Optimization of a LanthaScreen™ Kinase assay for FRAP1 (mTOR)

Overview

This protocol describes how to run a LanthaScreen™ kinase assay designed to detect and characterize inhibitors of FRAP1 (mTOR). Separately, we determined the ATP K_m to be 20 μM in a radiometric assay, so to maximize the ability of the assay to detect ATP-competitive inhibitors, we developed the assay using 10 μM ATP. However, the assay could be re-optimized for other ATP concentrations in an analogous manner. The procedure is as follows:

1. **Optimization of kinase concentration required for assay at 10 μM ATP**
   A kinase titration was performed in order to determine the concentration of kinase required to elicit an approximately 50% change between the minimum and maximum TR-FRET emission ratios at the desired ATP concentration (the EC_{50} value). This is the concentration of kinase that will be used in an assay to determine an IC_{50} value for an inhibitor. Note that in many other LanthaScreen™ assays the EC_{80} (rather than EC_{50}) concentration of kinase is used in the inhibitor titration. We have found that the FRAP1 assay is best performed using the lower EC_{50} concentration of kinase, and the signal change remains robust enough to provide excellent data quality and high Z' values.
2. **Titrations with inhibitor**
   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, perform step 1 at the chosen concentration of ATP (to determine the optimal concentration of kinase), 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	1X FRAP1 (mTor) Assay Buffer	PV4794 (20 mL)	(1)
Kinase	FRAP1 (mTOR)	PV4753 (10 µg)	
Antibody	LanthaScreen™ Tb-anti-p4E-BP1 (pThr46)	PV4757 (25 µg) PV4758 (1 mg)	(2)
Substrate	GFP-4E-BP1	PV4759 (20 nmol)	(3)
Antibody Dilution Buffer	TR-FRET Dilution Buffer	PV3574 (100 mL)	(4)
500 mM EDTA	Kinase Quench Buffer	P2832 (10 mL)	
10 mM ATP	10 mM ATP	PV3227 (500 µL)	
FKBP12	FKBP12	#	
Inhibitors	Staurosporine	PHZ1271 (100 µg)	
	PI-103	528100 (Calbiochem)	(5)
	LY294002	PHZ1144 (5 mg)	
	Rapamycin	PHZ1233 (1 mg)	

(1) DTT should be added to the 1x reaction buffer at a final concentration of 2 mM immediately prior to use.
(2) The 4E-BP1 (pThr46) 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.
(3) The substrate is supplied at a concentration of ~20 µM, see the tube for the exact concentration.
(4) The antibody dilution buffer does not contain EDTA. EDTA is added separately, prior to addition of antibody.
(5) Alternative name for PI-103: 3-(4-(4-morpholinyl)pyrido[3',2':4,5]furo[3,2-d]pyrimidin-2-yl)phenol

Preparing the 1x Kinase Reaction Buffer

The 1x kinase reaction buffer is stable at room temperature. DTT (dithiothreitol) should be added to the 1x reaction buffer at final concentration of 2 mM immediately prior to use.

General Assay Conditions

Kinase reactions are performed in a 10 µL volume in low-volume 384-well plates. Typically, Corning model 3676 (black) or 3673 (white) plates are used. The concentration of substrate in the assay is 400 nM, and the 1x kinase reaction buffer consists of 50 mM HEPES pH 7.5, 0.01% Polysorbate 20, 1 mM EGTA, 10 mM $MnCl_2$, and 2 mM DTT. Kinase reactions are allowed to proceed for 1 hour at room temperature before a 10 µL preparation of EDTA (20 mM) and Tb-labeled antibody (4 nM) in TR-FRET dilution buffer are added. The final concentration of antibody in the assay well is 2 nM, and the final concentration of EDTA is 10 mM. The plate is allowed to incubate at room temperature for at least 30 minutes before being read on a plate reader configured for LanthaScreen™ TR-FRET.

Plate Readers

The data presented in this document were generated using a Tecan Infinite F500 plate reader using the LanthaScreen™ filters. The assay can be performed on a variety of plate readers including those from Tecan (Ultra, Safire^2), Molecular Devices (Analyst and M5), BMG LABTECH PHERAstar and 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.

