

Optimization of a LanthaScreen™ Kinase assay for BRAF

Overview

This protocol describes how to develop a LanthaScreen™ kinase assay designed to detect and characterize inhibitors of BRAF 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 IC₅₀ value for an inhibitor.

2. Inhibitor IC₅₀ 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 IC₅₀) 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 Additives: <i>None</i> | PV3189 (4 mL of 5X) <i>n.a.</i> | (1) |
| Kinase Antibody | BRAF LanthaScreen™ Tb-anti- pMAP2K1 [pS ^{217/221}] Ab | PV3848 (10 µg) 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 H₂O 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 MgCl₂, 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, Safire², 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 IC₅₀ 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 μ L 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 μ L of this 2X kinase stock. In this example, 10 μ g/mL was the highest desired concentration of kinase to be tested, and the stock concentration of kinase was 460 μ g/mL. Mix by gentle pipetting up and down.

Calculations:

Kinase: Stock = 460 μ g/mL 1x = 10 μ g/mL 2x = 20 μ g/mL

| | | | | | | | |
|---------|--------------------------------------|------------------|----------------|---|-------------|-------------------|---------------|
| | | <u>[Initial]</u> | | | | <u>[Final 2x]</u> | |
| Kinase: | 8.7 μ L | * | 460 μ g/mL | = | 200 μ L | * | 20 μ g/mL |
| Buffer: | 191.3 μ L kinase reaction buffer | | | | | | |

Procedure:

Add 8.7 μ L of 460 μ g/mL kinase to 191.3 μ L kinase reaction buffer.

- 3) Prepare a 2 fold serial dilution of BRAF in 1X assay buffer as follows: take 100 μ L of enzyme from A1 and transfer to the 100 μ L of 1X assay buffer in B1. Mix by pipetting up and down. Transfer 100 μ L of enzyme from B1 to the 100 μ L of assay buffer in A2. Mix by pipetting up and down. Transfer 100 μ L of enzyme from A2 to the 100 μ L of assay buffer in B2. Mix by pipetting up and down. Repeat this pattern to well A12. Discard the final 100 μ L from well A12 (do not transfer to well B12 – this will be the no enzyme control). All wells should contain 100 μ L in volume. The 2 fold serial diluted BRAF enzyme at 2X concentration should now be arranged in the 96 well plate as follows (100 μ L/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 kinase 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 BRAF 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 H₂O.

| | | | <u>[Initial]</u> | | | <u>[Final 2x]</u> |
|------------|--|---|--------------------|---|-------------------|---------------------|
| 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 BRAF 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 | P o o l A (1 μM ATP) | | | | | | | | | | | | | | | | | | | | | | | | |
| B | | | | | | | | | | | | | | | | | | | | | | | | | |
| C | | | | | | | | | | | | | | | | | | | | | | | | | |
| D | P o o l B (10 μM ATP) | | | | | | | | | | | | | | | | | | | | | | | | |
| E | | | | | | | | | | | | | | | | | | | | | | | | | |
| F | | | | | | | | | | | | | | | | | | | | | | | | | |
| G | P o o l C (100 μM ATP) | | | | | | | | | | | | | | | | | | | | | | | | |
| H | | | | | | | | | | | | | | | | | | | | | | | | | |
| I | | | | | | | | | | | | | | | | | | | | | | | | | |
| J | P o o l D (500 μM ATP) | | | | | | | | | | | | | | | | | | | | | | | | |
| K | | | | | | | | | | | | | | | | | | | | | | | | | |
| L | | | | | | | | | | | | | | | | | | | | | | | | | |
| M | P o o l E (1000 μM ATP) | | | | | | | | | | | | | | | | | | | | | | | | |
| N | | | | | | | | | | | | | | | | | | | | | | | | | |
| 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

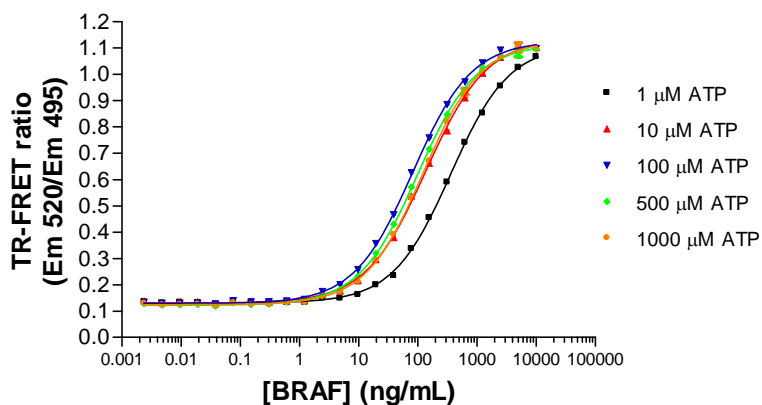
| | | | | | |
|-----------|--------------------------------------|---|------------------|----------------|-------------------|
| | | | <u>[Initial]</u> | | <u>[Final 2x]</u> |
| EDTA: | 200 μ L | * | 500 mM | = 5000 μ L | * 20 mM |
| Antibody: | 11.8 μ L | * | 1700 nM | = 5000 μ L | * 4 nM |
| Buffer: | 4788 μ L TR-FRET Dilution Buffer | | | | |

