Optimization of a LanthaScreen® Kinase assay for DNA-PK

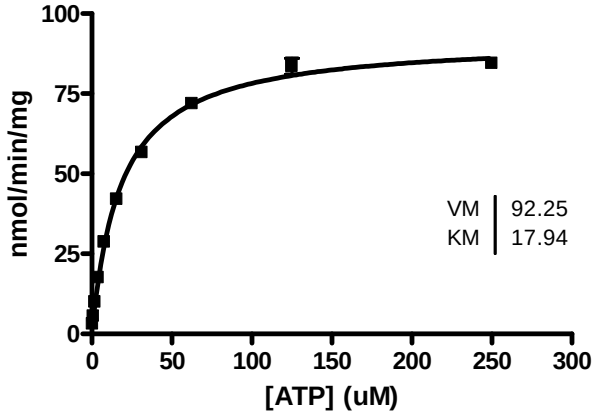
Overview

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

1. **Determination of ATP $K_{m,app}$**

   ATP $K_{m,app}$ is often determined in LanthaScreen® format. However, for DNA-PK the ATP $K_{m,app}$ value was determined using a radiometric, filter-binding assay using 1 µM unlabeled p53-derived peptide substrate in 1X Assay Buffer (50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM $MgCl_2$, 1 mM EGTA, 2.5 µg/mL DNA-PK Activator (CT-DNA), and 1 mM DTT. The enzyme concentration was 5µg/mL.

   Figure 1: ATP $K_{m,app}$ determination



   The ATP $K_{m,app}$ value was determined to be 18 µM and ATP was used at this concentration for the following LanthaScreen™ experiments.

2. **Optimization of kinase concentration required for assay at ATP $K_{m,app}$.**
   Using the ATP $K_{m,app}$ concentration of ATP determined in step 1, a kinase titration is performed in order to determine the amount of kinase required to elicit an approximately 50% change between the minimum and maximum TR-FRET emission ratios (the EC_{80} value).

Using the kinase concentration 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 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 2 is the only step that needs to be performed (at the chosen concentration of ATP) prior to performing the assay in the presence of inhibitors.

The data presented in this document is example data that was generated at Invitrogen. Specific results may vary based upon a variety of factors including the specific activity of the kinase, or the particular plate reader being used. In particular, the Emission Ratio measured can vary greatly between instruments. However, the quality of the data generated should be comparable to the data presented here. If you are reproducing the work presented in this document you should move between the various steps using the values determined in *your* experiments. If you are having trouble reproducing the data presented here, please do not hesitate to contact Invitrogen Technical Services or your Invitrogen representative.

Materials Required

Description	Part Name	Catalog #	Notes
Kinase Reaction Buffer	5X Kinase Buffer	PV3189 (4 mL of 5X)	(1)
Additives:	1M DTT	P2325 (1 mL)	
	1 mg/mL DNA-PK Activator (CT-DNA)	PV5884 (20 µL)	(2)
TE buffer	1X TE buffer (100 mL)	IVGN 12090-015	
Kinase	DNA-PK	PV5864 (10µg) PV5865 (1mg)	
Substrate	Fluorescein-p53 substrate	PV5132 (1 mL)	(3)
10 mM ATP	10 mM ATP	PV3227 (500 µL)	
Antibody	LanthaScreen® Tb-anti phospho-p53 [pSer15]	PV5130 (25 µg) PV5131 (1 mg)	(4)
Antibody Dilution Buffer	TR-FRET Dilution Buffer	PV3574 (100 mL)	(5)
500 mM EDTA	Kinase Quench Buffer	P2832 (10 mL)	
Inhibitors	DNA-PK inhibitor III (IC86621)	N/A	(6)
	PI-103	N/A	(7)
	PI3KA Inhibitor II	N/A	(8)
	TGX-221	N/A	(9)
	NU7026 (DNA-PK Inhibitor II)	N/A	(10)
	LY294002	N/A	(11)
	BEZ-235	N/A	(12)
	DNA-PK Inhibitor IV (IC60211)	N/A	(13)
	AMA37 (DNA-PK Inhibitor V)	N/A	(14)
	Ku55933 (ATM Kinase Inhibitor)	N/A	(15)
	Compound 401	N/A	(16)

