LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

Overview

This protocol describes how to perform a LanthaScreen™ Eu Kinase Bindng Assay designed to detect and characterize kinase inhibitors. Procedure 1 describes an experiment to optimize the concentration of tracer to use with a specific kinase target. Procedure 2 describes how to perform kinase inhibitor affinity (IC_{50}) measurements, using either the concentration of tracer determined by the user following Procedure 1 or using the concentration determined experimentally by Invitrogen. The protocol is accompanied by representative data generated at Invitrogen for both procedures.

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 in order to address a wide range of kinase targets. 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 both 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.

Because the kinase tracers are based on ATP-competitive kinase inhibitors, they are suitable for detection of any compounds that bind to the ATP site. This includes "Type II" inhibitors (e.g. Gleevec®/imatinib, sorafenib, BIRB-796), which bind to both the ATP site and a second site often referred to as the "allosteric" site.

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, the assay can be performed using either active or non-activated kinase preparations, which enables characterization of compounds that bind preferentially to non-activated kinases (e.g. Gleevec®/imatinib).
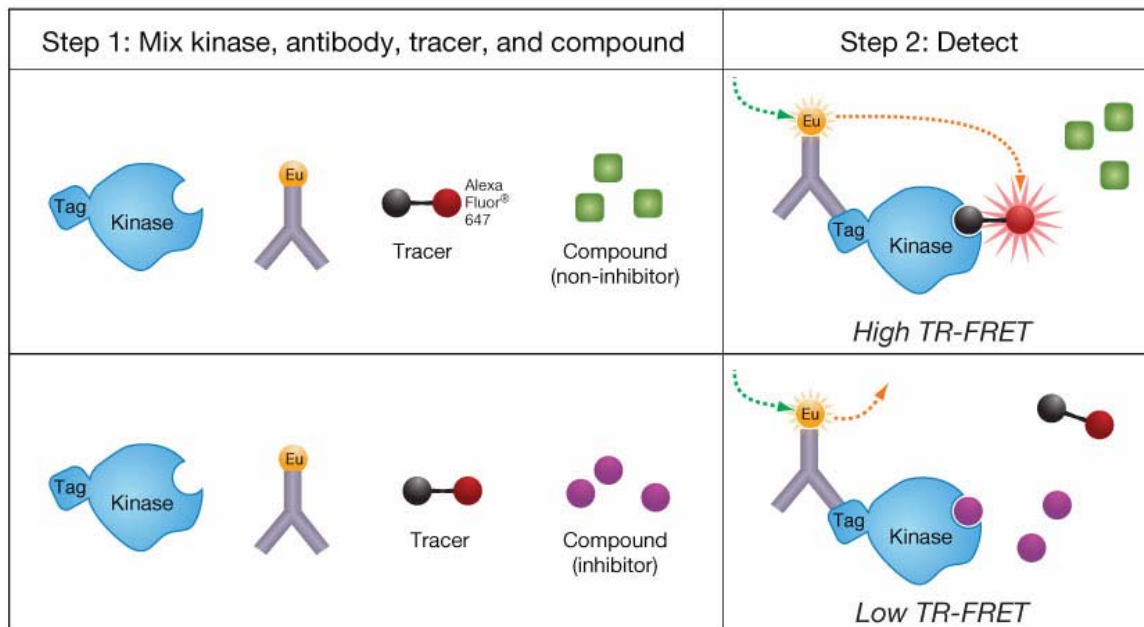

Figure 1. Schematic of LanthaScreen™ Eu Kinase Binding Assay

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200 Select option 3, ext. 40266 Email: drugdiscoverytech@invitrogen.com

Materials required

Product Name	Part Number	Concentration	Quantity	Notes
JAK2 JH1 JH2	PV4393	0.1 to 0.5 mg/mL	10 μg	(1)
5X Kinase Buffer A	PV3189	5X	4 mL	(2)
Kinase Tracer 236	PV5592	50 μM in DMSO	25 μL	(3)
LanthaScreen™ Eu-anti-GST Antibody	PV5594 or PV5595	0.22 to 0.28 mg/mL (1.5 to 1.8 μM)	25 μg or 1 mg	(4)
Staurosporine (optional)	PHZ1271	N/A	100 μg	(5)

