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1. Overview

This user guide describes how to perform a LanthaScreen® Eu Kinase Binding Assay designed to detect and characterize kinase inhibitors. The first procedure (Section 5.1) is an optional experiment to optimize the concentration of tracer to use with a specific kinase target. This step is not necessary when using conditions established for Invitrogen kinases (see www.invitrogen.com/bindingassaytable for a list). The second procedure (Section 5.2) describes how to perform kinase inhibitor affinity (IC_{50}) measurements using either the concentration of tracer determined by the user or using the concentration determined experimentally by Invitrogen. Each experimental method is accompanied by example data for a diverse set of kinases.

A growing list of Invitrogen kinases have been validated for use with the LanthaScreen® Eu Kinase Binding Assay. For detailed assay conditions and validation data for specific kinases, visit www.invitrogen.com/bindingassaytable.

2. Principle of LanthaScreen® Eu Kinase Binding Assays

LanthaScreen® 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. Tracers based on a variety of scaffolds have been developed 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 fluorescence resonance energy transfer (FRET) 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.

The kinase tracers are based on ATP-competitive kinase inhibitors, and are thus suitable for detecting any compound that binds to the ATP site (Type I or Type II inhibitor). They can even detect most compounds that are not ATP competitive (Type III/allosteric inhibitors). Almost all non-ATP competitive (Type III) inhibitors tested to date displace the tracers, indicating that they either bind close to the ATP site or induce a conformational change at the ATP site. Out of 15 diverse commercially available Type III compounds tested against their respective targets, 14 displaced the tracer with a competency comparable to published assays. Notable examples of Type III compounds detected include GNF-2 (Abl), BMS-345541 (IKK beta), and MK-2206 (AKT). The only compound class tested to date that was not detected, with a single example, is purely peptide/protein substrate competitive (e.g., MK2a inhibitor), and thus not truly allosteric.

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 inactive kinase preparations, which enables characterization of compounds that bind preferentially to inactive kinases (*e.g.* Gleevec®/imatinib).