(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 DNA-PK Activator (CT-DNA) is supplied as a 1 mg/mL stock. The final 1X concentration recommended per reaction is 2.5 µg/mL.
(3) The Fluorescein-p53 peptide substrate is supplied at a concentration of ~ 1.0 mg/mL (472 µM); see the tube for the exact concentration. The molecular weight of the fluoresceinp-53 peptide substrate is 2118.3 g/mol.
(4) The p53 (pSer15) 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.
(5) The antibody dilution buffer does not contain EDTA. EDTA is added separately, prior to addition of antibody.
(6) IC86621 = DNA-PK inhibitor III = 1-(2-Hydroxy-4-morpholin-4-yl-phenyl)ethanone. IC86621 can be purchased from Calbiochem (Part # 260962). CAS# 404009-40-1.
(7) PI-103 = 3-(4-(4-Morpholinyl)pyrido[3′,2′:4,5]furo[3,2-d]pyrimidin-2-yl)phenol. PI-103 can be purchased from Calbiochem (catalog # 528100). CAS# 371935-74-9.
(8) PI3KA Inhibitor II = 3-[4-(4-morpholinyl)thieno[3,2-d]pyrimidin-2-yl-phenol. PI3KA Inhibitor II can be purchased from Cayman Chemical (Part# 10010177). CAS# 371943-05-4.
(9) TGX-221 = PI 3-Kβ Inhibitor VI = (±)-7-Methyl-2-(morpholin-4-yl)-9-(1-phenylaminoethyl)-pyrido[1,2-a]-pyrimidin-4-one. TGX-221 can be purchased from Calbiochem (Part # 528113). CAS# 663619-89-4.
(10) NU7026 = DNA-PK Inhibitor II = LY293646 = 2-(Morpholin-4-yl)-benzo[h]chromen-4-one. NU7026 can be purchased from Calbiochem (Part# 260961) CAS# 154447-35-5.
(11) LY294002 = 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one. LY294002 can be purchased from Cayman Chemical (Part # 70920). CAS# 154447-36-6.
(12) BEZ-235 = NVP-BEZ235 = 2-Methyl-2-[4-(3-methyl-2-oxo-8-quinolin-3-yl-2,3-dihydro-imidazo[4,5-c]quinolin-1-yl)-phenyl]-propionitrile. BEZ-235 can be purchased from ChemieTek (Part # CT-BEZ). CAS# 915019-65-7.
(13) IC60211 = DNA-PK Inhibitor IV = 2-Hydroxy-4-morpholin-4-yl-benzaldehyde. IC60211 can be purchased from Calbiochem (Part# 260963)
(14) AMA37 = DNA-PK Inhibitor V = 1-(2-Hydroxy-4-morpholin-4-yl-phenyl)-phenyl-methanone. AMA37 can be purchased from Calbiochem (Part# 260964). CAS# 404009-46-7.
(15) Ku55933 = (ATM Kinase Inhibitor) = 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one. Ku55933 can be purchased from Calbiochem (Part# 118500). CAS# 587871-26-9.
(16) Compound 401= 2-(Morpholin-1-yl)pyrimido[2,1-a]isoquinolin-4-one. Compound 401 can be purchased from Calbiochem (Part# 234501).CAS# 168425-64-7.

Preparing the Activation Buffer

Before each experiment, thaw the 1 mg/mL stock of DNA-PK Activator (CT-DNA) (listed above) on ice. Once thawed, mix well by vortexing. Dilute the 1 mg/mL stock 10-fold to 100 µg/mL (0.1 mg/mL) in 1X TE buffer as follows: to 135 µL of 1X TE buffer, add 15 µL of 1 mg/mL DNA-PK Activator (CT-DNA) and mix well by vortexing. Use this 100 µg/mL stock of DNA-PK Activator (CT-DNA) in TE buffer (40X activation buffer) to prepare the 1X Kinase Reaction Buffer listed below.

Preparing the 1x Kinase Reaction Buffer

Before each experiment, prepare a fresh 1x solution of kinase reaction buffer from the 5x Kinase Buffer stock (listed above) by adding 1 mL of 5x stock, 5 µL of 1M DTT, and 125 µL of 40X activation buffer (100 µg/mL DNA_PK Activator (CT-DNA) in TE buffer) to 3870 µL H_2O to make 5 mL of 1x kinase reaction buffer supplemented with 1 mM DTT and 1X activation buffer (2.5 µg/mL DNA-PK Activator (CT-DNA)).

