Optimization of a LanthaScreen™ Kinase assay for cRAF

Overview

This protocol describes how to develop a LanthaScreen™ kinase assay designed to detect and characterize inhibitors of cRAF using fluorescein-labeled MAP2K1 as a substrate. In the experiments presented in this document, the amount of enzyme required for the assay was optimized for a variety of ATP concentrations, and then inhibitor titrations were performed under the conditions determined for that concentration of ATP.

1. **Optimization of kinase concentration required for assay at different concentrations of ATP**. A titration of kinase against 200 nM fluorescein-labeled MAP2K1 is performed using different concentrations of ATP 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 that concentration of ATP. This is the optimal concentration of kinase that will be used in an assay to determine an IC_{50} value for an inhibitor.
2. **Inhibitor IC_{50} Determination.**
   Using the ATP and kinase concentrations 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 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 and Equipment Required

Description	Part Name	Catalog #	Notes
Kinase Reaction Buffer	5X Kinase Buffer	PV3189 (4 mL of 5X)	(1)
	Additives: *None*	*n.a.*	
Kinase	cRAF	PV3805 (10 µg)	
Antibody	LanthaScreen™ Tb-anti-pMAP2K1 [pS$^{217/221}$] Ab	PV4813 (25 µg) PV4814 (1 mg)	(2)
Substrate	Fluorescein-MAP2K1, inactive	PV4812 (20 nmoles)	(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)	

(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 Tb-anti-pMAP2K1 [pS$^{217/221}$] 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 1.67 µM, or 1670 nM. The antibody concentration may vary slightly from lot-to-lot. Perform all calculations based on the actual stock concentration of antibody.
(3) The substrate is supplied at a concentration of 20 µM, but may vary from lot-to-lot. Perform all calculations based on the actual stock concentration of substrate.
(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. 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 BMG 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, Safire^2, and InfiniTE F500), Molecular Devices (Analyst and M5), 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.

Step 1: Optimization of kinase concentration required for assay at different concentrations of ATP

In this step, a titration of kinase is performed against 200 nM fluorescein-labeled MAP2K1 at 5 different concentrations of ATP. This is done 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 that concentration of ATP, which is the optimal concentration of kinase to use at that concentration of ATP in order to determine an inhibitor IC_{50} value.

This step is written to first prepare 24-point dilution series of kinase in a 96-well plate, that is then transferred to a 384-well assay plate. Separately, substrate + ATP solutions are prepared at 5 separate concentrations of ATP, which are then added to the assay plate to start the kinase reaction.

1) To a 96 well plate (for example, Nunc part # 249944), add 100 µL of 1X kinase buffer to columns 2 through 12 of row A and columns 1 through 12 of row B.

2) In well A1 of the 96-well plate, dilute the kinase stock into 1X kinase buffer to 2-times the highest concentration of kinase to be tested. Prepare 200 µL of this 2X kinase stock. In this example, 10 µg/mL was the highest desired concentration of kinase to be tested, and the stock concentration of kinase was 360 µg/mL. Mix by gentle pipetting up and down.

Calculations:

Kinase: Stock = 360 µg/mL 1x = 10 µg/mL 2x = 20 µg/mL

			[Initial]				[Final 2x]
Kinase:	11.1 µL	*	360 µg/mL	=	200 µL	*	20 µg/mL
Buffer:	189.9 µL kinase reaction buffer						

Procedure:

Add 11.1 µL of 360 µg/mL kinase to 189.9 µL kinase reaction buffer.

