

LanthaScreen™ Eu Kinase Binding Assay for NEK8

Catalog Number: A30982 Size: 10 µg Storage: -80°C, immediately upon receipt
 A32900 100 µg
 A33385 1 mg

Pub. No. MAN0017448 Rev. A.0

Overview

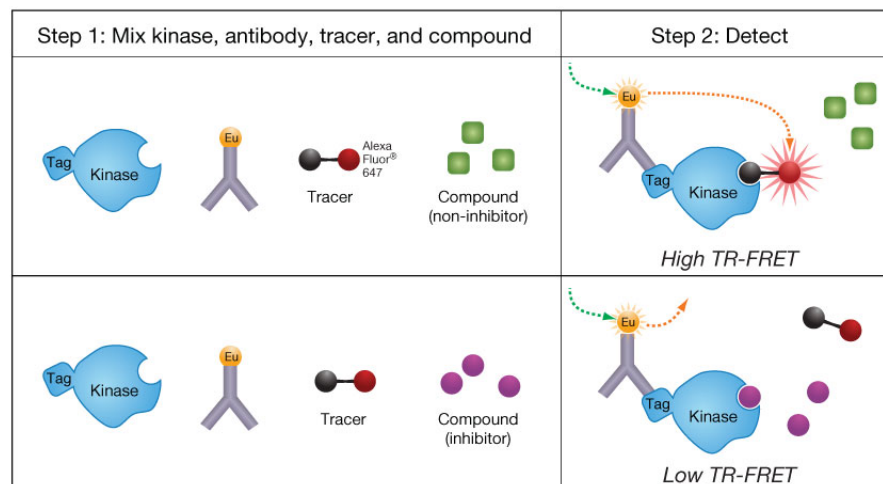
This user guide describes how to perform a LanthaScreen™ Eu Kinase Binding Assay designed to detect and characterize kinase inhibitors. It also provides representative IC₅₀ data on the control inhibitor (Figure 2, page 2) and percent inhibition data generated at Thermo Fisher Scientific for a panel of test compounds at concentrations of 1 µM and 10 µM (Table 1, page 3).

To prepare for the LanthaScreen™ Eu Kinase Binding Assay, determine the IC₅₀ of the recommended control inhibitor using the final assay conditions provided (page 2). The IC₅₀ of the control inhibitor from your measurements should fall within ±½ log of the reported value and the Z' for the assay should be >0.5.

Description of the LanthaScreen™ Eu Kinase Binding Assay

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 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.

Figure 1 Schematic of LanthaScreen™ Eu Kinase Binding Assay



Kinase Tracers from Thermo Fisher Scientific are based on ATP-competitive kinase inhibitors, making them suitable for detection of any compounds that bind to the ATP site or to an allosteric site altering the conformation of the ATP site. Inhibitors that bind the ATP site include both Type I kinase inhibitors, which bind solely to the ATP site, and Type II inhibitors (e.g., Gleevec™/Imatinib, Sorafenib, BIRB-796), which bind to both the ATP site and a hydrophobic site exposed in the DFG-out (non-active) conformation. Type III inhibitors are compounds that do not compete with ATP, and are loosely referred to as allosteric inhibitors. A study of 15 diverse Type III inhibitors demonstrated that all but one compound was detected in the binding assay with equivalent potency to activity assays. The sole exception was a substrate-competitive compound, and thus not a true allosteric inhibitor.

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Have a question? Contact our Technical Support Team
 NA: 800-955-6288 ext. 40266 or INTL: +1-760-603-7200
 Email: drugdiscoverytech@thermofisher.com

On/Off rates with the LanthaScreen™ Eu Kinase Binding Assay

In contrast to most fluorescence-based kinase activity assays, LanthaScreen™ Eu Kinase Binding Assays can be read continuously, which allows the evaluation of compounds with slow binding kinetics. Also, unlike most activity assays, binding assays can be performed using active or non-activated kinase preparations, enabling the characterization of compounds that bind preferentially to non-activated kinases, such as Gleevec™/imatinib and some allosteric inhibitors. See www.thermofisher.com/binding for more information.