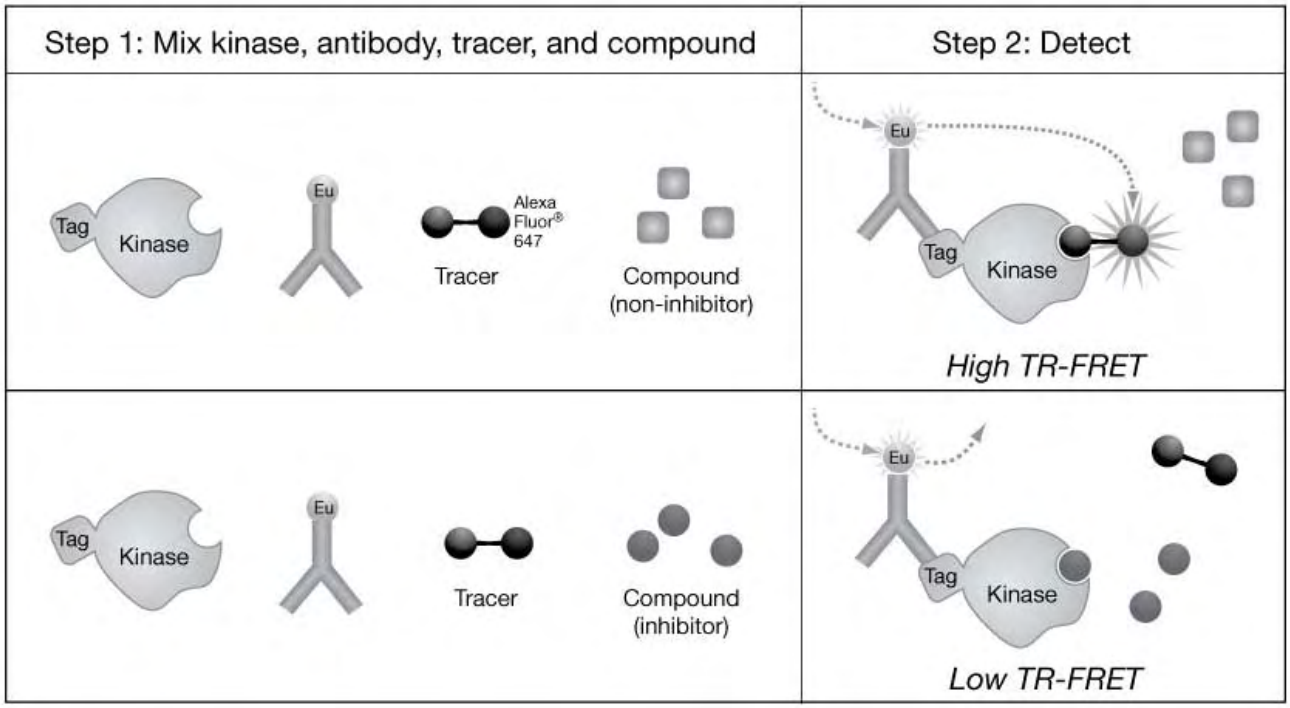

Figure 1. Schematic of LanthaScreen™ Eu Kinase Binding Assay.

3. Materials

3.1 Kinase Tracers

Product	Quantity	Composition	Catalog no.	Storage
Kinase Tracer 236	25 µL	Solution of 50 µM Tracer in DMSO	PV5592	–20°C
Kinase Tracer 178	25 µL	Solution of 25 µM Tracer in DMSO	PV5593	–20°C
Kinase Tracer 199	25 µL	Solution of 25 µM Tracer in DMSO	PV5830	–20°C
Kinase Tracer 314	25 µL	Solution of 25 µM Tracer in DMSO	PV6087	–20°C
Kinase Tracer 1710	25 µL	Solution of 25 µM Tracer in DMSO	PV6088	–20°C

3.2 Additional Assay Reagents

The Eu-anti-tag antibodies are supplied at approximately 0.22 to 0.28 mg/mL. The molecular weight of each antibody is 150 kD. The stock concentration of each antibody can be calculated by dividing the concentration in mg/mL by the molecular weight.

Important: Prior to use, the antibody tube should be centrifuged at approximately 10,000 × g for 10 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 due to any particulates in the product.

Product	Quantity	Composition	Catalog no.	Storage
LanthaScreen® Eu-anti-GST Antibody	25 µg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV5594	–20°C
LanthaScreen® Eu-anti-GST Antibody	1 mg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV5595	–20°C
LanthaScreen® Eu-anti-His Antibody	25 µg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV5596	–20°C
LanthaScreen® Eu-anti-His Antibody	1 mg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV5597	–20°C
Biotin anti-His Tag Antibody	25 µg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV6089	–20°C
Biotin anti-His Tag Antibody	1 mg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV6090	–20°C
LanthaScreen® Eu-anti-DYKDDDDK	25 µg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV6026	–20°C
LanthaScreen® Eu-anti-DYKDDDDK	1 mg	Approximately 0.25 mg/mL (see certificate of analysis for lot-specific concentration)	PV6027	–20°C
5X Kinase Buffer A	4 mL	250 mM HEPES pH 7.5, 0.05% Brij-35, 50 mM $MgCl_2$, and 5 mM EGTA	PV3189	20–30°C
Kinase	Visit www.invitrogen.com/kinase for ordering information for specific kinases			

3.3 Plate Readers

In general, instruments, instrument settings, and filters that work with other europium-based TR-FRET assay systems will perform well with the LanthaScreen® Eu Kinase Binding Assay. As with other TR-FRET systems, the europium donor is excited using a 340-nm excitation filter with a 30-nm bandpass. Energy transfer to the Alexa Fluor® 647 tracer is measured using a filter centered at 665 nm with a 10 nm bandpass, and this signal is referenced (or "ratioed") to the emission from europium peak, using a 615 nm, 10-nm bandpass filter. The "emission ratio" is calculated as the 665 nm signal divided by the 615 nm signal. The assay can be performed on a variety of plate readers including those from Tecan (Ultra, Infinite F-500, Safire2), Molecular Devices (Analyst and M5), BMG LABTECH (PHERAstar) and Perkin Elmer (EnVision, Victor, and ViewLux) or any other plate reader configured for LANCE® or HTRF® assays. Guidelines provided by the instrument manufacturer for Europium-based TR-FRET assays can also be used as a starting point for optimization.