3) Prepare a 2 fold serial dilution of cRAF in 1X assay buffer as follows: take 100 µL of enzyme from A1 and transfer to the 100 µL of 1X assay buffer in B1. Mix by pipetting up and down. Transfer 100 µL of enzyme from B1 to the 100 µL of assay buffer in A2. Mix by pipetting up and down. Transfer 100 µL of enzyme from A2 to the 100 µL of assay buffer in B2. Mix by pipetting up and down. Repeat this pattern to well A12. Discard the final 100 µL from well A12 (do not transfer to well B12 – this will be the no enzyme control). All wells should contain 100 µL in volume. The 2 fold serial diluted cRAF enzyme at 2X concentration should now be arranged in the 96 well plate as follows (100 µL/well in 1X kinase buffer, concentrations shown in ng/mL):

	1	2	3	4	5	6	7	8	9	10	11	12
A	20,000	5000	1250	312.5	78.125	19.53	4.88	1.22	0.305	0.076	0.019	0.0048
B	10,000	2500	625	156.25	39.1	9.77	2.44	0.61	0.153	0.038	0.0095	0

4) Using a 12 channel pipette, transfer 5 µL of 2X kinase from row A of the 96 well plate to the ODD columns (1, 3, 5, 7, 9, etc.) of a Corning 3676 black low-volume NBS round bottom 384 well plate in rows A - O. Using a 12 channel pipette, transfer 5 µL of 2X cRAF from row B of the 96 well plate to the EVEN columns (2, 4, 6, 8, 10, etc.) of the same 384 well plate in rows A - O. This will complete the 23 point 2 fold serial dilution of cRAF at 2X concentration across the 384 well plate.

5) In 5 separate reagent reservoirs (troughs), prepare 750 µL solutions of substrate + ATP solutions at 2-fold the concentration to be used in the assay. The 1x concentration of substrate will be 200 nM in all assays, and the 1x ATP concentrations will be 1, 10, 100, 500, and 1000 µM

Pool A: 1x ATP = 1 µM

Calculations:

Substrate:	Stock = 19.7 µM	1x = 0.2 µM	2x = 0.4 µM
ATP:	Stock = 1 mM*	1x = 1 µM	2x = 2 µM

* 1 mM ATP is made by adding 10 µL of 10 mM stock to 90 µL H_20.

			[Initial]				[Final 2x]
Substrate:	15.2 µL	*	19.7 µM	=	750 µL	*	0.4 µM
ATP:	1.5 µL	*	1 mM	=	750 µL	*	2 µM
Buffer:	733.3 µL kinase reaction buffer						

Add 15.2 µL of 19.7 µM substrate and 1.5 µL of 1 mM ATP to 733.3 µL kinase reaction buffer.

Pools B, C, D, and E are prepared analogously, except 10 mM ATP is used to prepare the solutions:

Pool B: 1x ATP = 10 µM

Add 15.2 µL of 19.7 µM substrate and 1.5 µL of 10 mM ATP to 733.3 µL kinase reaction buffer.

Pool C: 1x ATP = 100 µM

Add 15.2 µL of 19.7 µM substrate and 15 µL of 10 mM ATP to 719.8 µL kinase reaction buffer.

Pool D: 1x ATP = 500 µM

Add 15.2 µL of 19.7 µM substrate and 75 µL of 10 mM ATP to 659.8 µL kinase reaction buffer.

Pool E: 1x ATP = 1000 µM

Add 15.2 µL of 19.7 µM substrate and 150 µL of 10 mM ATP to 584.8 µL kinase reaction buffer.

6) Using a multi-channel pipette:

- add 5 µL of 2X substrate/ATP "pool A" to the 5 µL of 2X enzyme in rows A, B, and C
- add 5 µL of 2X substrate/ATP "pool B" to the 5 µL of 2X enzyme in rows D, E, and F
- Repeat for pools C, D, and E following the 384 well plate layout listed below.

The final kinase reaction will be 10 µL in volume and will contain 200 nM Fluorescein-MAP2K1 at 5 different ATP concentrations (1, 10, 100, 500, and 1000 µM).

The assay plate layout is shown below.