(1) JAK2 JH1 JH2 is supplied at a concentration between 0.1 to 0.5 mg/mL, with the exact concentration is printed on the product label. The molecular weight of the kinase is 97.8 kD, which can be found on the kinase Certificate of Analysis shipped with the product or at Invitrogen.com/kinase. The kinase molecular weight will be needed to convert the concentration to molarity as required in the following protocol.

(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 50mM HEPES pH 7.5, 10 mM MgCl2, 1 mM EGTA, 0.01% Brij-35.

(3) Kinase Tracer 236 is supplied as a 50 μM stock in DMSO.

(4) Prior to use, the antibody tube should be centrifuged at approximately 10,000 x g for 5 minutes, and the solution needed for the assay should be aspirated from the top of the solution. This centrifugation step will eliminate spurious data points that can arise on occasion due to any particulates in the product.

(5) A 1 mM stock of staurosporine can be prepared by dissolving 100 μg of staurosporine in 210 μL of DMSO.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

Basic protocol for inhibitor studies

LanthaScreen[TM] Kinase Binding Assays to evaluate inhibitors are typically performed by addition of 3 components each at 3X the final desired concentration as follows:

1. Add 5 µL of **test compound**.
2. Add 5 µL of **kinase/antibody** mixture.
3. Add 5 µL of **tracer.**
4. Incubate for 1 hour at room temperature and read plate.

Final assay conditions for inhibitor studies

5 nM kinase[1]
2 nM Eu-Anti-GST Antibody
10 nM Kinase Tracer 236[2]
1X Kinase Buffer A

[1]A kinase concentration of 5 nM kinase is recommended as a starting point for assay development as it 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. For specific test cases, successful assays have been performed with at little as 200 pM kinase, though the assay window may be lower.
[2]A tracer concentration of 10 nM is suggested by Invitrogen, but could also be experimentally determined or optimized in Procedure 1. Optimal tracer concentrations for all validated kinases typically fall within the 1 to 100 nM range.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

invitrogen™

LanthaScreen™ Eu Kinase Binding
Assay Validation Packet

Version No.:
1

Page 4 of 17

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

Plate readers

The data presented in this document were generated using a Tecan Infinite F-500 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, Safire[2]), Molecular Devices (Analyst and M5), BMG LABTECH (PHERAstar) 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.

Plates

Assays are typically performed in black, low-volume 384-well plates (Corning Part#3676). However, white plates may yield higher quality data (Corning Part#3673) for assays that give a relatively low assay window (1.5 to 2 fold) that are being measured on monochromater-based instruments or older filter-based instruments. For most kinases and most plate readers, plate color has little or no impact.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

Procedure 1. Optimization of tracer concentration

This step describes how to optimize the tracer concentration for use in subsequent inhibitor studies by performing binding assays with a 2-fold serial dilution of tracer. This experiment allows for approximation of the tracer dissociation constant (K_d) and evaluation of the signal strength or "assay window" as a function of tracer concentration. It is typically best to select a tracer concentration near K_d or below K_d to ensure sensitive detection of inhibitors. For example, the measured IC_{50} value from a simple compound titration will approach K_i (dissociation constant of a competitive inhibitor) if [tracer] < tracer K_d and [kinase] << [tracer]. The majority of kinase assays validated by Invitrogen yield a robust signal with the tracer no more than twice the K_d value. In many cases, the K_d value can also be used to calculate K_i from a compound titration experiment using the Cheng-Prusoff equation (see Procedure 2), which compensates for the tracer concentration being above K_d. The other factor to consider when selecting a tracer concentration is the signal strength or "assay window" as it correlates very well with assay robustness (i.e. Z' values). Although in many cases assay windows can exceed 10-fold, excellent Z' values are typically obtained with an assay window as low as 2-fold (See Appendix A). The specific end application may also impact the choice of tracer concentration, based on both the assay window, Z', and K_d value.