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 is1.6 µM, and the 1x kinase reaction buffer consists of 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM $MgCl_2$, and 1 mM EGTA, plus any additional additives that may be required for a specific kinase (1 mM DTT and 1X activation buffer required for DNA-PK). 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 60 minutes before being read on a plate reader configured for LanthaScreen™ TR-FRET.

Plate Readers

The data presented in this document were generated using a BMG LABTECH Pherastar plate reader using the LanthaScreen™ filter block available from BMG. The assay can be performed on a variety of plate readers including those from Tecan (Ultra, Infinite F500 and Safire^2), Molecular Devices (Analyst and M5), Perkin Elmer (EnVision, Victor, and ViewLux). Visit 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 for more information on performing LanthaScreen™ assays on your particular instrument.

Filter Selection

The terbium donor is excited using a 340 nM excitation filter with a 30 nm bandpass. However, the exact specifications of the excitation filter are not as critical, and filters with similar specifications will work well. In general, excitation filters that work with europium-based TR-FRET systems will perform well with the LanthaScreen® terbium chelates. Because it is important to measure energy transfer to the acceptor (fluorescein or GFP) without interference from terbium, a filter centered at 520 nm with a 25 nm bandpass is used for this purpose. In general, **standard "fluorescein" filters may not be used**, because such filters also pass light associated with the terbium spectra. The emission of fluorescein due to FRET is referenced (or "ratioed") to the emission of the first terbium peak, using a filter that isolates this peak. This is typically accomplished with a filter centered at 490 or 495 nm, with a 10 nm bandpass. In general, a 490 nm filter will reduce the amount of fluorescein emission that "bleeds through" into this measurement, although instrument dichroic mirror choices (such as those on the Tecan Ultra Evolution instrument) may necessitate the use of a 495 nm filter. The effect on the quality of the resulting measurements is minimal in either case.

LanthaScreen® Terbium:	
Excitation:	340 nm filter (30 nm bandwidth)
Fluroescein Emission:	520 nm filter (25 nm bandwidth)
Terbium Emission:	490 nm filter (10 nm bandwidth)

Visit www.invitrogen.com/instrumentsetup or contact Invitrogen Discovery Sciences technical support at 800-955-6288 (select option 3 and enter 40266), or email tech_support@invitrogen.com for more information on performing LanthaScreen® Terbium assays on your particular instrument.

Example Protocols

The following example protocols describe the various steps using 16-point dilutions of the variable reagent (kinase, ATP, or inhibitor) in triplicate.

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

Step 2: Titration of Kinase at ATP $K_{m,app}$.

(2.1) In an appropriate tube or vial, prepare 50 µL of kinase in kinase reaction buffer at 2 times the highest concentration of kinase to be tested. In this example, 50 µg/mL (50,000 ng/mL) was the highest concentration of kinase to be tested, and the stock concentration of kinase was 210 µg/mL.

Calculations:

Kinase: Stock = 210 µg/mL 1x = 50 µg/mL 2x = 100 µg/mL

			[Initial]				[Final 2x]
Kinase:	23.8 µL	*	210 µg/mL	=	50 µL	*	100 µg/mL
Buffer:	26.2 µL kinase reaction buffer						

Procedure:

Add 23.8 µL of 210 µg/mL kinase to 26.2 µL kinase reaction buffer.

Keep the diluted kinase on ice until needed.

(2.2) In a low-volume 384-well plate, fill each well in columns 1–3, rows 2 through 16 (B through P) with 5 µL of kinase reaction buffer. Place 10 µL of the kinase stock 100,000 ng/mL in the top well of each column, and then perform a 2-fold serial dilution down the plate by removing 5 µL of kinase from the top well, adding this to the well below, mixing, and repeating with the next well below. Discard 5 µL of solution from the bottom well such that each well contains 5 µL of kinase solution.

(2.3) In an appropriate container, prepare 1 mL of a solution of substrate and ATP in kinase reaction buffer at 2 times the final concentration of each reagent desired in the assay.

If a 1000 µL solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed with a multichannel pipette.