Final 1X cRAF concentration (ng/mL) per 10 µL kinase reaction

	10,000	5000	2500	1250	625	312.5	156.25	78.13	39.1	19.53	9.77	4.88	2.44	1.22	0.61	0.31	0.153	0.076	0.038	0.019	0.0095	0.0048	0.0024	0
<>	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
A																								
B							**P o o l A (1 µM ATP)**																	
C																								
D																								
E							**P o o l B (10 µM ATP)**																	
F																								
G																								
H							**P o o l C (100 µM ATP)**																	
I																								
J																								
K							**P o o l D (500 µM ATP)**																	
L																								
M																								
N							**P o o l E (1000 µM ATP)**																	
O																								

7) Briefly shake the plate and incubate at room temperature for 1 hour. Cover the plate loosely with aluminum foil in order to prevent evaporation.

8) Prior to completion of the kinase reaction, prepare 5 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:	200 µL	*	500 mM	=	5000 µL	*	20 mM
Antibody:	11.8 µL	*	1700 nM	=	5000 µL	*	4 nM
Buffer:	4788 µL TR-FRET Dilution Buffer						

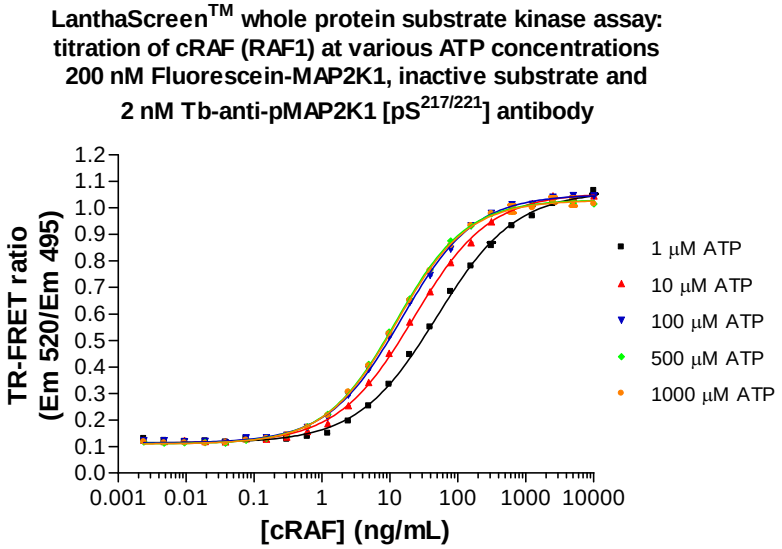
Procedure:

Add 200 µL of 500 mM EDTA and 11.8 µL of 1700 nM antibody to 4788 µL TR-FRET Dilution Buffer.

9) After the 1 hour kinase reaction, add 10 µL of the Tb-antibody + EDTA solution prepared in step 8 to each well of the assay plate and mix briefly, either by pipette or on a plate shaker.

10) Cover the assay plate and incubate for 1 hour at room temperature before reading on an appropriate plate reader.

11) 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.



	1 µM ATP	10 µM ATP	100 µM ATP	500 µM ATP	1000 µM ATP
BOTTOM	0.1135	0.1122	0.1127	0.1092	0.1090
TOP	1.063	1.057	1.050	1.031	1.029
LOGEC50	1.687	1.347	1.169	1.085	1.099
HILLSLOPE	0.7469	0.7746	0.8078	0.8358	0.8308
EC50	48.68	22.23	14.75	12.16	12.55

Step 2: IC_{50} determination of ATP competitive inhibitors at different ATP concentrations

In this step of the protocol, inhibitor IC_{50} values are determined using the ATP and kinase concentrations determined in step 1.