The relatively simple method to determine tracer K_d described in Procedure 1 is supported by data from an alternative method to calculate K_d (in addition to K_i) as described in Appendix B.   This alternative method is based on a series of inhibitor titrations performed at different tracer concentrations and the resulting K_d values correlate well with those derived using the more rapid method of Procedure 1.

Estimation of tracer K_d and assay window

(1.1) Reagent preparation

1.   A dilution series of tracer is prepared at 3 times the final concentration to be assayed.

   a.   Dilute tracer to 3000 nM by adding 3.6 µL of 50 µM stock tracer to 56 µL of 1X Kinase Buffer A.

   b.   Add 50 µL of 1X Kinase Buffer A to 6 wells in each of 2 columns of a 96-well plate.

   c.   Add 50 µL of 3000 nM tracer to well A1 and mix.

   d.   Remove 50 µL from well A1, transfer to well A2 and mix.

   e.   Remove 50 µL from well A2, transfer to well B1 and mix.

   f.   Continue process as depicted in Figure 2.
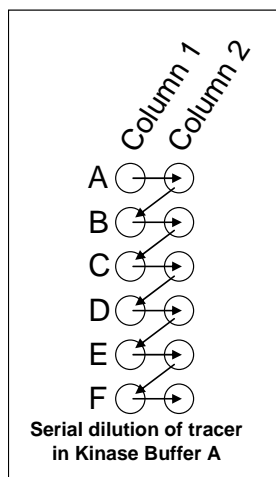

Figure 2. Serial dilution of tracer.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

invitrogen™

LanthaScreen™ Eu Kinase Binding
Assay Validation Packet

Version No.:
1

Page 6 of 17

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

2. Prepare kinase/antibody solution at 15 nM kinase and 6 nM antibody (3X the desired final assay concentration). Centrifuge the antibody tube at approximately 10,000 x g for 10 minutes and aspirate desired volume from the top of the solution. Add the volumes of reagents calculated below to calculated volume of Kinase Buffer A.

Calculations (for a 1000 µL solution):

Stock kinase conc. (nM) = $\dfrac{\text{stock conc. (mg/mL)} * 1{,}000{,}000{,}000 \text{ (nmol/mol)}}{\text{kinase MW (grams/mol)}}$

Kinase volume needed (µL) = $\dfrac{1000 \text{ µL} * 15 \text{ nM}}{\text{Stock kinase conc. (nM)}}$

Antibody volume needed (µL) = $\dfrac{1000 \text{ µL} * 6 \text{ nM}}{\text{Stock antibody conc. (µM)} * 1000 \text{ (nmol/µmol)}}$

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

3. Prepare 30 µM staurosporine ("competitor solution") by diluting 30 µL of 1 mM staurosporine (from a stock in DMSO) into 970 µL Kinase Buffer A.

4. Prepare 3% DMSO control solution by adding 30 µL DMSO to 970 µL Kinase Buffer A.

(1.2) Experimental procedure

1. Add 5 µL of each concentration of serially diluted tracer to six replicate assay wells in a 384-well plate (columns 1-6) as depicted in Figure 3.
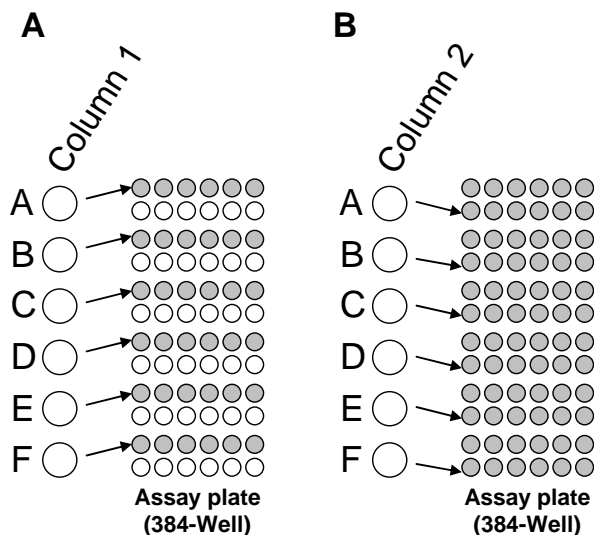