Calculations:

Substrate: Stock = 472 µM 1x = 1.6 µM 2x = 3.2 µM

ATP: Stock = 10,000 µM 1x = 18 µM 2x = 36 µM

			[Initial]				[Final 2x]
Substrate:	6.8 µL	*	472 µM	=	1000 µL	*	3.2 µM
ATP:	3.6 µL	*	10,000 µM	=	1000 µL	*	36 µM
Buffer:	989.6 µL kinase reaction buffer						

Procedure:

Add 6.8 µL of 472 µM substrate and 3.6 µL of 10,000 µM ATP to 989.6 µL kinase reaction buffer.

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

(2.5) Cover the assay plate and allow 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

			[Initial]				[Final 2x]
EDTA:	40 µL	*	500 mM	=	1000 µL	*	20 mM
Antibody:	1.2 µL	*	3700 nM	=	1000 µL	*	4 nM
Buffer:	958.8 µL TR-FRET Dilution Buffer						

Procedure:

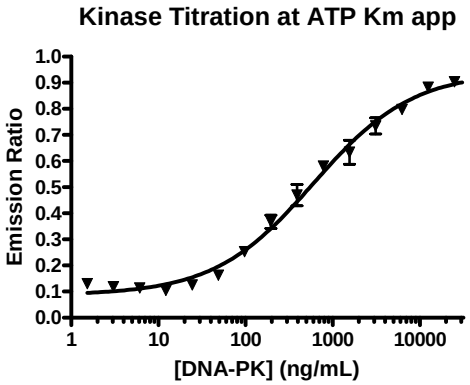
Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer.

(2.7) Add 10µL 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 60 minutes at room temperature before reading on an appropriate plate reader.

(2.9) 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_{50} concentration from the curve. Alternatively, the amount of kinase needed to elicit an 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.

Example of Kinase Titration at ATP $K_{m,app}$



The EC_{50} value determined from the example data was 600 ng/mL kinase. Based on this result, 600 ng/mL kinase was used to determine inhibitor IC_{50} values when performing the assay at 18 µM ATP.

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

Step 3: Determination of Inhibitor IC_{50} Value.

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

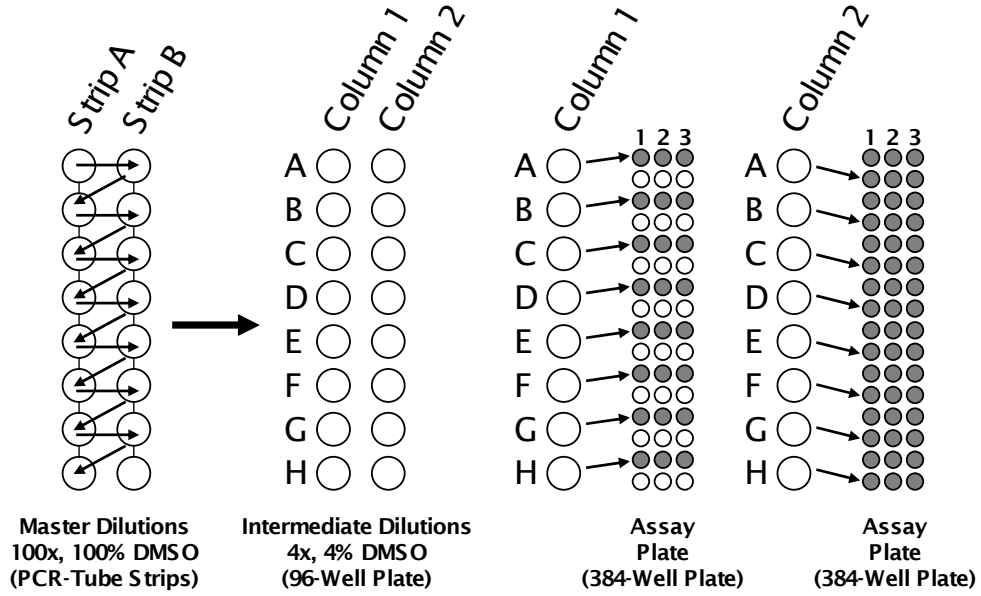
1. Add 2.5 µL of inhibitor in 4% DMSO at 4-fold the final assay concentration to triplicate assay wells.
2. To start the reaction, add 2.5 µL of kinase at 4-fold the final assay concentration, followed by 5.0 µL of substrate + ATP at 2-fold the final reaction concentrations.
3. The remainder of the protocol is similar to previous steps.

