AQT1076 – MAPK3/1 (ERK1/2) Assay Validation

PhosphoSens®–Lysate Assay Format

Outline for this Study

PhosphoSens-Lysate Assay Validation

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Lysate Source:

NIH-3T3 Cells (ATCC, CRL-1658.2) +/- 25 ng/mL PDGF-bb (ThermoFisher, 100-14B-10UG)

See slides 3-4 for Preparation of Crude Cell Lysates from NIH3T3 Cells Treated +/- PDGF to Show Activation of ERK1/2

Reference Compound Information:

SCH772984 (MedChemExpress, HY-50846)

Vx-11e (MedChemExpress, HY-14178)

Experimental Validation at AssayQuant:

NIH-3T3 cell lysate (activated with 25 ng/mL PDGF) titration

Detecting ERK1/2 Activity Across a Variety of Cell Types

AQT1076 substrate K_m determination

DMSO Tolerance Test

Reference Compound IC_{50} Determinations

Preparation of Crude Cell Lysates from NIH3T3 Cells Treated +/- PDGF to Show Activation of ERK1/2

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1) NIH-3T3 Cells (ATCC, CRL-1658.2) were plated in 6-well tissue culture-treated plates and incubated for 48 hours at 37°C in DMEM Medium with 10% FBS and PenStrep in an atmosphere of 5% CO_2. Cells were then serum-starved in culture medium with 0.1% FBS (ThermoFisher, A56708-01) for 24 hours and incubated for 15 minutes with or without 25 ng/mL PDGF-bb (ThermoFisher, 100-14B-10UG). Cells were then washed with PBS, and lysed with lysis buffer containing:

   - 50 mM HEPES, pH 7.4
   - 150 mM NaCl
   - 2 mM EGTA
   - 1 mM DTT
   - 1% Triton X-100
   - 30 mM NaF
   - 10 mM $Na_4P_2O_7$
   - 100 µM Na_3VO_4
   - 50 mM β-glycerophosphate
   - Protease Inhibitor Cocktail diluted 60-fold into lysis buffer (Sigma-Aldrich product no. #P8340)
   - Phosphatase Inhibitor Cocktail diluted 60-fold into lysis buffer (Sigma-Aldrich product no. #P2850)

2) The DNA strands were broken by briefly sonicating on ice for 2 seconds on low power. Lysates were used immediately or aliquoted and frozen at –80 °C. 1 µg of each Lysate was run on a gel, transferred to a nitrocellulose membrane, and the signal developed by Western blotting using antibodies to total ERK1/2 (Cell Signaling, #4696) and phospho-ERK1/2 (T202/Y204) (Cell Signaling, #4370).

Basal vs Stimulated and Lysate Titration

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

1 mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.54 mM EGTA

10 mM $MgCl_2$

15 µM AQT1076

Crude Lysate in µg/well of total protein (determined by the Bradford method) from NIH-3T3 cells activated treated +/- 25 ng/mL PDGF:

- Single dose: 0.25 µg/well
- Titration: 0, 0.039, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, 5, 10 µg/well NIH3T3 lysates (from cells activated with 25 ng/mL PDGF)

Reaction Set Up:

20 µL Reaction Mix with AQT1076, ATP, & DTT
15 minutes incubation at 30°C (in the reader)
5 µL Lysate (diluted to 5x in Enzyme dilution buffer, EDB) or EDB with lysate buffer)
25 µL Final reaction volume

Reaction was run at 30 ˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 20 or 25 µL final well volume or in in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 µL final well volume after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

Notes:

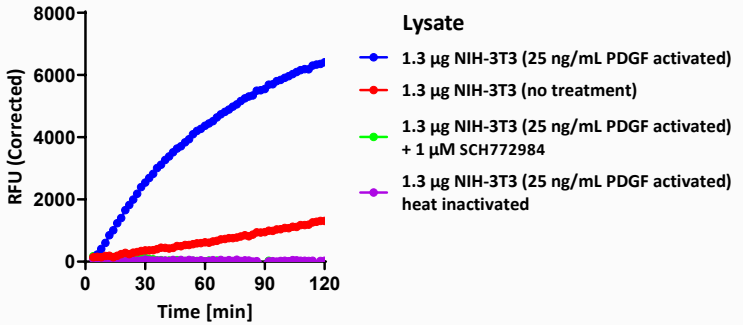
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