Figure 3. Transfer of tracer dilutions from 96-well to 384-well plate.

2. Add 5 µL of competitor solution to three wells for each tracer concentration (columns 1-3).

3. Add 5 µL of DMSO control solution to the other three wells for each tracer concentration (columns 4-6).

4. Add 5 µL of kinase/antibody solution to all wells in columns 1-6.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

5. Incubate the plate at room temperature for 60 min and read plate.

(1.3)   Data analysis

1. Divide the acceptor/tracer emission (665 nM) by the antibody/donor emission (615 nM) to calculate the "emission ratio".

2. Plot [tracer] versus emission ratio for the competitor (staurosporine) and control (DMSO only) (Figure 4). The sigmoidal dose-response curve with a variable slope can be fit to the data (optional). The following equation can be used with GraphPad™ Prism software:

    F=50

    logEC50=logECF-(1/HillSlope)*log(F/(100-F))

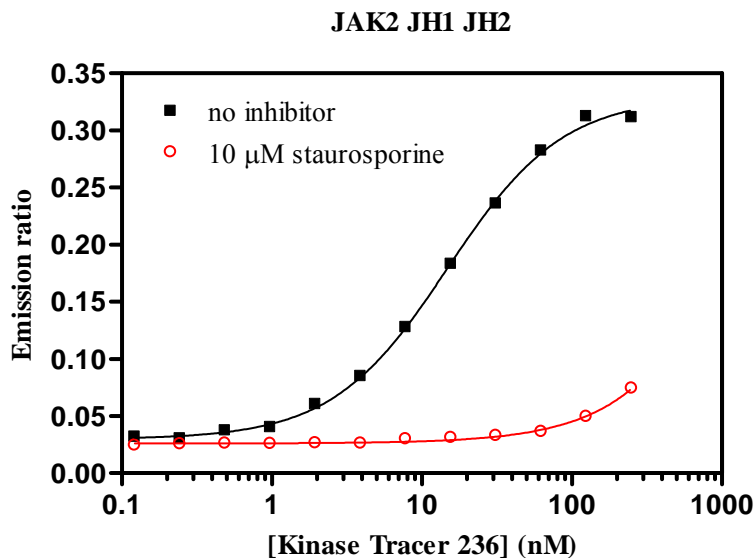   Y=Bottom+(Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

Figure 4.   Tracer titration curve.

3. The assay window for any given tracer concentration can be calculated by dividing the signal in the absence of competitor (+ staurosporine curve) by the signal in the presence of competitor. The assay window is one of two criteria (the other being the tracer K_d) that are typically used to select a tracer concentration for inhibitor studies. Assays windows of ≥ 2 usually result in high Z' values (see Appendix A).

4. Subtract the competitor curve (+ staurosporine) from the control curve (DMSO only) to correct for background signal, which is typically due to diffusion enhanced FRET from Eu to unbound tracer.

5. Plot the background-corrected emission ratios versus [tracer] and fit to the one site binding (hyperbola) equation to estimate the dissociation constant (Figure 5). In some cases data at the highest one or two tracer concentrations are excluded from curve fits due to relatively high "background" signal in presence of competitor. This can be observed as these data points deviate from the one site binding model, whereas the other points align.