Final assay conditions for NEK8

5 nM kinase¹
2 nM Eu-Anti-GST Antibody
30 nM Kinase Tracer 236²
1X Kinase Buffer A

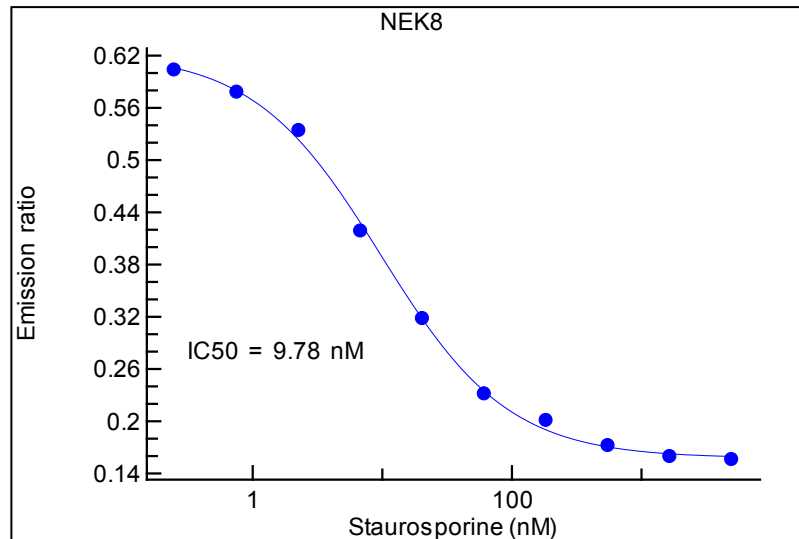
¹We recommend a kinase concentration of 5 nM, which typically results in a robust signal. Decreasing the kinase concentration may be necessary for accurate measurement of very tight-binding inhibitors, similar to kinase activity assays. For specific test cases, successful assays have been performed with as little as 200 pM kinase, though the assay window may be smaller.

²We recommend a tracer concentration of 30 nM for NEK8 obtained from Thermo Fisher Scientific. The K_d of the tracer was determined to be 53 nM. If the K_d value of the tracer is >100 nM, we recommend using the tracer at 100 nM. If using kinases from other sources, you may need to re-determine the tracer K_d for optimal tracer concentration (see "Optional: Determine the tracer K_d ", page 7).

IC₅₀ of control inhibitor

Control inhibitor: Staurosporine
IC₅₀ value for Staurosporine = 9.78 nM.

Figure 2. IC₅₀ of Staurosporine using final assay conditions



Percent inhibition with different control inhibitors

Table 1. Percent inhibition obtained with various control inhibitors compared to literature.

Compound	LanthaScreen™ Eu Kinase Binding Assay (KBA) percent inhibition		KBA IC ₅₀	Literature K _d values ¹
	1 μM compound	10 μM compound		
BIRB-796, Doramapimod	-4%	-2%	NA	NA
BMS-345541, IKK Inhibitor III	1%	5%	NA	NA
Dasatinib, BMS-354825	2%	23%	NA	NA
Erlotinib, Tarceva	-1%	2%	NA	NA
Gefitinib, Iressa	-1%	3%	NA	NA
Imatinib, Gleevec	0%	-3%	NA	NA
PI-103	-3%	-2%	NA	NA
SB-203580	-4%	-1%	NA	NA
Sorafenib, Bay 43-9006	10%	-6%	NA	NA
Staurosporine	99%	99%	9.78 nM	NA
Sunitinib, Sutent	10%	14%	NA	NA
TAE-684	43%	94%	NA	NA
Vandetanib, PTK787	-2%	3%	NA	NA

¹Davis, M.I., *et al.* Nat Biotechnology 29(11) 1046–1051 (2011). NA: Not available.

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

Required materials

Product	Part Number	Concentration	Amount	Storage	Notes
NEK8	A30982	Lot specific	10 µg	-80°C	(1)
5X Kinase Buffer A	PV3189	5X	4 mL	20°C–30°C	(2)
Kinase Tracer 236	PV5592	50 µM in DMSO	25 µL	-20°C	(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	-20°C	(4)
Staurosporine (optional)	Sigma, S-4400 or Fermentek, 62996-74-1	NA	varies	See manufacturer's COA	(5)

- NEK8 is supplied at the nM concentration found on the kinase Certificate of Analysis (CoA). Go to www.thermofisher.com/kinase and find your kinase in the Kinase Target Portfolio table. Click on the catalog number, then search for the CoA by the lot number of the kinase you have received, which is printed on the tube.
- 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 water. The 1X kinase reaction buffer is stable at room temperature. 1X Kinase Buffer A consists of 50 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM EGTA, and 0.01% Brij-35.
- Kinase Tracer 236 is supplied as a 50 µM stock in DMSO.
- Prior to use, centrifuge the antibody tube at approximately 10,000 × g for 5 minutes, and aspirate the volume needed for the assay from the top of the solution. This centrifugation step significantly reduces noise that can arise due to antibody aggregation.
- Stock solutions of control inhibitor or test compounds are typically prepared at mM concentrations in 100% DMSO (see Table 2).