ERK1/2 Lysate Activity Assay

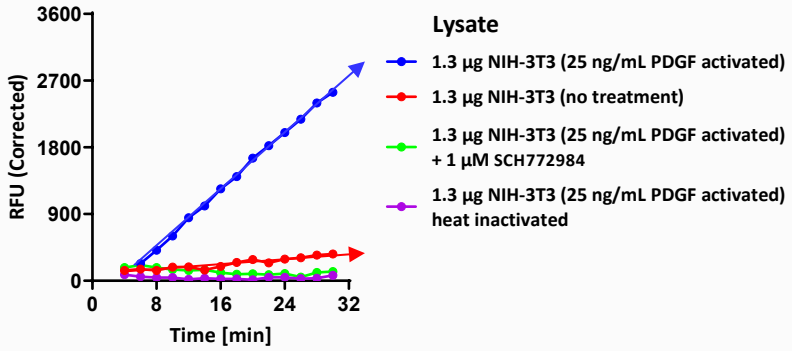
To Determine the Activation of ERK1/2 in Lysates from ± PDGF-treated Cells

A. Crude Lysate Samples

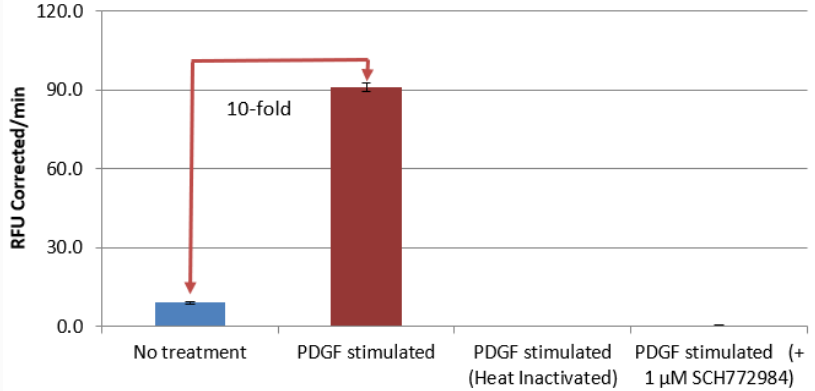
1) Full Time Course (0-120 min.)



2) Linear Range (4-30 min.)

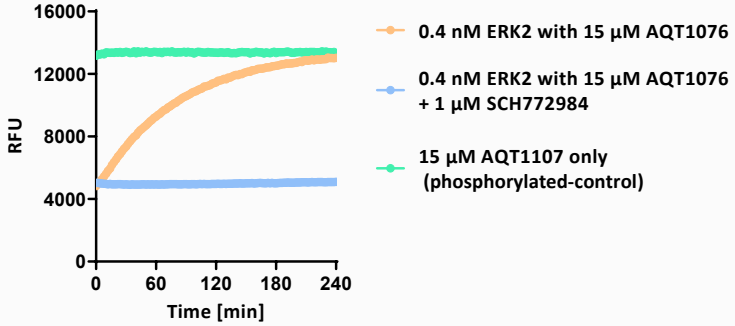


3) Histogram



B. Purified ERK2 & AQT1107 Control

1) Full Time Course (0-240 min.)



A. Crude lysate samples: The AQT1076 sensor peptide was used to generate RFU Corrected values (Total – Background) for **1)** Full progress curve time course (0-120 min.), and **2)** Linear range (4-30 min.), which was used to determine the slope for each condition and the results are shown as Reaction rates (RFU Corrected/min. +/- standard deviations) as a histogram in **3)**, highlighting a 10-fold activation of ERK1/2 kinase activity in lysates from NIH3T3 cells treated with PDGF at 25 ng/mL for 15 minutes. The signal was eliminated by heat inactivation of the lysate or by adding the selective ERK1/2 inhibitor SCH772984. The amount of activation depends on several factors, including cell type, serum concentration and duration of the pre-incubation to make cells quiescent, and the nature, concentration, and duration of the activating stimulus. These conditions can be varied to determine the effect on ERK1/2 activity. The total amount of ERK1 and 2 protein can be determined by Western Blotting or an ELISA using kits available; however, with the short stimulation times typically used, these levels are not expected to change.

