Buffer:	$958.8 \mu\text{L}$ TR-FRET Dilution Buffer					

#### Procedure:

Add  $40 \mu\text{L}$  of  $500 \text{ mM}$  EDTA and  $1.2 \mu\text{L}$  of  $3300 \text{ nM}$  antibody to  $958.8 \mu\text{L}$  TR-FRET Dilution Buffer.

- (3.7) Add  $10 \mu\text{L}$  of the Tb-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  $\text{EC}_{50}$  concentration from the curve. The following equation can be used with GraphPad<sup>TM</sup> Prism<sup>®</sup> software:

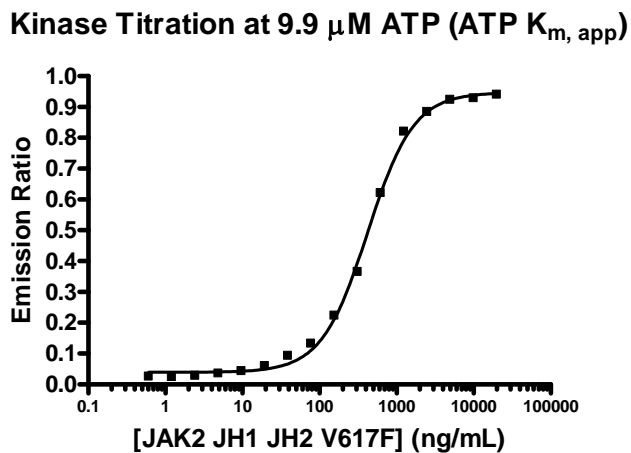
$$F=50$$

$$\log\text{EC}_{50} = \log\text{ECF} - (1/\text{HillSlope}) * \log(F/(100-F))$$

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\log\text{EC}_{50} - X) * \text{HillSlope})})$$

Alternatively, the amount of kinase needed to elicit a 50% 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  $EC_{50}$  concentration of the kinase determined from this graph.

**Figure 3: Example of Kinase Titration at ATP  $K_{m,app}$**



The  $EC_{50}$  value determined from the example data was 421 ng/mL kinase. Based on this result, 421 ng/mL kinase was used to determine inhibitor  $IC_{50}$  values when performing the assay at 9.9  $\mu$ M ATP.

## Step 4: Determination of Inhibitor IC<sub>50</sub> Value.

(4.1) The general procedure for determining an inhibitor IC<sub>50</sub> value is as follows:

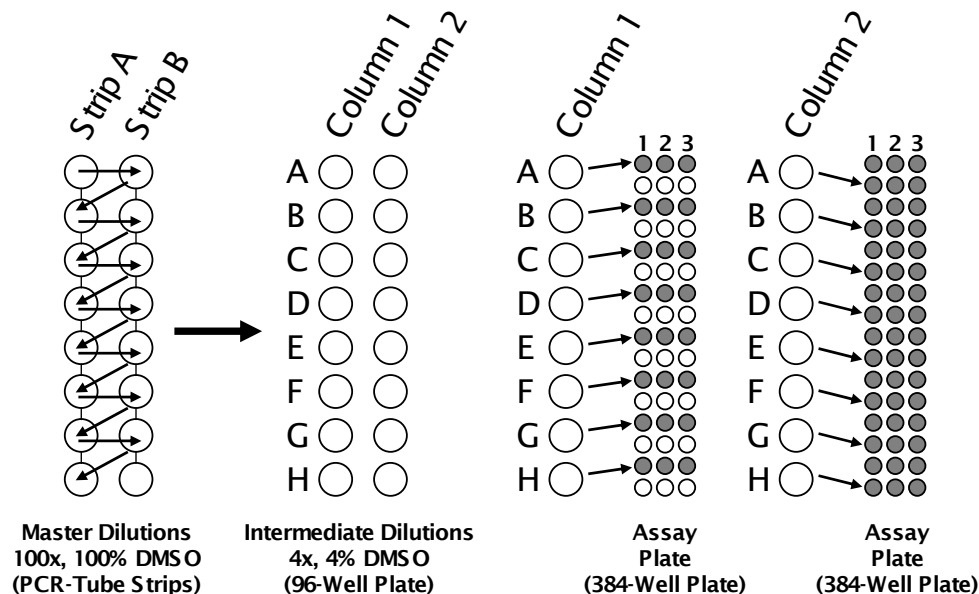
1. Add 2.5  $\mu$ L of inhibitor in 4% DMSO at 4-fold the final assay concentration to triplicate assay wells.
2. To start the reaction, add 5  $\mu$ L of kinase + substrate at 2-fold the final assay concentrations, followed by 2.5  $\mu$ L of ATP at 4-fold the final reaction concentration.
3. The remainder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