Table 2. Preparation of control inhibitor stock solutions

Control inhibitor	Source and Part No.	Stock concentration	Amount	DMSO
Staurosporine	Sigma, S-4400 or Fermentek, 62996-74-1	5 mM	20 mg	8575 µL
Dasatinib	LC Labs, D-3307	100 mM	100 mg	2050 µL
Sunitinib	LC Labs, S-8803	10 mM	5 mg	939 µL
PP121	Sigma, P0036 or Tocris, 3894	10 mM	5 mg	1565 µL
SB 202190	Sigma, S7067 or LC Labs, S1700	10 mM	5 mg	1509 µL

Plate readers

The data presented in this document were generated using a BMG LABTECH CLARIOstar™ plate reader using the appropriate filters and instrument settings for europium-based LanthaScreen™ assays. You can perform the assay on a variety of plate readers capable of TR-FRET.

Refer to www.thermofisher.com/instrumentsetup for instrument-specific settings and for a control test to determine the instrument response using your reagents. General instrument settings are listed in Table 3.

Table 3. General instrument settings used for europium-based LanthaScreen™ assays

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 questions, contact Technical Support at 800-955-6288 ext. 40266, or email drugdiscoverytech@thermofisher.com for more information on performing these assays on your instrument.

Plates

Assays are typically performed in white low-volume 384-well plates (Corning, Cat. No. 4513 or Greiner, Cat. No. 784207) or black, low-volume 384-well plates (Corning, Cat. No. 4514).

We generally recommend white plates because they yield higher quality data for many instruments, especially if running kinases with a relatively low assay window (1.5 to 2-fold) or those that are being measured on monochromator-based instruments.

Creation of the master dilution series requires plates tolerant to 100% DMSO, for example, Nalg™ Nunc™ 96-well polypropylene plate (Cat. No. 249944).

Determine the IC₅₀ of the control inhibitor

Overview

This procedure describes how to determine inhibitor potencies by generating a 10-point IC₅₀ curve from a 4-fold dilution series of the test compound using the final assay conditions. The control inhibitor is shown as an example. Under these conditions and using the control inhibitor, a Z' value of greater than 0.5 was obtained while keeping the tracer concentration close to the K_d value.

Basic outline of the protocol for inhibitor studies

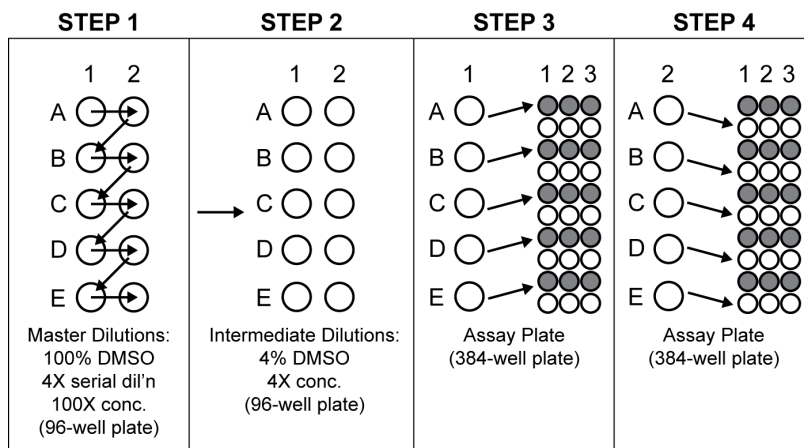
LanthaScreen™ Kinase Binding Assays to evaluate kinase inhibitors are typically performed by the addition of 3 components as follows:

1. Add 4 μL of 4X **control inhibitor** or **test compound**.
2. Add 8 μL of 2X **kinase/antibody** mixture.
3. Add 4 μL of 4X **tracer**.
4. Incubate for 1 hour at room temperature and read plate.

Prepare reagents

1. Prepare 1X kinase buffer according to Note (2) under the “Required materials” table (page 4). In addition to the volume of kinase buffer required to prepare the tracer and kinase/antibody reagents, 1.2 mL of kinase buffer is used per compound tested.
2. Prepare a 4X Master Dilution series of the control inhibitor or test compound in 100% DMSO in a DMSO-tolerant plate such that the top concentration is 1 mM (suggested starting point) (Figure 3, Step 1).
 - a. Prepare 4 mM control inhibitor or 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 control or test 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 3.