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 tech_support@invitrogen.com for more information on performing LanthaScreen® assays on your particular instrument.

3.4 Plate Selection

Assays are typically performed in white low-volume 384-well plates (Corning 3673 or Greiner 784207) or black, low-volume 384-well plates (Corning 3676). White plates are generally recommended as they yield higher quality data for many assays, especially those with a relatively low assay window (1.5 to 2 fold) or those that are being measured on monochromater-based instruments or some filter-based instruments (e.g. Perkin Elmer EnVision®). In other cases, black and white plates yield comparable data.

4. Basic Protocol for Inhibitor Studies

LanthaScreen® Kinase Binding Assays to evaluate inhibitors are typically performed by addition of three 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.

The final assay conditions are typically as follows:

5 nM kinase[1]
2 nM Eu-anti-tag Antibody[2,3]
1–100 nM Kinase Tracer 178, 199, 236, 314, or 1710[4]
1X Kinase Buffer A

Notes:

[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]Eu-anti-His, Eu-anti-GST, or Eu-anti-DYKDDDDK

[3]Alternatively, for his-tagged kinases, 2 nM Eu-streptavidin and 2 nM biotin-anti-His may be used

[4]Optimal tracer concentrations for all validated kinases typically fall within the 1–100 nM range

5. Procedures

5.1 Optimization of Tracer Concentration

Note: When using an Invitrogen kinase for which an assay protocol exists (see www.invitrogen.com/bindingassaytable), this procedure is not necessary. You can proceed directly to Section 5.2.

This procedure 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 as detailed in Section 5.2, 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 Section 6). 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 this section is supported by data from an alternative method to calculate K_d (in addition to K_i) as described in Section 6. 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 described below.

5.1.1 Tracer Preparation

1. ***For Kinase Tracers 178, 199, 314, or 1710***: Dilute tracer to 3000 nM by mixing 7.2 µL of 25 µM stock tracer with 53 µL 1X Kinase Buffer A.

   For Kinase Tracer 236: Dilute tracer to 3000 nM by mixing 3.6 µL of 50 µM stock tracer with 56 µL 1X Kinase Buffer A.
2. Add 50 µL of 1X Kinase Buffer A to 6 wells in each of 2 columns of a 96-well plate.
3. Add 50 µL of 3000 nM tracer to well A1 and mix.
4. Remove 50 µL from well A1, transfer to well A2 and mix.
5. Remove 50 µL from well A21, transfer to well B1 and mix.
6. Continue as depicted in Figure 2.

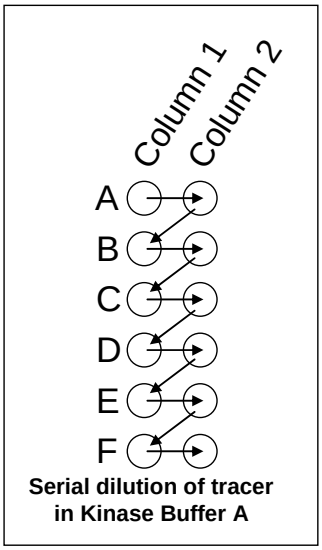

Figure 2. Serial dilution of tracer.

5.1.2 Kinase/Antibody Solution Preparation

1. Centrifuge antibody stock in a microfuge at approximately 10,000 × g for 10 minutes. Prepare a 1 mL solution of 6 nM antibody and 15 nM kinase. When using biotin-anti-His, 6 nM Eu-streptavidin should also be included.
2. Prepare competitor solution by diluting a known inhibitor to 30 µM in Kinase Buffer A, typically from a 10 mM stock in DMSO. A suitable competitor for specific kinases validated by Invitrogen can be found in the Validation Packets at www.invitrogen.com/bindingassaytable.
3. Prepare DMSO control solution by adding DMSO to Kinase Buffer to the same DMSO concentration as in the competitor solution (typically 0.3%).