(4.2) A 3-fold 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°C for use in future experiments. The dilutions are “staggered” between strips as shown in the left side of Figure 4:

1. Add 50  $\mu$ L of DMSO to tubes 2–8 of strip A, and all tubes of strip B.
2. Add 75  $\mu$ L of inhibitor in DMSO, at 100-fold the highest concentration to be tested in the experiment, to tube 1 of strip A.
3. Transfer 25  $\mu$ L of inhibitor from tube 1 of strip A to tube 1 of strip B.
4. After mixing, transfer 25  $\mu$ L 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  $\mu$ L of kinase reaction buffer into all wells of two columns of a 96-well plate.
2. Then, transfer 4  $\mu$ L of the master inhibitor stock from strip A into column 1 of the 96 well plate, and 4  $\mu$ L 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  $\mu$ L multichannel pipette.

4. Using an 8-channel pipette, add 2.5  $\mu\text{L}$  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) Prepare a 1 mL solution of kinase and substrate in kinase reaction buffer at 2x the final desired reaction concentration of each reagent. From Step 3, 421 ng/mL kinase was determined to be the concentration required for the assay.

**Calculation:**

Substrate:	Stock = 5.0 $\mu\text{M}$	1x = 0.1 $\mu\text{M}$	2x = 0.2 $\mu\text{M}$
Kinase:	Initial conc. = 310 $\mu\text{g/mL}$	1x = 421 ng/mL	2x = 842 ng/mL

			<u>[Initial]</u>			<u>[Final 2x]</u>
Kinase:	2.7 $\mu\text{L}$	*	310 $\mu\text{g/mL}$	=	1000 $\mu\text{L}$	* 0.842 $\mu\text{g/mL}$
Substrate:	40 $\mu\text{L}$	*	5.0 $\mu\text{M}$	=	1000 $\mu\text{L}$	* 0.2 $\mu\text{M}$
Buffer:	957.3 $\mu\text{L}$ kinase reaction buffer					

**Procedure:**

Add 40  $\mu\text{L}$  of 5.0  $\mu\text{M}$  substrate and 2.7  $\mu\text{L}$  of 310  $\mu\text{g/mL}$  kinase to 957.3  $\mu\text{L}$  kinase reaction buffer.

- (4.5) Add 5  $\mu\text{L}$  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 ATP in kinase reaction buffer at 4 times the final desired concentration in the assay.

Since you will be assaying your kinase at < 100  $\mu\text{M}$  ATP, first prepare 200  $\mu\text{L}$  of an intermediate dilution of ATP consisting of 1 mM of ATP in kinase reaction buffer by adding 20  $\mu\text{L}$  of 10 mM ATP to 180  $\mu\text{L}$  of kinase reaction buffer, and use this intermediate dilution of ATP to prepare the solution.

If a 1000  $\mu\text{L}$  solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed using a multichannel pipette.

**Calculations:**

ATP:	Stock = 1000 $\mu\text{M}$	1x = 9.9 $\mu\text{M}$	4x = 39.6 $\mu\text{M}$
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			<u>[Initial]</u>			<u>[Final 4x]</u>
ATP:	39.6 $\mu\text{L}$	*	1000 $\mu\text{M}$	=	1000 $\mu\text{L}$	* 39.6 $\mu\text{M}$
Buffer:	960.4 $\mu\text{L}$ kinase reaction buffer					

**Procedure:**

Add 39.6  $\mu\text{L}$  of 1000  $\mu\text{M}$  ATP to 960.4  $\mu\text{L}$  kinase reaction buffer.