Example Protocols

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

Step 1: Titration of Kinase at ATP K_m (or an alternative ATP concentration).

(1.1) In an appropriate tube or vial, prepare 100 µL of kinase in kinase reaction buffer at 2 times the highest concentration of kinase to be tested. In this example, 10 µg/mL (10,000 ng/mL) was the desired highest concentration of kinase to be tested, and the stock concentration of kinase was 450 µg/mL.

Calculation:

Kinase: Stock = 450 µg/mL 1x = 10 µg/mL 2x = 20 µg/mL

			[Initial]				[Final 2x]
Kinase:	4.4 µL	*	450 µg/mL	=	100 µL	*	20 µg/mL
Buffer:	95.6 µL kinase reaction buffer						

Procedure:

Add 4.4 µL of 450 µg/mL kinase to 95.6 µL kinase reaction buffer.

Keep the diluted kinase on ice until needed.

(1.2) 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 solution as prepared above 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.

(1.3) 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.

For this particular example, you will be assaying FRAP1 at 10 µM ATP.

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

Calculations:

Substrate: Stock = 20.09 µM 1x = 0.4 µM 2x = 0.8 µM

ATP: Stock = 10 mM 1x =0.01 mM 2x = 0.02 mM

			[Initial]				[Final 2x]
Substrate:	39.8 µL	*	20.09 µM	=	1000 µL	*	0.8 µM
ATP:	2 µL	*	10 mM	=	1000 µL	*	0.02 mM
Buffer:	958.2 µL kinase reaction buffer						

Procedure:

Add 39.8 µL of 20.09 µM substrate and 2 µL of 10 mM ATP to 958.2 µL kinase reaction buffer.

(1.4) Start the kinase reaction by adding 5 µL of the substrate + ATP solution prepared in step 1.3 to each well of the assay plate.

(1.5) Cover the assay plate and allow reaction to proceed for 1 hour at room temperature.

(1.6) Prior to completion of the kinase reaction, prepare 1 mL of a solution of EDTA and Tb-labeled antibody at 2 times the desired final concentrations of each reagent in TR-FRET dilution buffer. The antibody is stable in EDTA for several hours, but because it is sensitive to high concentrations of EDTA we recommend first adding the concentrated EDTA to the dilution buffer, mixing the solution well, and then adding the antibody before mixing further.

Calculations:

EDTA:	Stock = 500 mM	1x = 10 mM	2x = 20 mM
Antibody:	Stock = 3300 nM	1x = 2 nM	2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	40 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.

(1.7) Add 10µL of the Tb-antibody + EDTA solution prepared in step 1.6 to each well of the assay plate and mix briefly, either by pipette or on a plate shaker.

(1.8) Cover the assay plate and incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

(1.9) Plot the resulting TR-FRET emission ratio against the concentration of ATP, and fit the data to a sigmoidal dose-response curve with a variable slope. Calculate the EC_{50} concentration from the curve. The following equation can be used with GraphPad™ Prism® software:

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F=50
logEC50=logECF-(1/HillSlope)*log(F/(100-F))
Y=Bottom+(Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))
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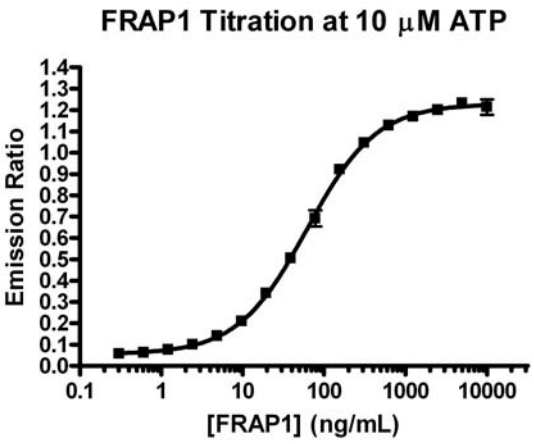
Alternatively, the amount of kinase needed to elicit a 50% change in TR-FRET response may be estimated from a visual inspection of the curve. It is important that the reactions performed to determine the IC_{50} value of an inhibitor be performed at or below the EC_{50} concentration of the kinase determined from this graph.