    The following equation can be used with GraphPad™ Prism software: Y=Bmax*X/(K_d + X)

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

JAK2 JH1 JH2

$K_d =$ 16 nM
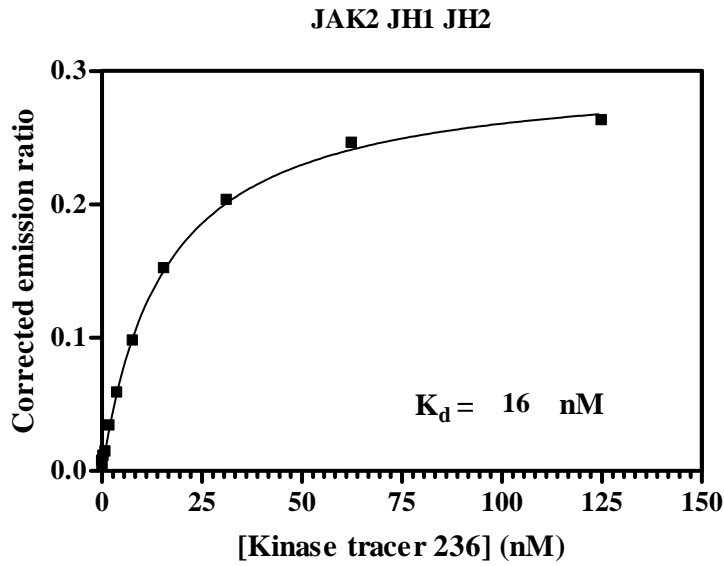
Figure 5. Tracer K_d determination.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

Procedure 2. 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. The concentration of tracer used in the below protocol is based on the tracer titration from Procedure 1.

10 nM Tracer 236 was chosen by Invitrogen for inhibitor studies. Under these conditions a high Z' value of 0.77 was obtained while keeping the tracer concentration close to the K$_d$ value (16 nM).

(2.1) Reagent preparation

1. Prepare an intermediate dilution series of each test compound by 4X serial dilution in DMSO such that the top concentration is 1 mM (suggested starting point) (Figure 6, 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 6.

Figure 6. Compound serial dilution.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

invitrogen™

LanthaScreen™ Eu Kinase Binding
Assay Validation Packet

Version No.:
1

Page 10 of 17

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

2. Dilute the "Master Dilution" series 33.3-fold into Kinase Buffer A. Remove 5 µL of each concentration of diluted compound, transfer to another 96-well plate, add 162 µL of Kinase Buffer A and mix (Figure 6, Step 2).

3. Prepare tracer solution in Kinase Buffer A at 30 nM tracer (3X the desired final assay concentration). Add the volumes of reagents calculated below to calculated volume of Kinase Buffer A.

   <u>**Calculations (for a 1000 µL solution)**</u>:

   $$\textbf{Tracer volume needed (µL)} = \frac{1000\ \mu L^1 * 30\ nM^2}{1000\ nM/\mu M * 50\ \mu M^3}$$

   [1] final volume of solution
   [2] desired 3X tracer concentration
   [3] stock tracer concentration

   Kinase Buffer A needed (µL) = 1000 µL – tracer volume needed (µL)

4. Prepare kinase/antibody solution at 15 nM kinase and 6 nM antibody (3X the desired final assay concentration). Centrifuge the antibody tube at approximately 10,000 x g for 10 minutes and aspirate desired volume from the top of the solution. Add volumes of reagents calculated below to calculated volume of Kinase Buffer A.

   <u>**Calculations (for a 1000 µL solution)**</u>:

   $$\textbf{Stock kinase conc. (nM)} = \frac{stock\ conc\ (mg/mL) * 1,000,000,000\ (nmol/mol)}{kinase\ MW\ (grams/mol)}$$

   $$\textbf{Kinase volume needed (µL)} = \frac{1000\ \mu L * 15\ nM}{Stock\ kinase\ conc.\ (nM)}$$

   $$\textbf{Antibody volume needed (µL)} = \frac{1000\ \mu L * 6\ nM}{Stock\ antibody\ conc\ (\mu M) * 1000\ (nmol/\mu mol)}$$

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

(2.2) 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 6.