Figure 3 Test compound serial dilution



3. Dilute the “Master Dilution” series 25-fold into 1X kinase buffer. Remove 5 μL of each concentration of diluted compound, transfer to another 96-well plate, then add 120 μL of 1X kinase buffer and mix (Figure 3, Step 2).
4. Prepare tracer solution in 1X kinase buffer at 120 nM tracer (4X the desired final assay concentration). Add the volumes of reagents calculated below to the calculated volume of 1X kinase buffer.

$$\begin{aligned} \text{Tracer volume needed } (\mu\text{L}) &= \frac{\text{Final volume of solution} \times \text{Desired 4X tracer concentration}}{1000 \text{ nM}/\mu\text{M} \times \text{Stock tracer concentration}} \\ &= \frac{1000 \mu\text{L} \times 120 \text{ nM}}{1000 \text{ nM}/\mu\text{M} \times 50 \mu\text{M}} \end{aligned}$$

$$\text{1X kinase buffer needed } (\mu\text{L}) = 1000 \mu\text{L} - \text{tracer volume needed } (\mu\text{L})$$

- Prepare 2X kinase/antibody solution at 10 nM kinase and 4 nM antibody (2X the desired final assay concentration).

IMPORTANT! Centrifuge the antibody tube at approximately 10,000 × g for 10 minutes and aspirate desired volume from the top of the solution. Add volumes of the reagents calculated below to the calculated volume of 1X kinase buffer.

$$\text{Kinase volume needed } (\mu\text{L}) = \frac{1000 \mu\text{L} \times 10 \text{ nM}}{\text{Stock kinase conc. (nM)}\dagger}$$

† Stock kinase concentration is provided in nM on the Certificate of Analysis (CoA). To obtain the CoA, go to www.thermofisher.com/kinase and find your kinase in the Kinase Target Portfolio table. Click on the catalog number, then search for the CoA by the lot number of the kinase you have received.

$$\text{Antibody volume needed } (\mu\text{L}) = \frac{1000 \mu\text{L} \times 4 \text{ nM}}{\text{Stock antibody conc. } (\mu\text{M}) \times 1000 \text{ (nmol}/\mu\text{mol})}$$

$$\text{1X kinase buffer needed } (\mu\text{L}) = 1000 \mu\text{L} - \text{kinase volume needed } (\mu\text{L}) - \text{antibody volume needed } (\mu\text{L})$$

Determine the IC₅₀

- Add 4 μL of each concentration of serially diluted control inhibitor or test compound to triplicate assay wells in a 384-well plate (columns 1–3) as depicted in Steps 2 and 3 of Figure 3 (page 5).
- Add 8 μL of kinase/antibody solution to all wells.
- Add 4 μL of tracer solution to all wells.
- Incubate the plate at room temperature for 60 minutes and read.

Note: 60 minutes is a general guideline for incubation. However, in some cases, multiple read times or continuous measurements can be used to examine the kinetics of binding reactions, which might be of interest for studies on slow-binding compounds.

Data analysis

- Divide the acceptor (tracer) emission at 665 nm by the antibody (donor) emission at 615 nm to calculate the “emission ratio”. As most donor Eu are not involved in signaling, this ratio is typically much less than 1.0.
- Plot [test compound] versus emission ratio. The sigmoidal dose-response curve with a variable slope can be fit to the data using the following equation with GraphPad™ Prism software:

$$F = 50$$

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

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

Note: Representative data generated by Thermo Fisher Scientific for various control inhibitor compounds are presented in Figure 2 and Table 1 (page 2).

Optional: Determine the tracer K_d

Note: When using kinases from Thermo Fisher Scientific, this step is not necessary.

Overview

This step describes how to optimize the tracer concentration for use in 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.

Signal strength or “assay window” 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”, page 10). The specific end application may also impact the choice of tracer concentration, based on both the assay window, Z' , and K_d value. For K_d values over 100 nM, tracer is typically used at a concentration of 100 nM.

It is best to select a tracer concentration near or below the K_d to ensure sensitive detection of inhibitors. For example, the measured IC_{50} value from a simple compound titration will approach the K_i (dissociation constant of a competitive inhibitor) if $[tracer] < tracer K_d$ and $[kinase] \ll [tracer]$. The majority of kinase assays validated by Thermo Fisher Scientific 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 equation¹ (below), which compensates for the tracer concentration being above K_d .