B.1. Purified recombinant ERK2 enzyme & AQT1107 Control: The ERK2 protein (0.4 nM) fully phosphorylated the AQT1076 sensor peptide substrate by 240 min., as shown by convergence with the signal obtained with the AQT1107 phosphopeptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). The signal with ERK2 enzyme was eliminated by adding the SCH772984 inhibitor. The signal with AQT1107 is used to convert RFU (Corrected) values to nmoles of Phosphate.

ERK1/2 Lysate Western Blots

A Commonly Used Method to Assess ERK1/2 Activation

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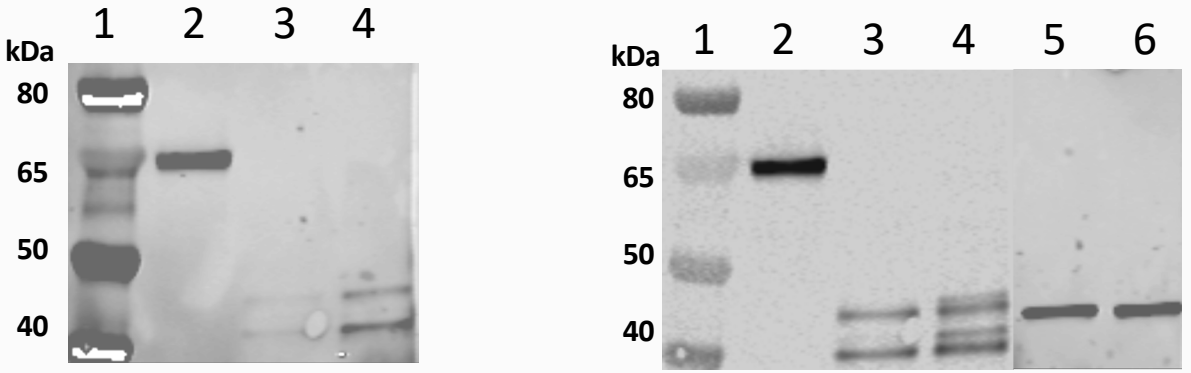
Phospho-ERK1/2

Loading Controls

Total ERK1/2 **Actin**



Lane Description:

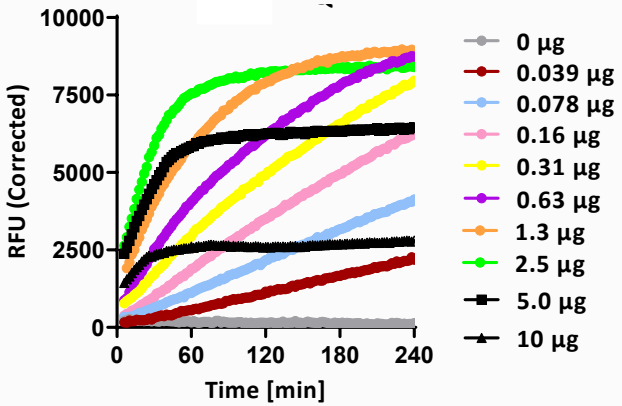
1. MW Markers
2. Recombinant ERK2 (8 ng)
3. NIH-3T3 (no activation)
4. NIH-3T3 (+ 25 ng/mL PDGF for 15 minutes)
5. NIH-3T3 (no activation) – Loading Control
6. NIH-3T3 (+ 25 ng/mL PDGF for 15 minutes) – Loading Control

Membranes were blocked in TBST with 5% BSA for 2 hours and then incubated for 2 hours with anti-ERK1/2 (Cell Signaling Technology, 4696) and anti-phospho-ERK1/2 (Cell Signaling Technology, 4370) diluted 2,000-fold in TBST with 2% BSA (Lanes 2-4) or anti-beta actin (Cell Signaling Technology, 4970) diluted 5000-fold in TBST with 2% BSA (Lanes 5-6). Membranes were washed 3 times for 10 minutes each in TBST and then incubated for 2 hours with goat anti-mouse antibody (LI-COR, IR Dye 800CW 926-32210) and/or donkey anti-rabbit antibody (LI-COR, IR Dye 680RD 926-68073) diluted 20,000-fold in TBST with 2% BSA. Membranes were washed 3 times for 10 minutes each in TBST and then read in a LI-COR Odyssey.