Figure 1: Example of Kinase Titration at 10 µM ATP.



The EC_{50} value determined from the example data was 61.7 ng/mL kinase. Based on this result, 61.7 ng/mL FRAP1 was used to determine inhibitor IC_{50} values when performing the assay at 10 µM ATP.

Step 2: Determination of Inhibitor IC_{50} Value.

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

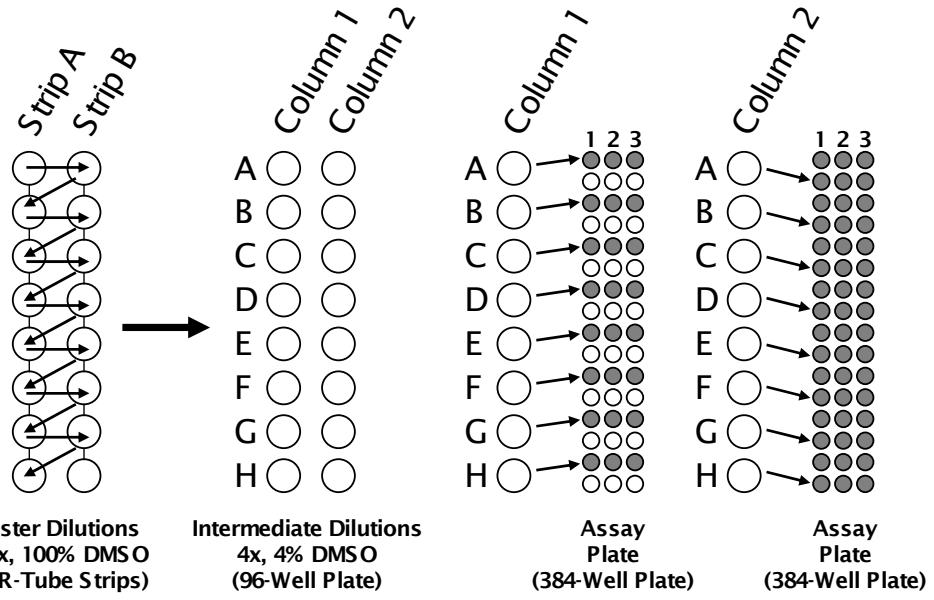
1. Add 2.5 µL of inhibitor in 4% DMSO at 4-fold the final assay concentration to triplicate assay wells.
2. To start the reaction, add 2.5 µL of kinase at 4-fold the final assay concentration, followed by 5 µL of substrate + ATP at 2-fold the final reaction concentrations.
3. The remainder of the protocol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

(2.2) 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 2:

1. Add 50 µL of DMSO to tubes 2–8 of strip A, and all tubes of strip B.
2. Add 100 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 50 µL of inhibitor from tube 1 of strip A to tube 1 of strip B.
4. After mixing, transfer 50 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).

Figure 2: Preparing a Dilution Series of Inhibitor



(2.3) 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 2. 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.

(2.4) Prepare a solution of kinase in kinase reaction buffer at 4x the final desired reaction concentration of the kinase. From Step 1, 61.7 ng/mL FRAP1 was determined to be the concentration required for the assay.

Calculation:

Kinase: Initial conc. = 450 µg/mL 1x = 61.7 ng/mL 4x = 246.8 ng/mL

			[Initial]				[Final 4x]
Kinase:	2 µL	*	450 µg/mL	=	3.6 mL	*	0.247 µg/mL
Buffer:	3.6 mL kinase reaction buffer						

Procedure:

Add 2 µL of 450 µg/mL kinase to 3.5 mL kinase reaction buffer. (Note: you are preparing more kinase than you need, but it is difficult to accurately pipette less than 2 µL volumes. We recommend that the kinase be prepared fresh, and not from the dilution made in step 1.1)

(2.5) Add 2.5 µL of the kinase solution prepared in step 2.4 to each well of the assay plate.