5.1.3 Experimental Procedure

1. Add 5 µL each concentration of serially diluted tracer to six replicate assay wells (columns 1–6) (Fig. 3).
2. Add 5 µL competitor solution to three wells for each tracer concentration (columns 1–3).
3. Add 5 µL Kinase Buffer (with 3% DMSO) to the other three wells for each tracer concentration (columns 4–6).
4. Add 5 µL kinase/antibody solution to all wells in columns 1–6.
5. Incubate the plate at room temperature for 60 minutes and read.

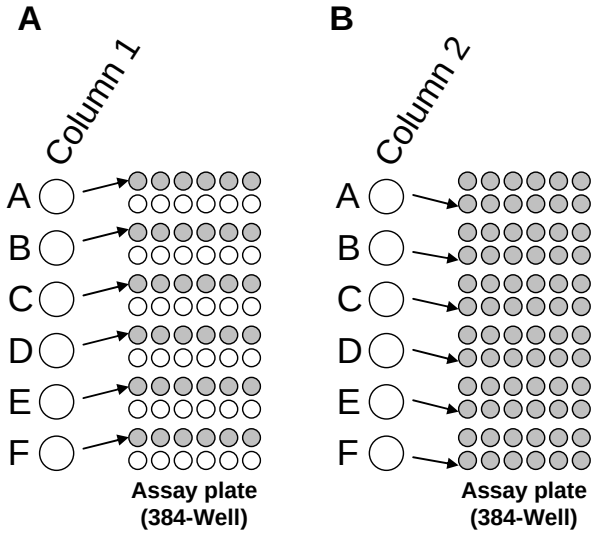

Figure 3. Transfer of tracer dilutions from 96-well to 384-well plate.

5.1.4 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 and control (DMSO only). 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

   $logEC_{50}$=logECF-(1/HillSlope)*log(F/(100-F))

   Y=Bottom+(Top-Bottom)/(1+10^(($LogEC_{50}$-X)*HillSlope))
3. Divide the signal in the absence of competitor by the signal in the presence of competitor to calculate the "assay window" at each concentration of tracer. The assay window correlates well with assay robustness (Figure 4), with assay windows of greater than 1.5 generally yielding Z'-factors of >0.5 while the majority of assays have Z'-factors of >0.75. Assay windows beyond 3 or 4 typically do not result in an increase Z'-factor. These data, combined with the K_d determined below, are helpful in selecting an optimal tracer concentration.

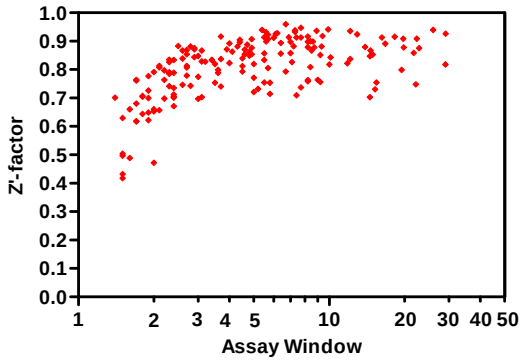

Figure 4. Assay Window Plotted Against Z'-Factor.

4. Subtract the competitor curve (+competitor) 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. The following equation can be used with GraphPad™ Prism software:

$$Y=Bmax*X/(Kd+X)$$

6. Select a tracer concentration for inhibitor studies based on the tracer K_d and the assay window. Ideal assays are performed with the tracer and or below the tracer K_d and at a tracer concentration such that the assay window is at least 2-fold.

Figure 5 shows representative data for kinase:tracer combinations with a range of K_d values. Some data points at the higher tracer concentrations are removed from curve fits on a case-by-case basis due to deviation from the one site binding equation caused by a high degree of background signal. For example, for MAPK14 and AURKA, the 250 and 500 nM data points were discarded.