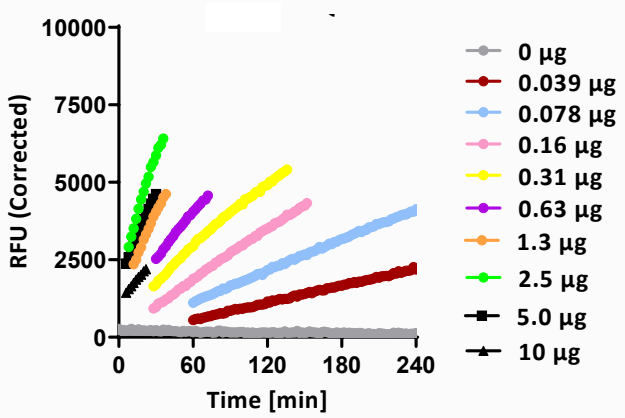
Lysate Titration

To Determine the Linearity and Sensitivity Using Lysate from + PDGF-treated Cells

1. Full Time Course Progress Curves

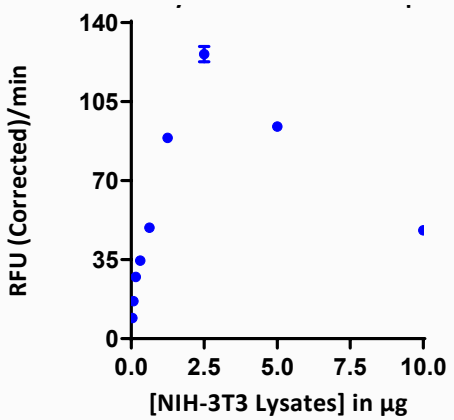


2. Linear Range of Progress Curves

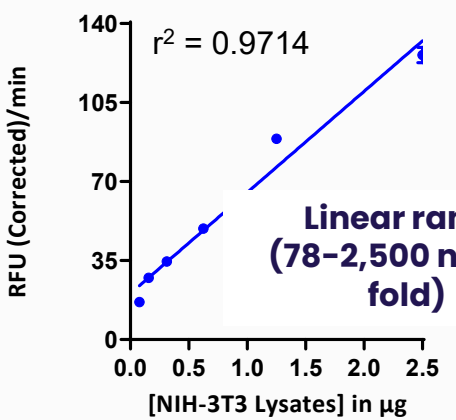


3. Reaction Rate vs Amount of Lysate per Well

A. All Lysate Amounts



B. Lysate Amounts in Linear Range



The AQT1076 sensor peptide was used at 15 µM with an increasing amount of lysate from NIH3T3 cells treated with PDGF to activate the ERK1/2 MAPK enzymes. RFU Corrected values (Total – Background) were determined for each condition. The results are presented for each amount of lysate for **1)** Full time course of each progress curve (0-240 min.), and **2)** Linear range of each progress curve, which was used to determine the slope for each amount of lysate. The results were then plotted as Reaction rates (RFU Corrected/min. +/- standard deviations) for all lysate amounts **3A)**, or those within the linear range as determined by an r^2 value > 0.95. Having the concentration of crude lysate samples at 1 mg/mL or higher, ensures that the amount of CEB in the reaction is minimized, even at the highest concentrations to avoid any inhibition of the kinase activity that can reduce the linear range.

The PhosphoSens-Lysate kinase activity assay for ERK1/2 provides a selective, highly quantitative, and accurate measure of kinase activity in a complex sample.