The general format for determination of an inhibitor IC_{50} value is as follows:

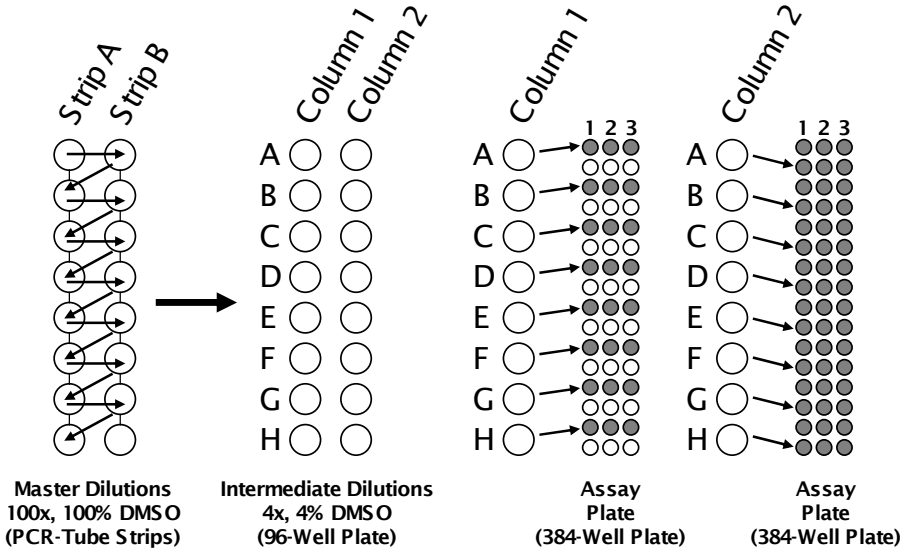
- 2.5 µL of inhibitor, at 4-fold the final assay concentration in 4% DMSO, is added to triplicate assay wells.
- A 5 µL solution of kinase and substrate, at 2-fold the final assay concentration, is then added and allowed to incubate with the inhibitor for 15 minutes.
- 2.5 uL of ATP, at 4-fold the final reaction concentration, is added to start the reaction.
- The remainder of the protocol is similar to previous steps.

First, a dilution series of inhibitor in 100% DMSO is 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 the figure below. For example, to perform a 15 point 3-fold titration of compound,

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. In this example, the RAF inhibitor GW 5074 titration is started at 1X = 50 µM, therefore the 100X master titration is started with 60 µL of 5 mM GW 5074.
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).

Schematic for Preparing a Dilution Series of Inhibitor:



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.

Once the intermediate dilutions of inhibitor have been prepared, the assay protocol is as follows:

1) Using a multi-channel pipette, add 2.5 µL of inhibitor to the assay plate as shown above. Column 1 of the intermediate stock is used to fill rows A, C, E, etc. of the 384-well assay plate (columns 1-15), and column 2 of the intermediate stock is used to fill the alternating rows B, D, F, etc. of the 384-well assay plate (columns 1-15).

2) In 5 separate reagent reservoirs (troughs), prepare 750 µL solutions of substrate + kinase solutions at 2-fold the concentration to be used in the assay. The 1x concentration of substrate will be 200 nM in all assays, and the kinase concentrations will be the EC_{50} value that was determined for each ATP concentration in step 1.

Since the concentration of kinase to be used is low, first prepare an intermediate 1:100 dilution of kinase by adding 2 µL of kinase to 198 µL kinase buffer. With a stock concentration of kinase of 360 µg/mL, this prepares an intermediate dilution of 3.6 µg/mL.

Pool A: 1x kinase = 48 ng/mL (For use with 1x ATP = 1 µM)

Calculations:

Substrate:	Stock =	19.7 µM	1x = 0.2 µM	2x = 0.4 µM
Kinase:	Intermediate =	3.6 µg/mL	1x = 48 ng/mL	2x = 96 ng/mL

			[Initial]				[Final 2x]
Substrate:	15.2 µL	*	19.7 µM	=	750 µL	*	0.4 µM
Kinase:	20 µL	*	3.6 µg/mL	=	750 µL	*	96 ng/mL
Buffer:	714.8 µL kinase reaction buffer						

Procedure:
Add 15.2 µL of 19.7 µM substrate and 20 µL of 3.6 µg/mL kinase to 714.8 µL kinase reaction buffer.