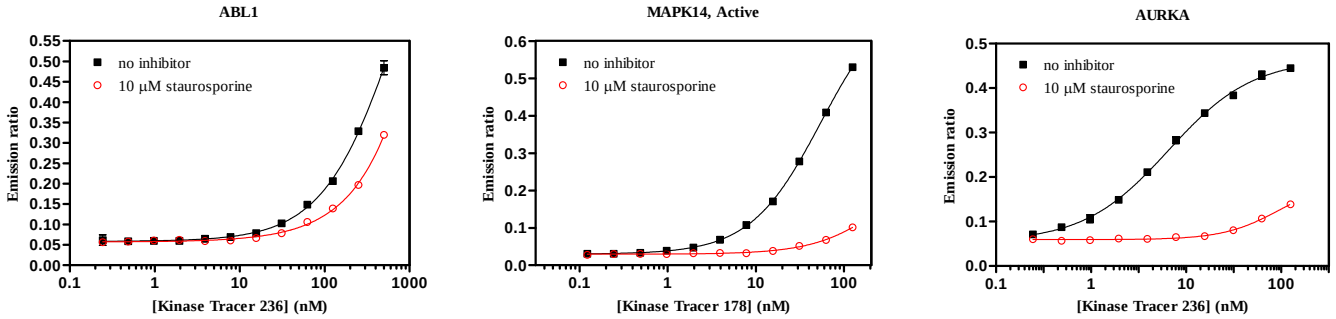

Figure 5. Representative selection of tracer titrations for kinases with varying degrees of tracer affinity.

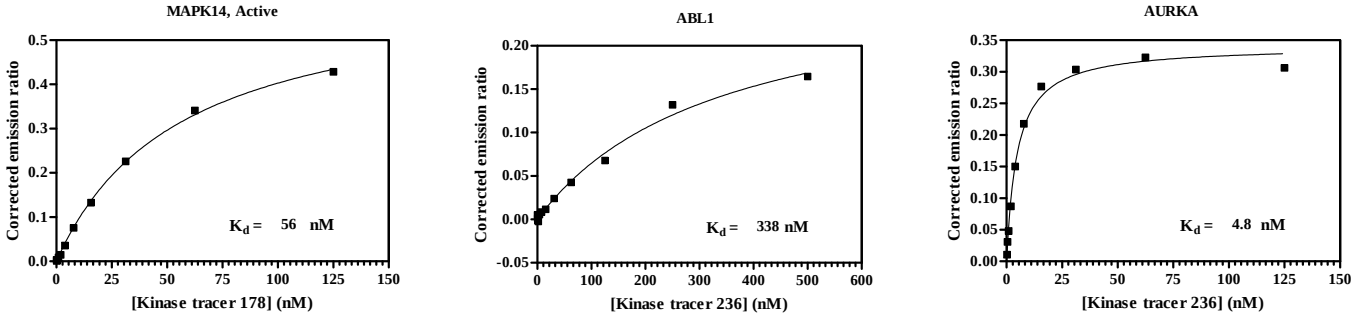

Figure 6. Representative selection of K_d determinations for kinases with varying degrees of tracer affinity.

5.2 Inhibitor Titration

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 assay may be performed using either the concentration of kinase tracer determined by the user (Section 5.1) or, for Invitrogen kinases, using the optimal concentration determined experimentally by Invitrogen.

5.2.1 Reagent Preparation

1. Prepare intermediate dilution series of test compound(s) in DMSO. Serially dilute compound(s) (4X dilutions) across 10 wells of a 96-well plate into DMSO such that the top concentration is 1 mM.
2. Dilute intermediate compound dilution series 33.3-fold into Kinase Buffer.
3. Prepare the kinase tracer solution. Dilute the tracer to 3X the desired final concentration.
4. Prepare the kinase/antibody solution. Centrifuge the antibody stock in a microfuge for 10 minutes. To the Kinase Buffer, add the kinase to 15 nM and antibody to 6 nM.