Procedure:

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

- 9) After the 1 hour kinase reaction, add 10 μ L 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 EC₅₀ concentration from the curve.

**LanthaScreen™ whole protein substrate kinase assay:
 titration of BRAF at various ATP concentrations
 200 nM Fluorescein-MAP2K1, inactive substrate and
 2 nM Tb-anti-pMAP2K1 [pS^{217/221}] antibody**



| | 1 μ M ATP | 10 μ M ATP | 100 μ M ATP | 500 μ M ATP | 1000 μ M ATP |
|-----------|---------------|----------------|-----------------|-----------------|------------------|
| BOTTOM | 0.1308 | 0.1302 | 0.1286 | 0.1221 | 0.1237 |
| TOP | 1.113 | 1.139 | 1.127 | 1.116 | 1.128 |
| LOGEC50 | 2.561 | 2.143 | 1.918 | 1.998 | 2.093 |
| HILLSLOPE | 0.8804 | 0.8467 | 0.8732 | 0.8730 | 0.8860 |
| EC50 | 364.0 | 138.8 | 82.73 | 99.55 | 123.9 |

Step 2: IC₅₀ determination of ATP competitive inhibitors at different ATP concentrations

In this step of the protocol, inhibitor IC₅₀ values are determined using the ATP and kinase concentrations determined in step 1.

The general format for determination of an inhibitor IC₅₀ value is as follows:

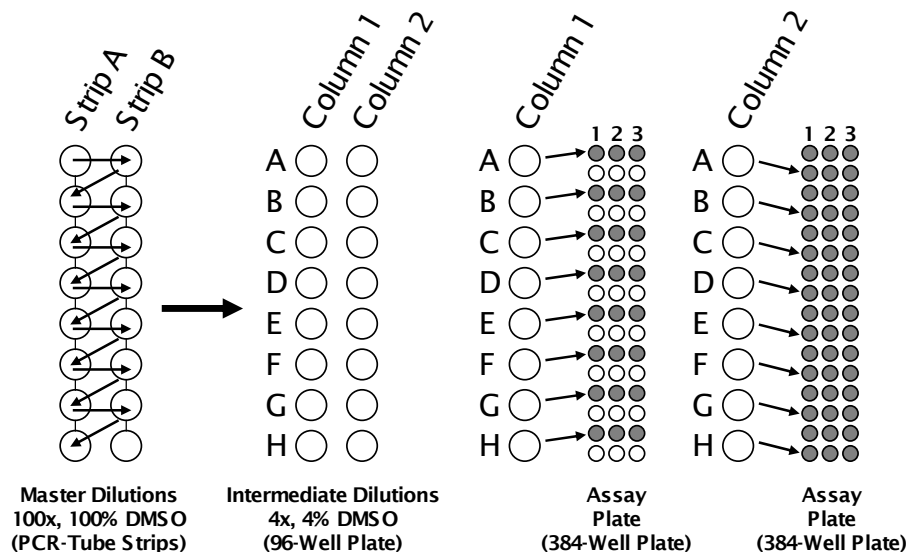
- 2.5 µL of inhibitor, at 4-fold the final assay concentration in 4% DMSO, is added to triplicate assay wells.
- A 5 µL solution of kinase and substrate, at 2-fold the final assay concentration, is then added and allowed to incubate with the inhibitor for 15 minutes.
- 2.5 µL of ATP, at 4-fold the final 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 1:10 dilution of kinase by adding 4 μL of kinase to 36 μL kinase buffer. With a stock concentration of kinase of 460 $\mu\text{g}/\text{mL}$, this prepares an intermediate dilution of 46 $\mu\text{g}/\text{mL}$.