Pools B, C, D, and E are prepared analogously:

Pool B: 1x kinase = 22 ng/mL (For use with 1x ATP = 10 µM)
Add 15.2 µL of 19.7 µM substrate and 9.2 µL of 3.6 µg/mL kinase to 725.6 µL kinase reaction buffer.

Pool C: 1x kinase = 15 ng/mL (For use with 1x ATP = 100 µM)
Add 15.2 µL of 19.7 µM substrate and 6.25 µL of 3.6 µg/mL kinase to 728.6 µL kinase reaction buffer.

Pool D: 1x kinase = 12 ng/mL (For use with 1x ATP = 500 μM)
Add 15.2 μL of 19.7 μM substrate and 5 μL of 3.6 μg/mL kinase to 729.8 μL kinase reaction buffer.

Pool E: 1x kinase = 12 ng/mL (For use with 1x ATP = 1000 μM)
Add 15.2 μL of 19.7 μM substrate and 5 μL of 3.6 μg/mL kinase to 729.8 μL kinase reaction buffer.

3) Using a multi-channel pipette:

- add 5 μL of 2X substrate/kinase "pool A" to the 2.5 μL of 4X inhibitor in columns 1-3
- add 5 μL of 2X substrate/kinase "pool B" to the 2.5 μL of 4X inhibitor in columns 4-6
- Repeat for pools C, D, and E following the 384 well plate layout shown below.

		1X ATP:	1 μM (pool 1)			10 μM (pool 2)			100 μM (pool 3)			500 μM (pool 4)			1000 μM (pool 5)		
		1X cRAF:	49 ng/mL (pool A)			22 ng/mL (pool B)			15 ng/mL (pool C)			12 ng/mL (pool D)			12 ng/mL (pool E)		
1X [GW 5074] (nM)			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
50000	A																
16666.67	B																
5555.56	C																
1851.85	D																
617.28	E																
205.76	F																
68.59	G																
22.86	H																
7.62	I																
2.540	J																
0.847	K																
0.282	L																
0.094	M																
0.031	N																
0.010	O																
0	P																

4) If desired, the kinase should be allowed to incubate for 15 minutes with the inhibitor before adding ATP (described below) to start the reaction.

5) In reagent reservoirs, prepare 5 separate 500 uL solutions of ATP at 4x the concentration to be used in the assay.

Pool 1: 1x ATP = 1 μM (4x ATP = 4 μM)
For Pool 1 only, first prepare an intermediate dilution of 1 mM ATP by adding 10 μL of 10 mM stock to 90 μL H_20.
Add 2 uL of 1 mM ATP to 498 uL kinase reaction buffer

Pool 2: 1x ATP = 10 μM (4x ATP = 40 μM)
Add 2 uL of 10 mM ATP to 498 uL kinase reaction buffer

Pool 3: 1x ATP = 100 μM (4x ATP = 400 μM)
Add 20 uL of 10 mM ATP to 480 uL kinase reaction buffer

Pool 4: 1x ATP = 500 μM (4x ATP = 2000 μM)
Add 100 uL of 10 mM ATP to 400 uL kinase reaction buffer

Pool 5: 1x ATP = 1000 μM (4x ATP = 4000 μM)
Add 200 uL of 10 mM ATP to 300 uL kinase reaction buffer

6) Using a multi-channel pipette:

- add 2.5 μL of 4X ATP "pool 1" to 7.5 μL of enzyme+substrate in columns 1, 2, and 3.
- add 2.5 μL of 4X ATP "pool 2" to 7.5 μL of enzyme+substrate in columns 4, 5, and 6.
- Repeat for remaining pools following the plate layout above.

7) Briefly shake the plate and incubate at room temperature for 1 hour. Cover the plate loosely with aluminum foil in order to protect from light.