Note: A kinase concentration of 15 nM in the 3X kinase/antibody solution is recommended as a starting point, as it typically results in a robust signal. However, it may be desirable to vary or optimize the concentration of kinase for some applications.

5.2.2 Experimental Procedure

1. Add 5 µL of each concentration of serially diluted compound to three replicate assay wells.
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 minutes and read.

Note: A general guideline for incubation is 60 minutes. However, some binding reactions with kinase inhibitors rapidly come to equilibrium (less than 5 minutes). In addition, 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/slow-off rate compounds.

5.2.3 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.

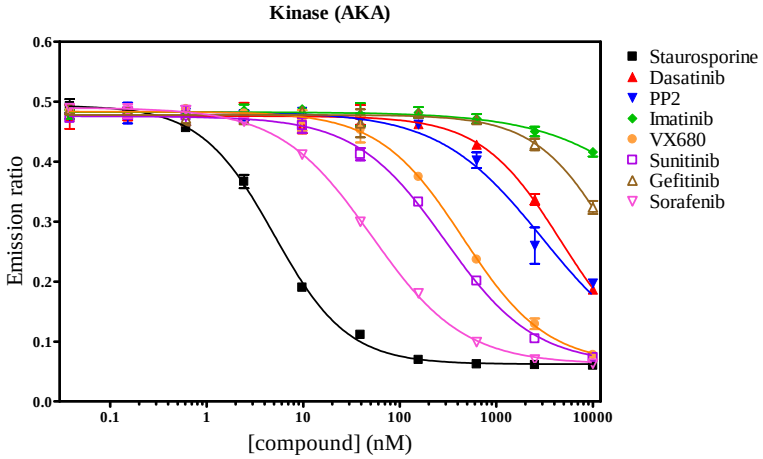

Figure 7. Representative data generated at Invitrogen

3. In some cases the Cheng-Prusoff equation can be used to convert IC_{50} to K_i based on the concentration of tracer and an accurate tracer K_d, as determined by following the procedure in Section 5.1 or Section 6 (Cheng and Prusoff).

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

This relationship holds true when the following criteria are met:

- [kinase] << [tracer] and [kinase] << IC_{50}
- [kinase] < tracer K_d
- There is only a single class of binding sites.

Note: An alternative method to determine K_i from a series of inhibitor titrations performed at different tracer concentrations is described in Section 6.

6. 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 Section 5.1. This section describes an alternate method based on IC_{50} curves performed at various tracer concentrations followed by analysis with the Cheng-Prusoff equation (Newton et al). In addition to determining tracer K_d values, this method also enables calculation of K_i values (dissociation constant for the inhibitor). Rearrangement of the Cheng-Prusoff equation (*i.e.,* the form $y = mx + b$) results in a linear relationship, which is 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.

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

Application of the Cheng-Prusoff equation is valid if the following criteria are met:

- There is a single class of ligand binding site
- The is no ligand depletion (i.e. [tracer] >> [kinase])
- The receptor concentration < K_d

This method was applied to calculate the tracer K_d for representative kinase:tracer interactions with a range affinities and compared to the tracer titration method (Section 5.1). 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 A1). Data for all kinases compared with both methods are in Table B1, showing close agreement between both methods and supporting use of the more simple method based on a single tracer titration.