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.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

(2.3) Data analysis

1. Divide the acceptor/tracer emission (665 nM) by the antibody/donor emission (615 nM) to calculate the "emission ratio".

2. Plot [test compound] versus emission ratio. The sigmoidal dose-response curve with a variable slope can be fit to the data (see section 1.3, step 2 for equation). Representative date generated by Invitrogen for a set of well-characterized kinase inhibitors is presented in Figure 7. A comparison of IC_{50} values to literature and in some cases internally generated data from activity-based assays (SelectScreen[TM] profiling service) are provided as a reference in Table 1.

3. In some cases, the Cheng-Prusoff equation[1] (equation 1) can be used to convert IC_{50} to K_i based on the concentration of Tracer and an accurate Tracer K_d (determined by following Procedure 1 or Appendix B).

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

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

In order 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).

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2
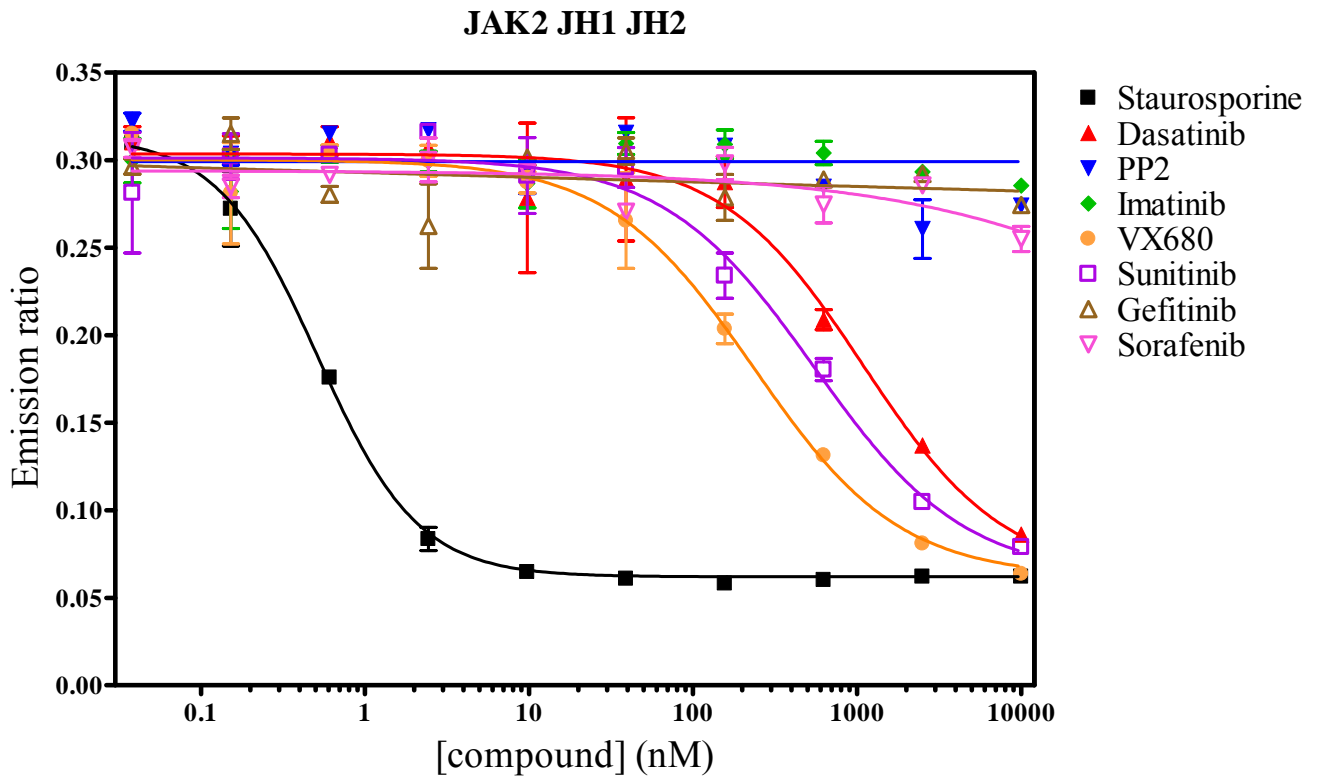
Figure 7. Representative data generated at Invitrogen.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