(3.2) A dilution series of inhibitor in 100% DMSO is first prepared at 100 times the concentrations to be assayed. By performing the initial dilutions in 100% DMSO, solubility problems associated with dilutions into aqueous buffer can be minimized.

This "master" dilution series of inhibitor can be prepared in two separate 8-tube PCR strips, and stored at -20˚ or -80˚ for use in future experiments. The dilutions are "staggered" between strips as shown in the left side of Figure 4:

1. Add 40 µL of DMSO to tubes 2–8 of strip A, and all tubes of strip B.
2. Add 60 µL of inhibitor in DMSO, at 100-fold the highest concentration to be tested in the experiment, to tube 1 of strip A.
3. Transfer 20 µL of inhibitor from tube 1 of strip A to tube 1 of strip B.
4. After mixing, transfer 20 µL from tube 1 of strip B to tube 2 of strip A.
5. This process is repeated for all but the final tube of strip B, which contains only DMSO (no inhibitor).

Preparing a Dilution Series of Inhibitor



(3.3) From the master dilutions of inhibitor in 100% DMSO, intermediate dilutions are then prepared in two columns of a 96-well plate. The 96-well plate is used only as a convenient vessel for preparing the intermediate dilutions.

1. First, place 96 µL of kinase reaction buffer into all wells of two columns of a 96-well plate.
2. Then, transfer 4 µL of the master inhibitor stock from strip A into column 1 of the 96 well plate, and 4 µL of the master inhibitor stock from strip B into column 2 of the 96-well plate.
3. Mix the solutions well, either with a plate shaker or by mixing with a 20 µL multichannel pipette.

4. Using an 8-channel pipette, add 2.5 µL of inhibitor from the intermediate dilution in the 96-well plate to the 384-well assay plate as shown in figure 4. Use column 1 of the intermediate stock to fill rows A, C, E, etc. of the 384-well assay plate, and column 2 to fill the alternating rows B, D, F, etc.

(3.4) Prepare a 1 mL solution of kinase in kinase reaction buffer at 2x the final desired reaction concentration of the kinase. From Step 2, 4 units/well kinase was determined to be the concentration required for the assay.

Calculation:

Kinase: Initial conc. = 210 µg/mL 1x = 0.6 µg/mL 4x = 2.4 µg/mL

			[Initial]				[Final 4x]
Kinase:	11.4 µL	*	210 µg/mL	=	1000	*	2.4 µg/mL
Buffer:	988.6 µL kinase reaction buffer						

Procedure:

Add 11.4 µL of 210,000ng/mL kinase to 988.6 µL kinase reaction buffer.

(3.5) Add 2.5 µL of the kinase solution prepared in step 3.4 to each well of the assay plate.

(3.6) In an appropriate container, prepare 1 mL of a solution of substrate and ATP in kinase reaction buffer at 2 times the final concentration of each reagent desired in the assay.

If a 1000 µL solution is prepared in a plastic reagent reservoir (trough), then the next addition step can be performed using a multichannel pipette.

Calculations:

Substrate: Stock = 472 µM 1x = 1.6 nM 2x = 3.2 µM

ATP: Stock = 10,000 µM 1x = 18 µM 2x = 36 µM

			[Initial]				[Final 2x]
Substrate:	6.8 µL	*	472 µM	=	1000 µL	*	3.2 µM
ATP:	3.6 µL	*	10,000 µM	=	1000 µL	*	36 µM
Buffer:	989.6 µL kinase reaction buffer						

Procedure:

Add 6.8 µL of 472 µM substrate and 3.6 µL of 10,000 µM ATP to 989.6 µL kinase reaction buffer.

(3.7) Start the kinase reaction by adding 5.0 µL of the substrate + ATP 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 allow reaction to proceed for 1 hour at room temperature.