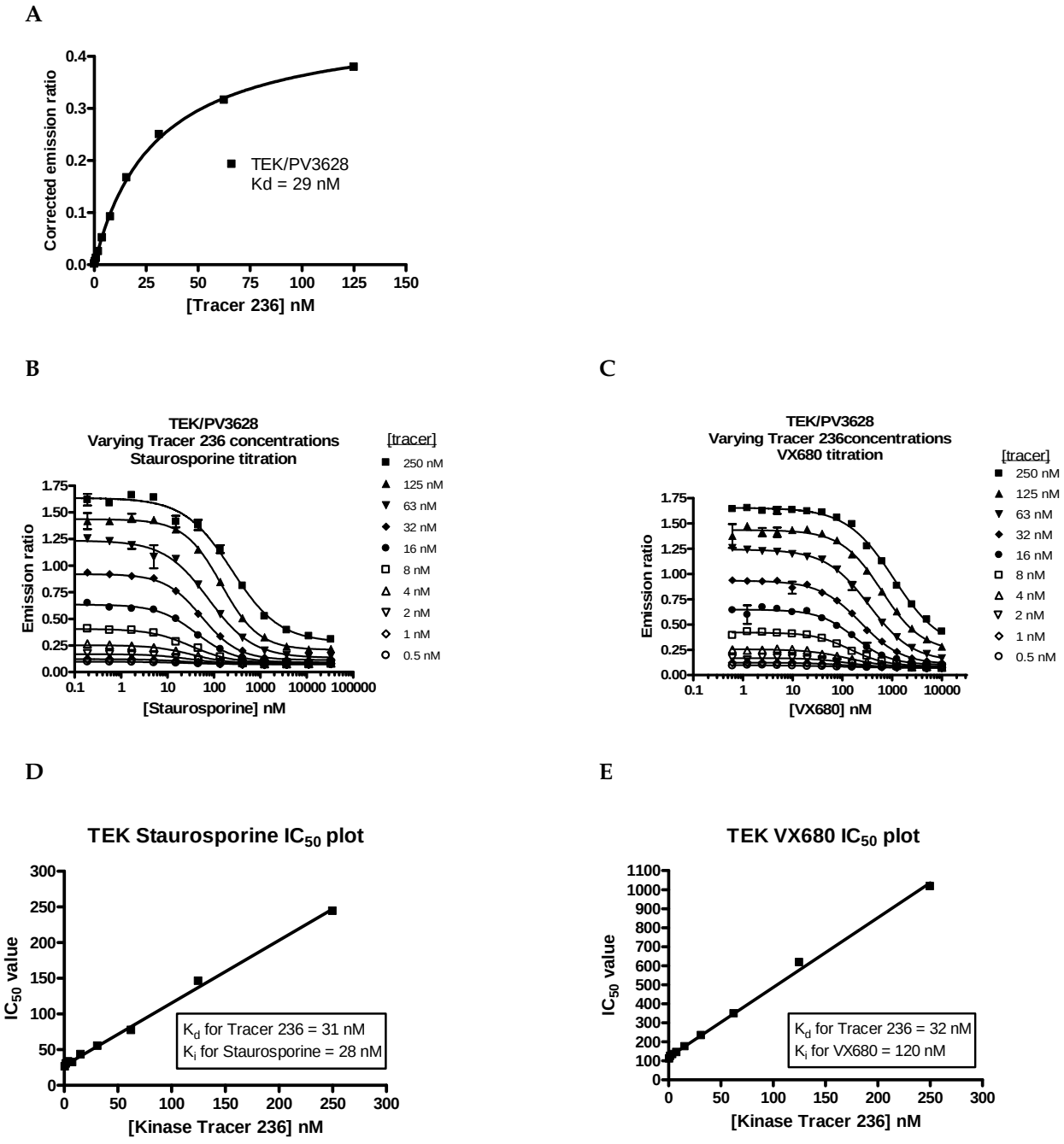

Figure 8. Determination of tracer K_d values by linearized Cheng-Prusoff equation and tracer titration method. The tracer K_d value was determined by the tracer titration method essentially as described in Section 5.1 with Kinase Tracer 236 and Eu-anti-GST antibody (A). IC_{50} curves were determined for TEK for the inhibitors staurosporine (B) and VX680 (C) essentially as described in Section 5.2 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.

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

	Tracer K_d values (nM)		
Kinase	**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.

7. References

Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol. 22*, 3099-3108.

Newton, P., Harrison, P., and Clulow, S. (2008) A novel method for determination of the affinity of protein: protein interactions in homogeneous assays. *J Biomol Screen 13*, 674-682.