Pool A: 1x kinase = 360 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 = 46 $\mu\text{g}/\text{mL}$ 1x = 360 ng/mL 2x = 720 ng/mL

| | | | [Initial] | | [Final 2x] |
|------------|--|---|----------------------------|---------------------|---------------------|
| Substrate: | 15.2 μL | * | 19.7 μM | = 750 μL | * 0.4 μM |
| Kinase: | 11.7 μL | * | 46 $\mu\text{g}/\text{mL}$ | = 750 μL | * 720 ng/mL |
| Buffer: | 714.8 μL kinase reaction buffer | | | | |

Procedure:

Add 15.2 μL of 19.7 μM substrate and 11.7 μL of 46 $\mu\text{g}/\text{mL}$ kinase to 723.1 μL kinase reaction buffer.

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

Pool B: 1x kinase = 135 ng/mL (For use with 1x ATP = 10 μM)

Add 15.2 μL of 19.7 μM substrate and 4.4 μL of 46 $\mu\text{g}/\text{mL}$ kinase to 730.4 μL kinase reaction buffer.

Pool C: 1x kinase = 80 ng/mL (For use with 1x ATP = 100 μM)

Add 15.2 μL of 19.7 μM substrate and 2.6 μL of 46 $\mu\text{g}/\text{mL}$ kinase to 732.2 μL kinase reaction buffer.

Pool D: 1x kinase = 95 ng/mL (For use with 1x ATP = 500 μ M)

Add 15.2 μ L of 19.7 μ M substrate and 3.1 μ L of 46 μ g/mL kinase to 731.7 μ L kinase reaction buffer.

Pool E: 1x kinase = 120 ng/mL (For use with 1x ATP = 1000 μ M)

Add 15.2 μ L of 19.7 μ M substrate and 3.9 μ L of 46 μ g/mL kinase to 730.9 μ L kinase reaction buffer.

3) Using a multi-channel pipette:

- add 5 μ L of 2X substrate/kinase “pool A” to the 2.5 μ L of 4X inhibitor in columns 1-3
- add 5 μ L of 2X substrate/kinase “pool B” to the 2.5 μ L 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 μ M (pool 1) | | | 10 μ M (pool 2) | | | 100 μ M (pool 3) | | | 500 μ M (pool 4) | | | 1000 μ M (pool 5) | | |
|-------------------|---|-----------------------------|---|---|---------------------|---|---|----------------------|---|---|----------------------|----|----|-----------------------|----|----|
| | | 1X BRAF: 360 ng/mL (pool A) | | | 135 ng/mL (pool B) | | | 80 ng/mL (pool C) | | | 95 ng/mL (pool D) | | | 120 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 μ M (4x ATP = 4 μ M)

For Pool 1 only, first prepare an intermediate dilution of 1 mM ATP by adding 10 μ L of 10 mM stock to 90 μ L H₂O.

Add 2 uL of 1 mM ATP to 498 uL kinase reaction buffer

Pool 2: 1x ATP = 10 μ M (4x ATP = 40 μ M)

Add 2 uL of 10 mM ATP to 498 uL kinase reaction buffer

Pool 3: 1x ATP = 100 μ M (4x ATP = 400 μ M)

Add 20 uL of 10 mM ATP to 480 uL kinase reaction buffer

Pool 4: 1x ATP = 500 μ M (4x ATP = 2000 μ M)

Add 100 uL of 10 mM ATP to 400 uL kinase reaction buffer

Pool 5: 1x ATP = 1000 μ M (4x ATP = 4000 μ M)

Add 200 μ L of 10 mM ATP to 300 μ L kinase reaction buffer

- 6) Using a multi-channel pipette:
- add 2.5 μ L of 4X ATP “pool 1” to 7.5 μ L of enzyme+substrate in columns 1, 2, and 3.
 - add 2.5 μ L of 4X ATP “pool 2” to 7.5 μ L 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