(2.6) 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 the 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 = 20.09 µM 1x = 0.4 µM 2x = 0.8 µM

ATP: Stock = 10 mM 1x = 0.01 mM 2x = 0.02 mM

			[Initial]				[Final 2x]
Substrate:	39.8 µL	*	20.09 µM	=	1000 µL	*	0.8 µM
ATP:	2 µL	*	10 mM	=	1000 µL	*	0.02 mM
Buffer:	958.2 µL kinase reaction buffer						

Procedure:

Add 39.8 µL of 20.09 µM substrate and 2 µL of 10 mM ATP to 958.2 µL kinase reaction buffer.

(2.7) Start the kinase reaction by adding 5 µL of the substrate + ATP 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 allow reaction to proceed for 1 hour at room temperature.

(2.9) 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:

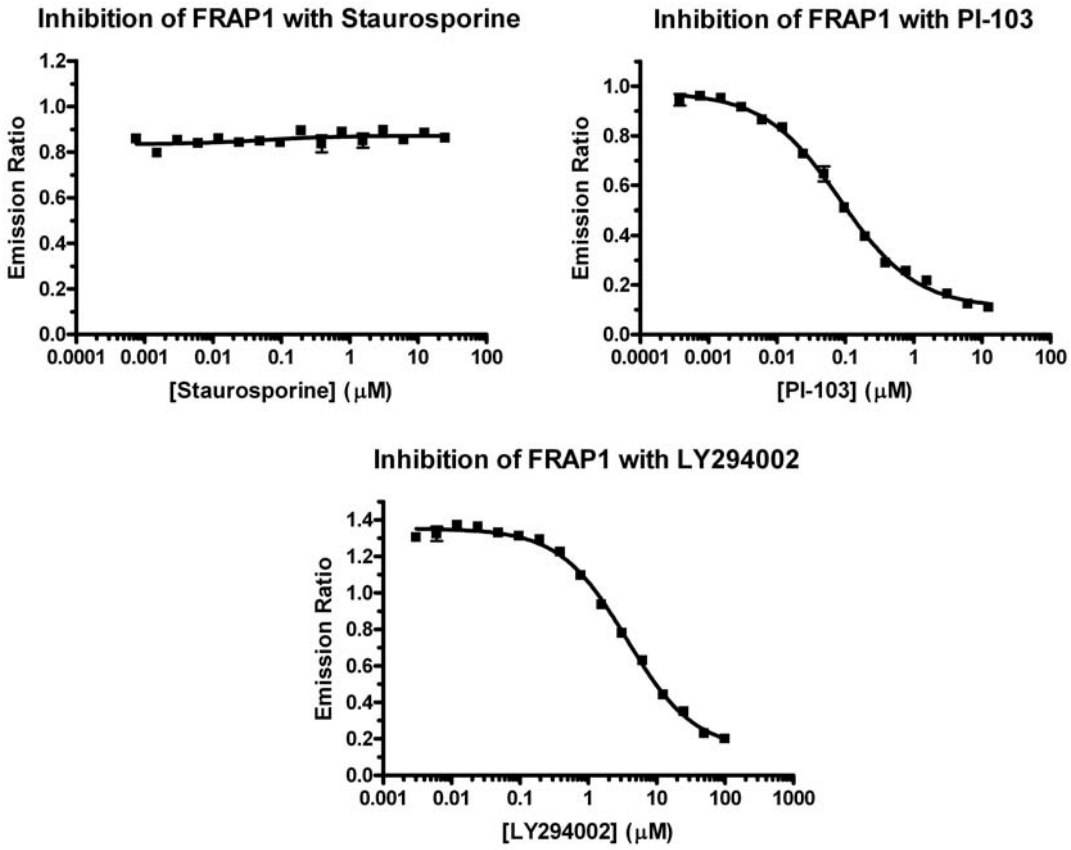
Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer

(2.10) Add 10 µL of the Tb-antibody + EDTA solution prepared in step 2.9 to each well of the assay plate.

(2.11) Cover the assay plate and incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

(2.12) 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.

Figure 3: Example IC_{50} Curves



The IC_{50} values for PI-103, and LY294002 determined from the example data was 0.085 μM and 3.6 μM, respectively. As expected, the titration of staurosporine with FRAP1 does not show any inhibition.

Step 3: Rapamycin Inhibitor IC_{50} Value Determination with FKBP12.

(3.1) A Rapamycin master mix and intermediate dilution series should be made in a similar fashion as outlined in step 2.2 – 2.3 above.

(3.2) Prepare a 246.8 ng/mL solution of kinase as in step 2.4.

(3.3) Add 2.5 μL of the kinase solution prepared in step 3.2 to each well of the assay plate.

(3.4) In an appropriate container, prepare 1 mL of a solution of substrate, FKBP12, and ATP in kinase reaction buffer at 2 times the final concentration of each reagent desired in the assay.

If the 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 = 20.09 μM	1x = 0.4 μM	2x = 0.8 μM
FKBP12:	Stock = 100 μM	1x = 0.1 μM	2x = 0.2 μM
ATP:	Stock = 10 mM	1x = 0.01 mM	2x = 0.02 mM

			[Initial]				[Final 2x]
Substrate:	39.8 µL	*	20.09 µM	=	1000 µL	*	0.8 µM
FKBP12	2 µL	*	100 µM	=	1000 µL	*	0.2 µM
ATP:	2 µL	*	10 mM	=	1000 µL	*	0.02 mM
Buffer:	956.2 µL kinase reaction buffer						

Procedure:

Add 39.8 µL of 20.09 µM substrate, 2 µL of 100 µM FKBP12, and 2 µL of 10 mM ATP to 956.2 µL kinase reaction buffer.

(3.5) Start the kinase reaction by adding 5 µL of the substrate, FKBP12, and ATP solution prepared in step 3.4 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 allow reaction to proceed for 1 hour at room temperature.

(2.9) 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:

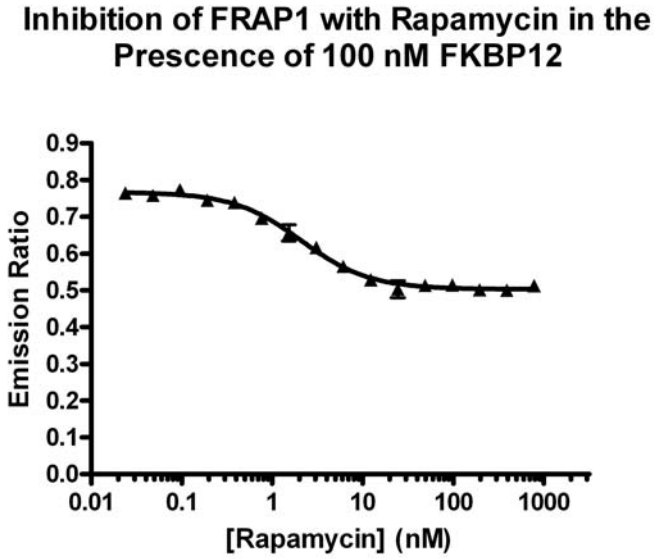
Add 40 µL of 500 mM EDTA and 1.2 µL of 3300 nM antibody to 958.8 µL TR-FRET Dilution Buffer

(2.10) Add 10 µL of the Tb-antibody + EDTA solution prepared in step 2.9 to each well of the assay plate.

(2.11) Cover the assay plate and incubate for 30 minutes at room temperature before reading on an appropriate plate reader.

(2.12) 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.

Figure 4: Example of IC_{50} Curves



The IC_{50} value for Rapamycin with 100 nM FKBP12 determined from the example data was 2.2 nM. The incomplete inhibition is consistent with literature reports showing that rapamycin-FKBP12 does not fully inhibit phosphorylation of all phosphorylation sites on 4E-BP1. See: L.P. McMahon *et al.* Mol Cell Biol 22 (2002) 7428-7438.