- (4.7) Start the kinase reaction by adding 2.5  $\mu\text{L}$  of the 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.
- (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 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 = 3300 nM	1x = 2 nM	2x = 4 nM

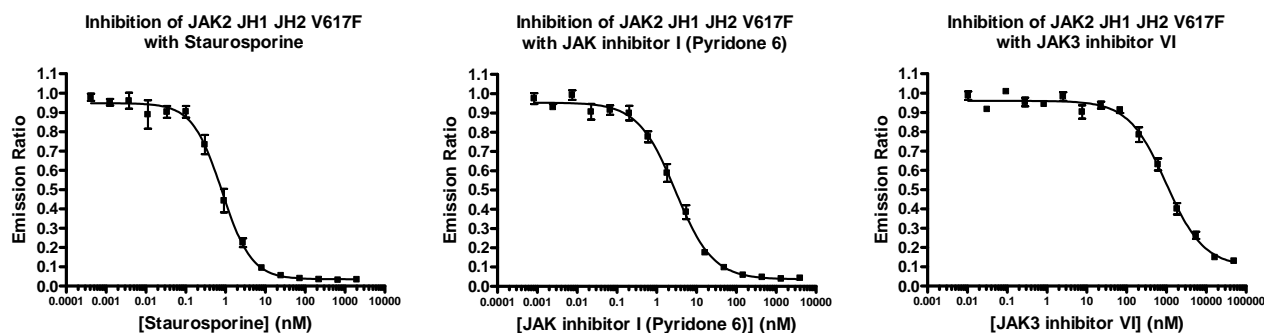
			[Initial]			[Final 2x]
EDTA:	40 $\mu$ L	*	500 mM	=	1000 $\mu$ L	* 20 mM
Antibody:	1.2 $\mu$ L	*	3300 nM	=	1000 $\mu$ L	* 4 nM
Buffer:	958.8 $\mu$ L TR-FRET Dilution Buffer					

### Procedure:

Add 40  $\mu$ L of 500 mM EDTA and 1.2  $\mu$ L of 3300 nM antibody to 958.8  $\mu$ L TR-FRET Dilution Buffer.

- (4.10) Add 10  $\mu$ L of the Tb-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<sub>50</sub> concentration from the curve. This is equal to the IC<sub>50</sub> value for the inhibitor.

Figure 5: Example IC<sub>50</sub> Curves



The inhibition of JAK2 JH1 JH2 V617F with the following inhibitors: staurosporine, JAK inhibitor I (pyridone 6), and JAK3 inhibitor VI. The IC<sub>50</sub> values for inhibition of JAK2 JH1 JH2 V617F with the above inhibitors are shown in the table below.

Kinase	Inhibitor	IC <sub>50</sub> (nM)	
		Literature	LanthaScreen™ Assay
JAK2 JH1 JH2 V617F	Staurosporine	NR	0.8
	Pyridone 6	1*	2.9
	JAK3 Inhibitor VI	>430**	991.4

NR = No value reported

\*JAK inhibitor I references: Pedranzini, L., et al. 2006. *Cancer Res.* **66**, 9714. Lucet, I.S., et al. 2005. *Blood* **107**, 176. Thompson, J.E., et al. 2002. *Bioorg. Med. Chem. Lett.* **12**, 1219.

\*\*JAK3 inhibitor VI references: Chen, J., et al. 2006. *Bioorg. Med. Chem. Lett.* **16**, 5633. Adams, C., et al. 2003. *Bioorg. Med. Chem. Lett.* **13**, 3105.

## Results

This kinase and a set of related kinases were assayed against a common set of inhibitors using GFP-STAT1 as the substrate and the results are tabulated below:

<u>Inhibitor</u>	<u>Kinase</u>							IC <sub>50</sub> (nM)
	<u>JAK1</u>	<u>JAK2</u>	<u>JAK2 JH1 JH2</u>	<u>JAK2 JH1 JH2 V617F</u>	<u>JAK3</u>	<u>TYK2</u>		
staurosporine	2.1	0.8	0.9	0.8	1.9	0.8		
JAK inhibitor I (pyridone 6)*	3.3	2.5	2.2	2.9	10.9	3.6		
JAK3 inhibitor VI**	1025	721.5	720.7	991.4	50.7	1628		

\* Literature reference IC<sub>50</sub> values: JAK1 (15 nM), JAK2 (1 nM), JAK3 (5 nM), TYK2 (1 nM)

\*\* Literature reference IC<sub>50</sub> values: JAK3 (27 nM), ~ 16 fold greater selectivity over JAK2