invitrogen™

LanthaScreen™ Eu Kinase Binding
Assay Validation Packet

Version No.:
1

Page 13 of 17

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

Table 1. IC_{50} values (nM) and comparison to literature and activity-based data.

Compound	LanthaScreen™ Eu Kinase Binding Assay	SelectScreen™ Kinase Profiling Service	Literature K_d values (Karaman et al.)
Staurosporine	0.5	0.55	11
Dasatinib	1000	1500	1000
PP2	>10000	>5000	n.d.
Imatinib	>10000	>5000	>10000
VX680	250	530	190
Sunitinib	530	2400	410
Gefitinib	>10000	n.d.	>10000
Sorafenib	>10000	>5000	>10000

Karaman, M.Z., *et al. Nat. Biotechnol.* 26(1) 127-132 (2008)

Note: These data are provided for references purposes. It is important to consider that the source of enzymes and method of detection (activity assay vs. binding assay) will affect whether measurements of IC_{50} values are due to active kinase, non-activated kinase, or a combination of both.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

	LanthaScreen™ Eu Kinase Binding Assay Validation Packet	Version No.: 1	Page 14 of 17

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

Appendix A. Assay robustness as a function of assay window

The Z'-factors for 215 kinase:tracer pairs were determined and plotted as a function of assay window (Figure A1). The data demonstrate excellent Z' values are typically obtained with an assay window of ≥ 2. Whereas, assay windows in the 1.5 to 2-fold range yield Z' values between 0.4 and 0.7, which may be suitable for some applications.

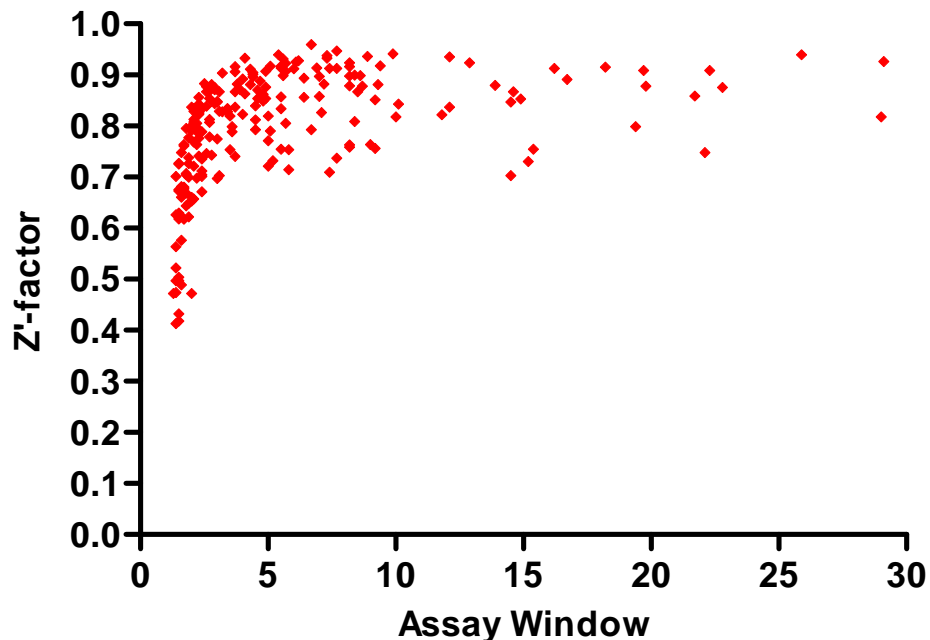**Figure A1. Z'-factor as a function of assay window for 215 kinases.**