Detecting ERK1/2 Activity with AQT1076 Across a Variety of Cell Types

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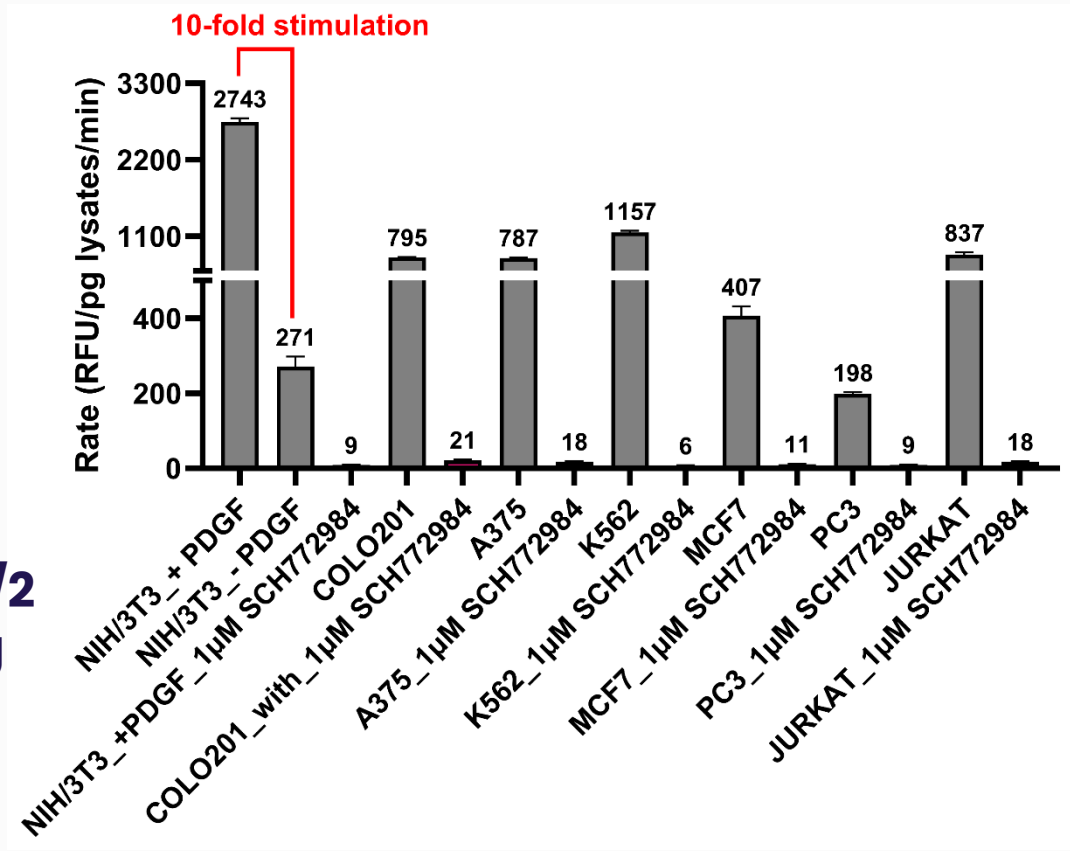
- AQT1076 was used to assay ERK1/2 activity in lysates from seven cell lines: NIH/3T3, COLO201, A375, K562, MCF7, PC3, and JURKAT.
- Normal NIH/3T3 cells could be made quiescent with serum deprivation, followed by induction with growth factor (PDGF) stimulation. In contrast, ERK1/2 was constitutively active in each of the other cell lines with no change with stimulus (data not shown).
- A tenfold increase in the rate of AQT1076 phosphorylation over the basal activity was observed with PDGF-stimulated NIH/3T3 cells.
- Incorporation of the ERK1/2-selective inhibitor, SCH77984, blocked the signal with AQT1076, highlighting the selectivity of the sensor peptide for evaluating ERK1/2 activity in these complex samples.

Selective sensor peptide substrate AQT1076 enables ERK1/2 activity monitoring across different cell types, providing a powerful tool for evaluating pathway activation in complex samples from normal and disease states

AQT1076 (15 µM) Sensor Peptide Activity in Lysates from 7 Cell Lines ± PDGF (NIH/3T3 only) and ERK1/2 Inhibitor



ERK1/2 Sensor Peptide K_m Determination

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Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

1 mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.54 mM EGTA

10 mM $MgCl_2$

0, 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 µM AQT1076

1.0 µg/well NIH-3T3 crude cell lysate (from cells activated with 25 ng/mL PDGF)

Reaction Set Up:

5 µl 5X AQT1076 Substrate dilutions
15 µL Reaction Mix with ATP & DTT
15 minutes incubation at 30 °C (in the reader)
5 µL Lysate (diluted to 5x in Enzyme dilution buffer, EDB) or EDB with lysate buffer
25 µL Final reaction volume