(3.9) 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 µL	*	500 mM	=	1000 µL	*	20 mM
Antibody:	1.2 µL	*	3300 nM	=	1000 µL	*	4 nM
Buffer:	958.8 µL TR-FRET Dilution Buffer						

Procedure:

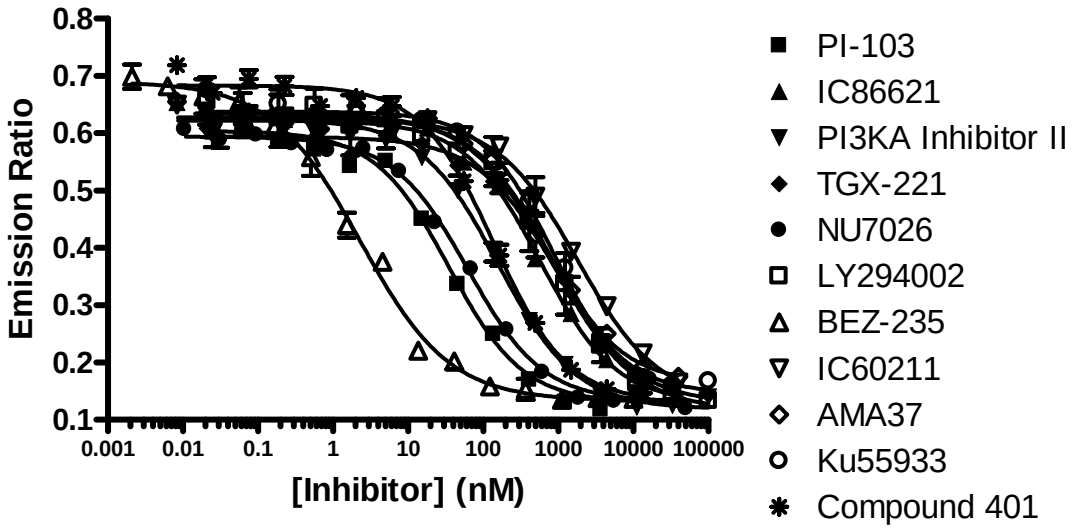
Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer

(3.10) Add 10 µL of the Tb-antibody + EDTA solution prepared in step 3.9 to each well of the assay plate.

(3.11) Cover the assay plate and incubate for 60 minutes at room temperature before reading on an appropriate plate reader.

(3.12) Plot the resulting TR-FRET emission ratio against the concentration of inhibitor, and fit the data to a sigmoidal dose-response curve with a variable slope. Calculate the EC_{50} concentration from the curve. This is equal to the IC_{50} value for the inhibitor.

Example IC_{50} Curves



The inhibition of DNA-PK with the following inhibitors: DNA-PK inhibitor III (IC86621), PI-103, PI3KA Inhibitor II, TGX-221 (PI3-Kβ Inhibitor VI), NU7026 (DNA-PK Inhibitor II), LY294002, BEZ-235, IC60211 (DNA-PK Inhibitor IV), AMA37 (DNA-PK Inhibitor V), Ku55933 (ATM Kinase Inhibitor), and Compound 401 is displayed above.

The IC_{50} values for inhibition of DNA-PK with the above inhibitors are shown in the table below.

		IC_{50} (nM)	
Kinase	**Inhibitor**	**Literature**	**LanthaScreen® Assay**
DNA-PK	IC86621 (DNA-PK inhibitor III)	120[a]/170[d]	380
	PI-103	2[b]	23
	PI3KA Inhibitor II	NR	120
	TGX-221 (PI3-Kβ Inhibitor VI)	NR	880
	NU7026 (DNA-PK Inhibitor II)	230[c]	46
	LY294002	360[a]/660[d]	350
	BEZ-235	NR	2.0
	IC60211 (DNA-PK Inhibitor IV)	400[a]/430[d]	1000
	AMA37 (DNA-PK Inhibitor V)	270[d]	650
	Ku55933 (ATM Kinase Inhibitor)	2500[e]	610
	Compound 401	280[f]	100

NR = No value reported

a) Kashishian, A., et al. 2004. *Mol. Cancer Ther.*, **2**, 1257. b) Knight, Z.A., et al. 2006. *Cell* **125**, 733.
c) Veuger, S.J., et al. 2003. *Cancer Res.* **63,** 6008. d) Knight, Z.A., et al. 2004. *Bioorg. Med. Chem.* **12,** 4749.
e) Hickson, I., et al. 2004. *Cancer Res.* **64,** 9152. f) Ballou, L.M., et al. 2007. *J. Biol. Chem.* **282,** 24463.

For convenience, titration curves can be normalized by dividing all values in the curve by the ratio obtained at the bottom of the curve. This will normalize the titration curves making data comparison between various instruments and gain settings easier. Normalizing the data will have no effect on the IC_{50} values or Z prime.