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

Appendix B. Alternate method to determine tracer K_d and K_i values

A simple method to determine tracer K_d values from a tracer titration is described in Procedure 1, whereas an alternate method is described here based on IC_{50} curves performed at various tracer concentrations followed by analysis with the Cheng-Prusoff equation. In addition to determination of tracer K_d values, this method also enables calculation of K_i values (dissociation constant for the inhibitor). Rearrangement of the Cheng-Prusoff equation results in a linear relationship (i.e. the form $y = mx + b$) useful for analysis of binding data from homogenous assays (equation 2)[2]. When plotted with the IC_{50} value on the y-axis and the tracer concentration of the x-axis, the K_i is equal to the y-intercept and the slope equals $[K_i]/[K_d]$. Thus, the y-intercept divided by the slope equals the tracer K_d. This method enables calculation of the tracer K_d from IC_{50} curves performed at various concentrations of tracer.

$$(2)\ IC_{50} = \left(\left(\frac{K_i}{K_d} \right) \times [Tracer] \right) + K_i$$

Application of the Cheng-Prusoff equation is valid if the following criteria are met:
1. There is a single class of ligand binding site
2. The is no ligand depletion (i.e. [tracer] >> [kinase])
3. The receptor concentration < K_d

This method was applied to calculated the tracer K_d for representative kinase:tracer interactions with a range affinities and compared to the tracer titration method (Procedure 1). Example data are presented for the kinase TEK. The K_d value calculated from a tracer titration is 29 nM whereas that calculated using the linearized Cheng-Prusoff equation using staurosporine as the inhibitor is 31 nM and using VX680 is 30 nM, in close agreement (Figure B1). Data for all kinases compared with both methods is in Table B1, showing close agreement between both methods and supporting use of the more simple method based on a single tracer titration.

[2]Newton, P., et al. *J Biomol Screen.* 13(7) 674-682 (2008).

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

ö invitrogen™

LanthaScreen™ Eu Kinase Binding
Assay Validation Packet

Version No.:
1

Page 16 of 17

Optimization of a LanthaScreen™ Eu Kinase Binding Assay for JAK2 JH1 JH2

A
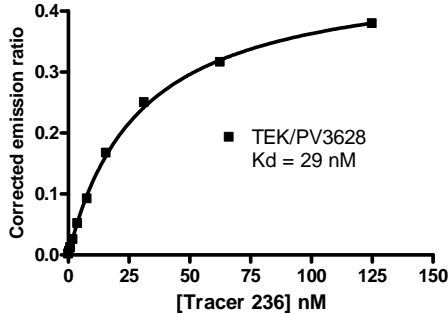
B

C

D

E

Figure B1. Determination of tracer K_d values by linearized Cheng-Prusoff equation and tracer titration method. The tracer K_d value was determined by the tracer titration method essentially as described in Procedure 1 with Kinase Tracer 236 and Eu-anti-GST antibody (A). IC_{50} curves were determined for TEK for the inhibitors staurosporine (B) and VX680 (C) essentially as described in Procedure 2 with various concentrations of Kinase Tracer 236. IC_{50} values were then plotted against the tracer concentration and the K_d values for the Tracer and Kinase and the K_i values for the inhibitor and the Kinase were determined from the slope and y-intercept.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com

Table B1. Comparison of K_d determination by linearized Cheng-Prusoff equation and tracer titration method.

Kinase	Tracer K_d values (nM)		
	Tracer titration	**Linearized Cheng-Prusoff with Staurosporine**	**Linearized Cheng-Prusoff with VX-680**
TEK	29	31	30
TAOK2	60	82	71
ITK	46	45	n.d.
MAP3K3	184	230	n.d.
MYLK2	237	299	n.d.

Have a question? Contact our Technical Support Team

NA: 800-955-6288 or INTL: 760-603-7200   Select option 3, ext. 40266   Email: drugdiscoverytech@invitrogen.com