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

This relationship holds true when the following criteria are met:

1. $[kinase] \ll [tracer]$ and $[kinase] \ll IC_{50}$
2. $[kinase] < Tracer K_d$
3. There is a single class of binding sites

To determine K_i values for very tight-binding compounds, it may be necessary to perform assays at lower kinase concentrations.

¹Cheng, Y.C., Prusoff, W.H. *Biochem Pharmacol.* (22) 3099-3108 (1973).

Prepare reagents

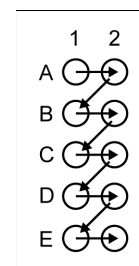
1. Prepare a dilution series of the tracer at 4X the final concentration to be assayed.
 - a. Dilute the tracer to 4 μ M in 1X kinase buffer (see Table 4).

Table 4. Tracer dilutions

Tracer	Initial Tracer concentration	Tracer volume	1X kinase buffer volume	Final Tracer concentration	Fold dilution
Tracer 236	50 μ M	5 μ L	57.5 μ L	4 μ M	1:12.5
Tracer 222	50 μ M	5 μ L	57.5 μ L	4 μ M	1:12.5
Tracer 178	25 μ M	10 μ L	52.5 μ L	4 μ M	1:6.25
Tracer 314	25 μ M	10 μ L	52.5 μ L	4 μ M	1:6.25
Tracer 199	25 μ M	10 μ L	52.5 μ L	4 μ M	1:6.25
Tracer 1710	25 μ M	10 μ L	52.5 μ L	4 μ M	1:6.25

- b. Add 50 μ L of 1X kinase buffer to 6 wells in each of the 2 columns of a 96-well plate.
- c. Add 50 μ L of 4 μ M tracer to well A1 and mix.
- d. Remove 50 μ L from well A1, transfer to well A2 and mix.
- e. Remove 50 μ L from well A2, transfer to well B1 and mix.
- f. Continue the process for the remaining wells as shown in Figure 4.

Figure 4 Serial dilution of the tracer in a 96-well plate



2. Prepare kinase/antibody solution at 2X the desired final assay concentration.

IMPORTANT! Centrifuge the antibody tube at approximately $10,000 \times g$ for 10 minutes and aspirate the desired volume from the top of the solution.

Add the volumes of the reagents calculated to the calculated volume of kinase buffer.

Calculations (for a 1000 μL solution):

$$\text{Kinase volume needed } (\mu\text{L}) = \frac{1000 \mu\text{L} \times 2\text{X kinase concentration}}{\text{Stock kinase concentration (nM)}}$$

$$\text{Antibody volume needed } (\mu\text{L}) = \frac{1000 \mu\text{L} \times 4 \text{ nM (2X antibody conc.)}}{\text{Stock antibody concentration } (\mu\text{M}) \times 1000 \text{ (nmol}/\mu\text{mol)}}$$

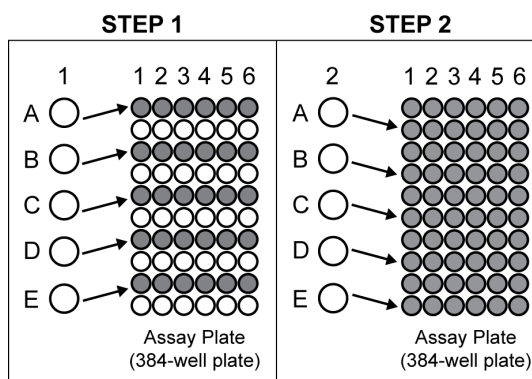
$$\text{Kinase buffer needed } (\mu\text{L}) = 1000 \mu\text{L} - \text{kinase volume needed } (\mu\text{L}) - \text{antibody volume needed } (\mu\text{L})$$

3. Prepare 40 μM control inhibitor solution in 4% DMSO.
4. Prepare 4% DMSO control solution by adding 40 μL DMSO to 960 μL kinase buffer.

Determine the K_d

1. Add 4 μL of each concentration of the serially diluted tracer to six replicate assay wells in a 384-well plate (Columns 1–6) as shown in Figure 5.

Figure 5 Transfer of tracer dilutions from 96-well to 384-well plate, first from Column 1 of the 96-well plate into alternate rows of the 384-well plate, then from Column 2 of the 96-well plate to the remaining rows of the 384-well plate.



2. Add 4 μL of competitor solution to three wells for each tracer concentration (Columns 1–3).
3. Add 4 μL of DMSO control solution to the other three wells for each tracer concentration (Columns 4–6).
4. Add 8 μL of kinase/antibody solution to all wells in Columns 1–6.
5. Incubate the plate at room temperature for 60 minutes, then read the plate.