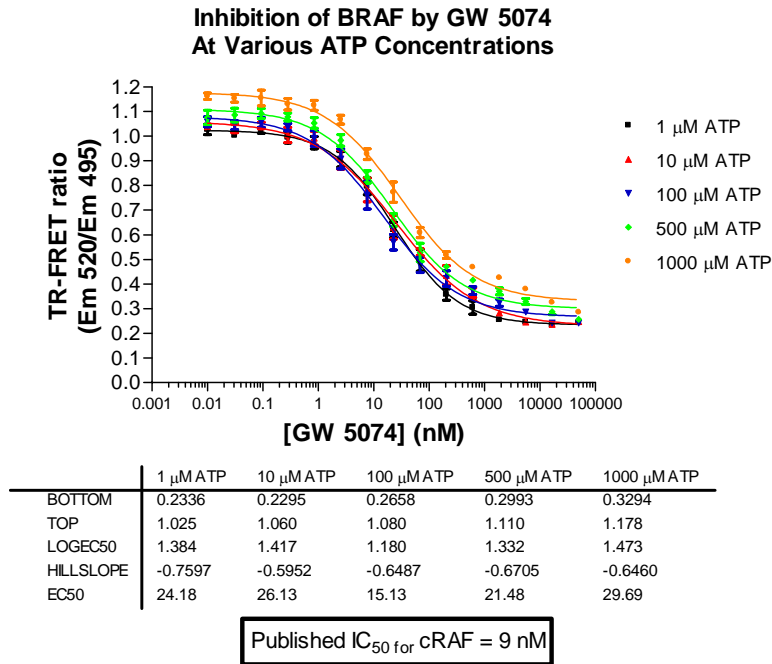
| | | | | | | |
|-----------|--------------------------------------|---|------------------|---|--------------|-------------------|
| | | | <u>[Initial]</u> | | | <u>[Final 2x]</u> |
| EDTA: | 140 μ L | * | 500 mM | = | 3500 μ L | * 20 mM |
| Antibody: | 8.2 μ L | * | 1700 nM | = | 3500 μ L | * 4 nM |
| Buffer: | 3352 μ L TR-FRET Dilution Buffer | | | | | |

Procedure:

Add 140 μ L of 500 mM EDTA and 8.2 μ L of 1700 nM antibody to 3352 μ L TR-FRET Dilution Buffer.

- 9) After the 1 hour kinase reaction, add 10 μ L 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 EC₅₀ concentration from the curve.

Example data:



Conclusions

Based upon the results obtained, an ATP concentration of 100 μ M should be used to assay BRAF in this assay format. 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 BRAF assay:

- (1) In a manner analogous to step one above, perform a titration of BRAF against a fixed concentration of fluorescein-MAP2K1 using 100 μ M 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 μ L of 2X enzyme titration

Addition 2: 5 μ L of 2X substrate / 2X ATP

Incubate for 60 minutes at room temperature.

Addition 3: 10 μ L 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 EC₅₀ value. This is the concentration of kinase to be used in the IC₅₀ experiment.

IC₅₀ determination of ATP competitive compounds:

Addition 1: 2.5 μ L of 4X inhibitor / 4% DMSO

Addition 2: 5 μ L of 2X substrate / 2X kinase

Incubate for 15 minutes at room temperature.

Addition 3: 2.5 μ L of 4X ATP

Incubate for 60 minutes at room temperature.

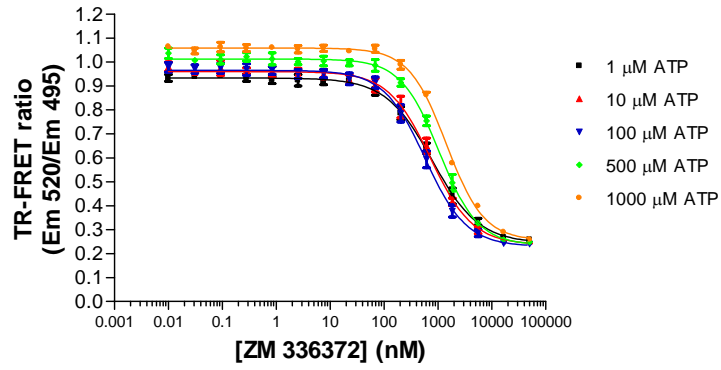
Addition 4: 10 μ L 2X EDTA / 2X Antibody in TR-FRET dilution buffer

Incubate for 60 minutes at room temperature, read plate using the appropriate LanthaScreen™ settings. Determine the IC₅₀ value.

Supplemental Data

IC₅₀ value determination for the ATP competitive RAF inhibitor ZM 336372 (Biomol EI-298) against BRAF. The assay was run as outlined in section 2 of this protocol:

Inhibition of BRAF by ZM 336372



| | 1 μM ATP | 10 μM ATP | 100 μM ATP | 500 μM ATP | 1000 μM ATP |
|-----------|----------|-----------|------------|------------|-------------|
| BOTTOM | 0.2437 | 0.2372 | 0.2298 | 0.2356 | 0.2550 |
| TOP | 0.9336 | 0.9601 | 0.9655 | 1.013 | 1.059 |
| LOGEC50 | 2.897 | 2.863 | 2.763 | 3.027 | 3.160 |
| HILLSLOPE | -1.010 | -1.113 | -1.128 | -1.217 | -1.251 |
| EC50 | 788.0 | 730.2 | 580.0 | 1064 | 1444 |

Published IC₅₀ for cRAF = 70 nM
10-fold less potent against BRAF (~ 700 nM)