8) Prior to completion of the kinase reaction, prepare 3.5 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:	140 μL	*	500 mM	=	3500 μL	*	20 mM
Antibody:	8.2 μL	*	1700 nM	=	3500 μL	*	4 nM
Buffer:	3352 μL TR-FRET Dilution Buffer						

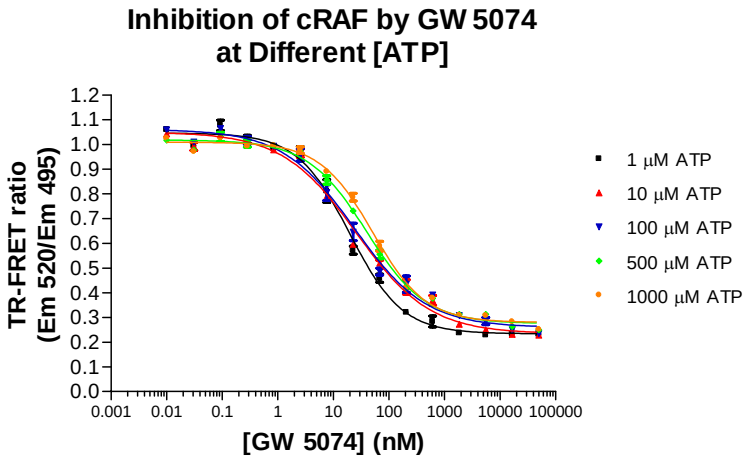
Procedure:
Add 140 μL of 500 mM EDTA and 8.2 μL of 1700 nM antibody to 3352 μL TR-FRET Dilution Buffer.

9) After the 1 hour kinase reaction, add 10 μL of the Tb-antibody + EDTA solution prepared in step 8 to each well of the assay plate and mix briefly, either by pipette or on a plate shaker.

10) Cover the assay plate and incubate for 1 hour at room temperature before reading on an appropriate plate reader.

11) 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.

Example data:



	1 μM ATP	10 μM ATP	100 μM ATP	500 μM ATP	1000 μM ATP
BOTTOM	0.2337	0.2336	0.2593	0.2751	0.2797
TOP	1.047	1.051	1.062	1.018	1.010
LOGEC50	1.295	1.414	1.382	1.603	1.709
HILLSLOPE	-0.9156	-0.6551	-0.6755	-0.8169	-0.9116
EC50	19.72	25.94	24.09	40.04	51.22

Published IC_{50} = 9 nM

Conclusions

Based upon the results obtained, an ATP concentration of 1 μM should be used to assay for inhibitors of cRAF in this assay format, as this concentration of ATP will provide greatest sensitivity towards inhibitors. Because kinase activity can vary between different lots of kinase, or because of laboratory temperature or other factors, it is recommended that the following experiments be performed to optimize a cRAF assay:

(1) In a manner analogous to step one above, perform a titration of cRAF against a fixed concentration of fluorescein-MAP2K1 using 1 μM ATP in the reaction. Determine the amount of kinase required to elicit a 50% change in the TR-FRET signal.

(2) Using the concentration of kinase determined in the first step, run the assay against a dilution series of inhibitor in manner analogous to step two above.

Quick Reference

Optimization of kinase concentration:

Addition 1: 5 µL of 2X enzyme titration

Addition 2: 5 µL of 2X substrate / 2X ATP

Incubate for 60 minutes at room temperature.

Addition 3: 10 µL 2X EDTA / 2X Antibody in TR-FRET dilution buffer

Incubate for 60 minutes at room temperature, read plate using the appropriate LanthaScreen™ settings. Determine the EC_{50} value. This is the concentration of kinase to be used in the IC_{50} experiment.

IC_{50} determination of ATP competitive compounds:

Addition 1: 2.5 µL of 4X inhibitor / 4% DMSO

Addition 2: 5 µL of 2X substrate / 2X kinase

Incubate for 15 minutes at room temperature.