Reaction was run at 30˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 20 or 25 µL final well volume or in in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 µL final well volume after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

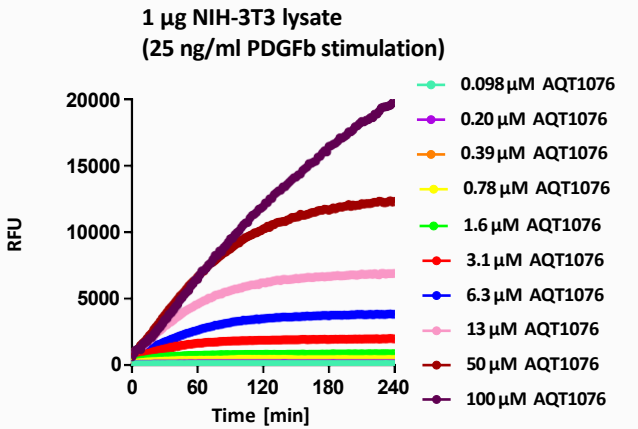
Notes:

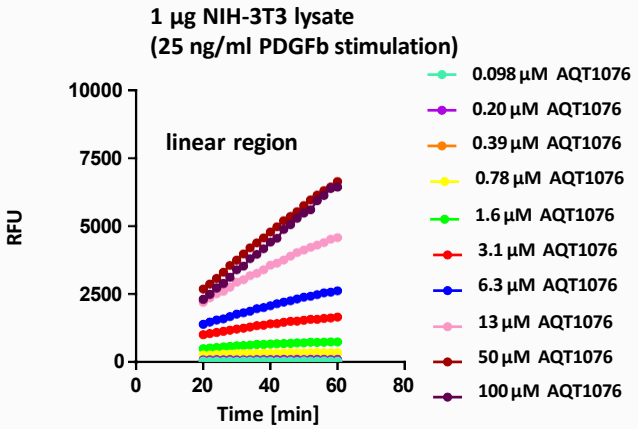
Enzyme Dilution Buffer (EDB):20 mM HEPES, pH 7.5,0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

Sensor Peptide K_m Determination

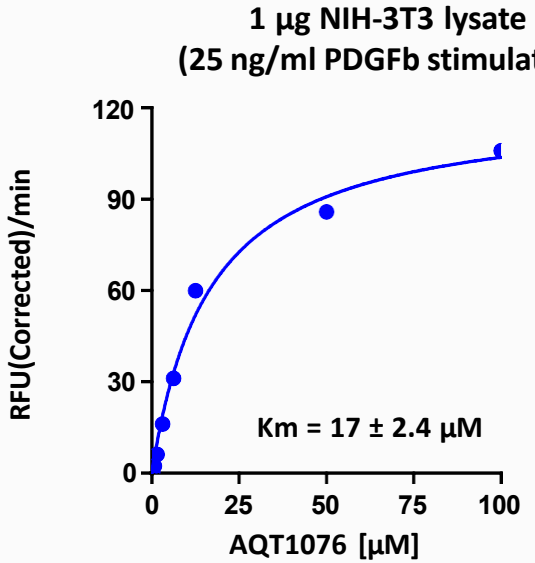
Titration Curves, K_m Plot and Tables Using Lysate from + PDGF-treated Cells

Sensor Peptide Titration Curves





Sensor Peptide K_m Plot



Sensor Peptide K_m Table

Michaelis-Menten Best-fit values	
Vmax	121.0
Km	16.65
Std. Error	
Vmax	5.875
Km	2.430
95% CI (asymptotic)	
Vmax	107.5 to 134.6
Km	11.04 to 22.25
Goodness of Fit	
Degrees of Freedom	8
R squared	0.9898
Sum of Squares	142.2
Sy.x	4.216

	AQT1076 K_m (µM)
NIH-3T3 (25 ng/mL stimulated) Lysate	17
Recombinant FL tagless ERK1 (Sino, M29-10U)	9.3
Recombinant FL GST-ERK1 (Sino, M29-10G)	9.3
Recombinant FL GST-ERK2 (Sino, M28-10G)	14

The K_m value for AQT1076 is 17 µM, similar to the K_m values observed for recombinant ERK1 and ERK2.