Data analysis

1. Divide the acceptor (tracer) emission at 665 nm by the antibody (donor) emission at 615 nm to calculate the "emission ratio". As most donor Eu are not involved in signaling, this ratio is typically much less than 1.0.
2. Plot [tracer] versus emission ratio for the control inhibitor and the no inhibitor control (DMSO only), as shown for CDK9 Inactive as an example (Figure 6). The sigmoidal dose-response curve with a variable slope can be fit to the data (optional).

You can use the following equation with the GraphPad™ Prism software:

$$F = 50$$

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

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

3. You can calculate the assay window for any given tracer concentration by dividing the signal of the no inhibitor control by the signal in the presence of the control inhibitor (staurosporine in the example). 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", page 10).
4. To obtain background-corrected emission values, subtract the no inhibitor control curve (DMSO only) from the control inhibitor curve. Background signal is the diffusion enhanced FRET from the unbound Eu-labeled antibody and tracer as they pass by each other in solution. This signal arises due to the extended excited state lifetime of the Eu chelate.
5. To obtain the K_d , plot the background-corrected emission ratios versus [tracer], then fit to the one site binding (hyperbola) equation to estimate the dissociation constant (Figure 7). In some cases, data at the highest one or two tracer concentrations are excluded from the curve fits due to the relatively high "background" signal in the presence of the competitor. This can be observed as these data points deviate from the one site binding model, whereas the other data points align.

You can use the following equation with the GraphPad™ Prism software:

$$Y = B_{\max} \times X / (K_d + X)$$

Figure 6 Tracer titration curve example using CDK9 Inactive

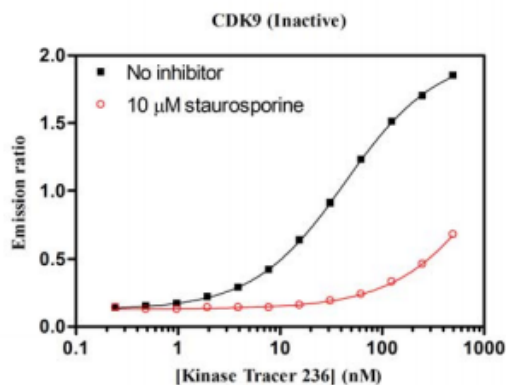
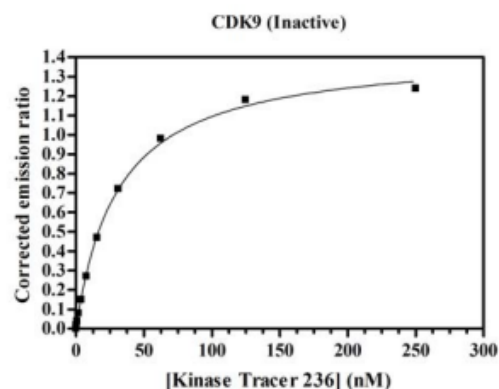


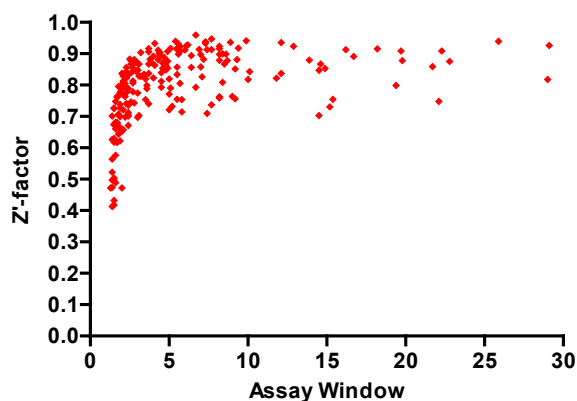
Figure 7 Tracer K_d determination example using CDK9 Inactive, ($K_d = 26$ nM)



Appendix

The Z'-factors for 215 kinase:tracer pairs were determined and plotted as a function of assay window (Figure 8). The data demonstrate excellent Z' values are typically obtained with an assay window of ≥ 2 . 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 8. Z'-factors for kinase:tracer pairs as a function of assay window.



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Explanation of symbols

Symbol	Description	Symbol	Description	Symbol	Description
	Manufacturer		Catalog number		Batch code
	Use by		Temperature limitation		
	Consult instructions for use		Caution, consult accompanying documents		

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