Addition 3: 2.5 µL of 4X ATP

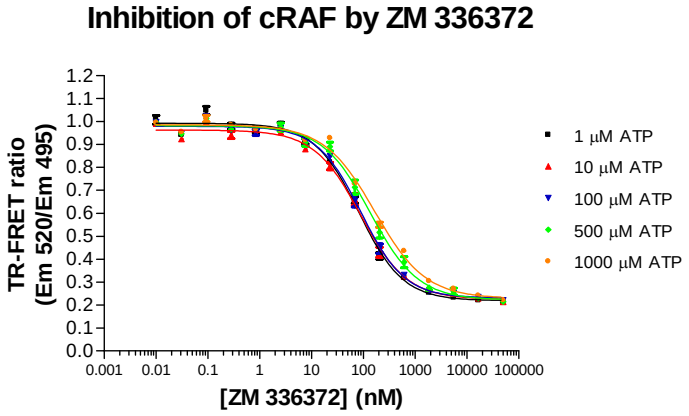
Incubate for 60 minutes at room temperature.

Addition 4: 10 µL 2X EDTA / 2X Antibody in TR-FRET dilution buffer

Incubate for 60 minutes at room temperature, read plate using the appropriate LanthaScreen™ settings. Determine the IC_{50} value.

Supplemental Data

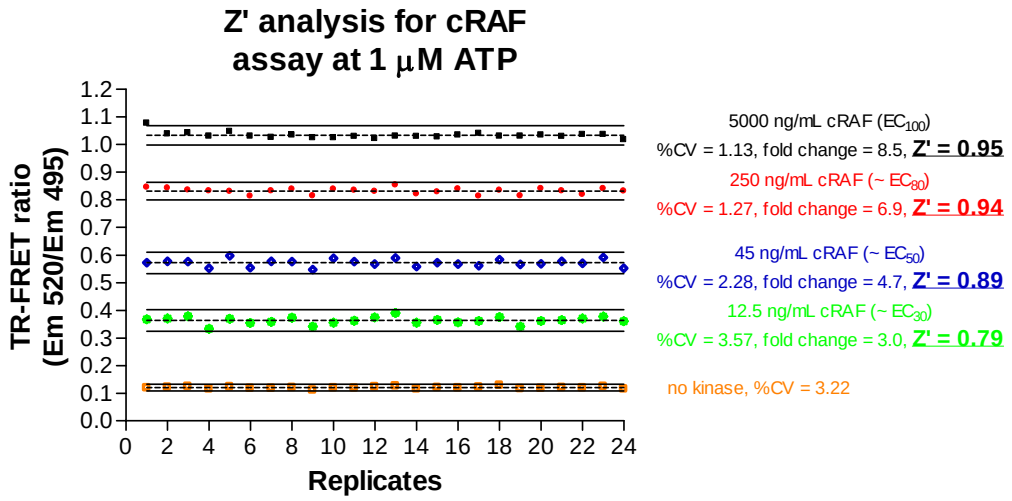
IC_{50} value determination for the ATP competitive RAF inhibitor ZM 336372 (Biomol EI-298) against cRAF (RAF1). The assay was run as outlined in section 2 of this protocol:



	1 µM ATP	10 µM ATP	100 µM ATP	500 µM ATP	1000 µM ATP
BOTTOM	0.2190	0.2250	0.2306	0.2220	0.2291
TOP	0.9922	0.9628	0.9794	0.9821	0.9858
LOGEC50	1.911	1.947	1.954	2.135	2.222
HILLSLOPE	-1.013	-0.9670	-1.031	-0.9380	-0.8865
EC50	81.52	88.49	89.96	136.5	166.8

Published IC_{50} = 70 nM

Z′ Determination at various [cRAF] when using 1 µM ATP:



ng/mL cRAF	5000 (~ EC_{100})	250 (~ EC_{80})	45 (~ EC_{50})	12.5 (~ EC_{30})
fold change	8.5	6.9	4.7	3.0
%CV	1.13	1.27	2.28	3.57
Z' value	**0.95**	**0.94**	**0.89**	**0.79**