DMSO Tolerance Test

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

1 mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.54 mM EGTA

10 mM $MgCl_2$

15 µM AQT1076

1.0 µg/well NIH-3T3 crude cell lysate (from cells activated with 25 ng/mL PDGF)

0-10% DMSO

Reaction Set Up:

2.5 µL 10X DMSO Titration
17.5 µL Reaction Mix with CSx Substrate, ATP & DTT
15 minutes incubation at 30 °C (in the reader)
5 µL Lysate (diluted to 5x in Enzyme dilution buffer, EDB) or EDB with lysate buffer
25 µL Final reaction volume

Reaction was run at 30˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 20 or 25 µL final well volume or in in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 µL final well volume after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

Notes:

Enzyme Dilution Buffer (EDB):20 mM HEPES, pH 7.5,0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

DMSO Tolerance Test

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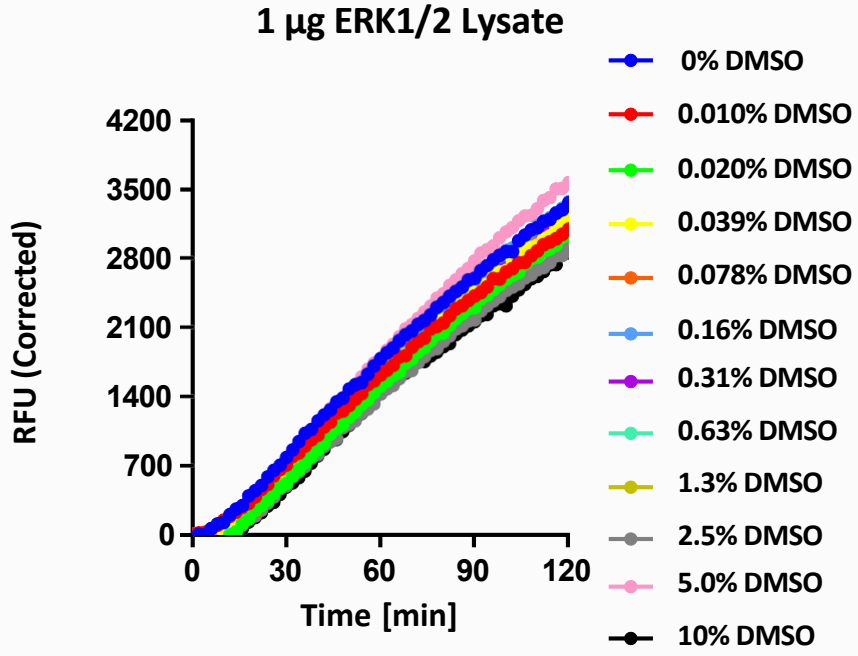
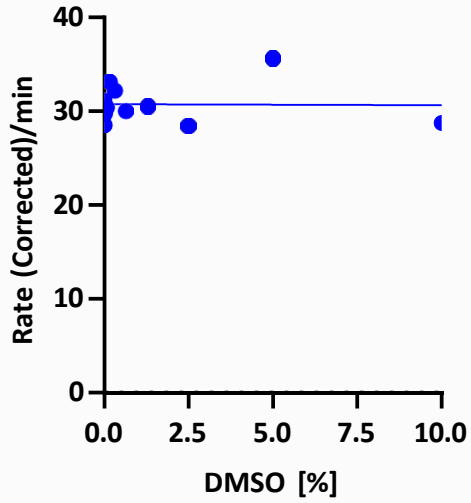
Titration Curves and Inhibition Plot Using Lysate from + PDGF-treated Cells

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No change in enzyme activity out to 10% DMSO

IC_{50} Determination

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5
1.0 mM ATP
1.2 mM DTT
0.012% Brij-35
1% glycerol
0.2 mg/ml BSA
0.54 mM EGTA
10 mM $MgCl_2$
15 µM AQT1076
2% DMSO
0-1.0 µM SCH772984 (MedChemExpress, HY-50846)
0-1.0 µM Vx-11e (MedChemExpress, HY-14178)
1.0 µg/well NIH-3T3 crude cell lysate (from cells activated with 25 ng/mL PDGF)

Reaction Set Up:

0.5 µL 50X Staurosporine dilutions in 100% DMSO
19.5 µL Reaction Mix with CSx Substrate, ATP & DTT
15 minutes incubation at 30°C (in the reader)
5 µL Lysate (diluted to 5x in Enzyme dilution buffer, EDB) or EDB with lysate buffer
25 µL Final reaction volume

Reaction was run at 30˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 20 or 25 µL final well volume or in in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 µL final well volume after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

Notes:

Enzyme Dilution Buffer (EDB):20 mM HEPES, pH 7.5,0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

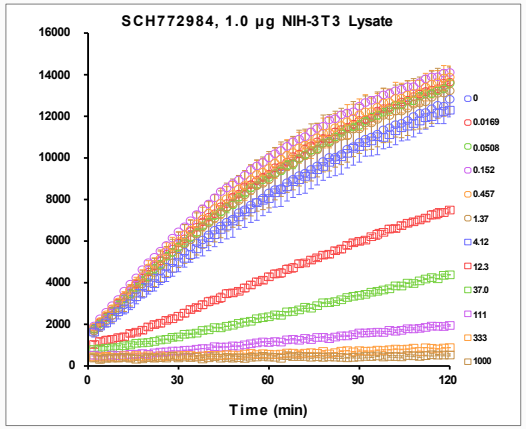
IC_{50} Determination

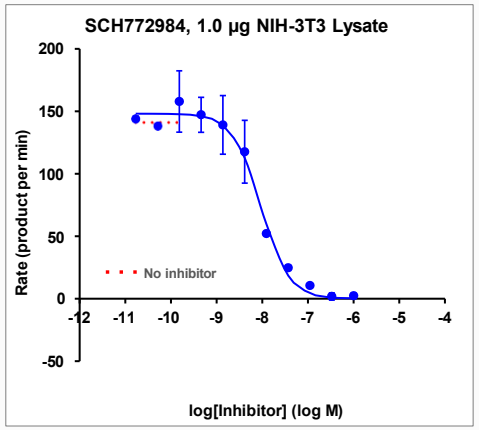
Assess Compound Potency Using Lysate from + PDGF-treated Cells

Progress Curves

IC_{50} Curve

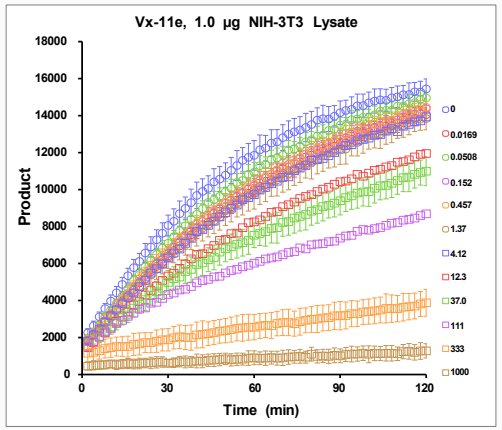
SCH772984

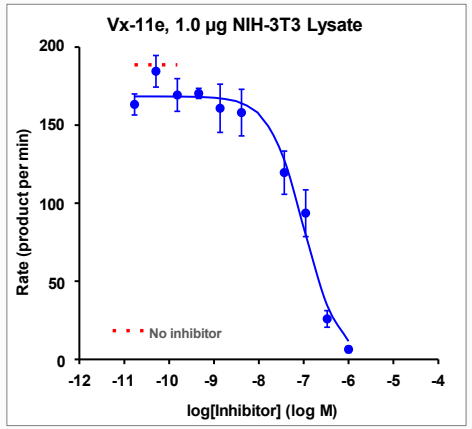




SCH772984 IC_{50} value is 9.3 nM

Vx-11e





Vx-11e IC_{50} value is 101 nM

Summary

NIH-3T3 cell activation with 25 ng/mL PDGF-bb increases ERK1/2 kinase activity 92-fold above the unstimulated control and is inhibited fully by 1 µM of both SCH772984 and Vx-11e reference compounds. This PhosphoSens-Lysate Assay for ERK1/2 enables direct and highly quantitative measurements in an easy-to-use format. