Initial Optimization of a LanthaScreen™ Kinase assay for MAP3K10 (MLK2)

Optimization of a LanthaScreen™ kinase assay for a particular kinase consists of determining the amount of kinase required to perform inhibitor IC_{50} determinations at a specific ATP concentration. In order to maximize assay sensitivity towards ATP competitive inhibitors, the ATP concentration used is typically at or below the ATP K_m value.

In this example protocol, no attempt was made to determine the ATP K_m value or to perform an inhibitor IC_{50} experiment. Instead, as an initial proof-of-principle experiment, a kinase titration was performed using 500 µM ATP, and fluorescein-MAP2K1 as the substrate. The EC_{50} value of this kinase titration was determined, which is the concentration of kinase that would then be used to perform an inhibitor IC_{50} titration when assaying the kinase ay 500 µM ATP. In an analogous manner, the experiment described in this document could be repeated at a different concentration of ATP in order to determine assay conditions at that concentration of ATP.

Full assay validation data including the determination of inhibitor IC_{50} values has been generated for bRAF and cRAF using the substrate an antibody combination used here. This data can be found at www.invitrogen.com/ lanthascreenkinase.

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: *None*	*n.a.*	
Kinase	MAP3K10 (MLK2)	PV3877	
Antibody	LanthaScreen™ Tb-anti-pMAP2K1 (pSer217/221)	PV4813 (25 µg) PV4814 (1 mg)	(2)
Substrate	Fluorescein-MAP2K1	PV4812 (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	

(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 MAP2K1 (pSer217/221) antibody is supplied at approximately 0.25 mg/mL. The molecular weight of the antibody is 150 kD. Thus, the stock concentration of the antibody is 1.7 µM, or 1700 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.

Preparing the 1x Kinase Reaction Buffer

Prepare a 1x solution of kinase reaction buffer from the 5x Kinase Buffer stock (listed above) by adding 4 mL of 5x stock to 16 mL H_2O to make 20 mL of 1x kinase reaction buffer.

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 200 nM, 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. 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.

Experimental Protocol

(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 240 µg/mL.

Calculation:

Kinase: Stock = 240 µg/mL 1x = 10 µg/mL 2x = 20 µg/mL

			[Initial]				[Final 2x]
Kinase:	8.3 µL	*	240 µg/mL	=	100 µL	*	20 µg/mL
Buffer:	91.7 µL kinase reaction buffer						

Procedure:

Add 8.3 µL of 240 µg/mL kinase to 91.7 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 MAP3K10 (MLK2) at 500 µM ATP.

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 = 19.7 µM 1x = 0.2 µM 2x = 0.4 µM

ATP: Stock = 10 mM 1x =0.5 mM 2x = 1 mM

			[Initial]				[Final 2x]
Substrate:	20.3 µL	*	19.7 µM	=	1000 µL	*	0.4 µM
ATP:	100 µL	*	10 mM	=	1000 µL	*	1 mM
Buffer:	879.7 µL kinase reaction buffer						

Procedure:

Add 20.3 µL of 19.7 µM substrate and 100 µL of 10 mM ATP to 879.7 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 = 1700 nM	1x = 2 nM	2x = 4 nM

			[Initial]				[Final 2x]
EDTA:	40 µL	*	500 mM	=	1000 µL	*	20 mM
Antibody:	2.4 µL	*	1700 nM	=	1000 µL	*	4 nM
Buffer:	957.6 µL TR-FRET Dilution Buffer						

Procedure:

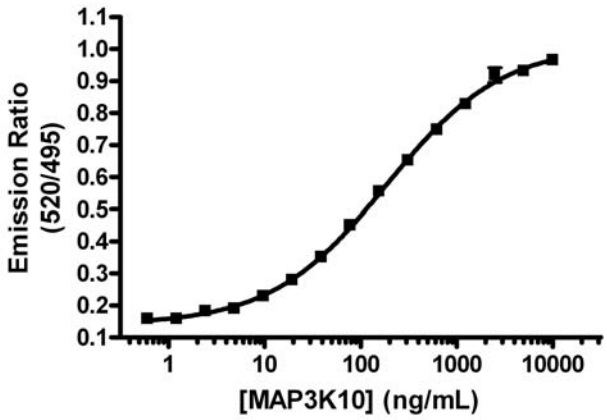
Add 40 µL of 500 mM EDTA and 2.4 µL of 1700 nM antibody to 957.6 µL TR-FRET Dilution Buffer.

(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. 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 500 µM ATP.



The EC_{50} value determined from the example data was 186.5 ng/mL MAP3K10 (MLK2). This is the concentration of kinase that would be used in an experiment to determine an IC_{50} value for an inhibitor when running the assay at 500 µM ATP. To determine an IC_{50} value at a different concentration of ATP, the above experiments would be conducted at that concentration of ATP in order to determine the appropriate concentration of kinase to use in such an experiment.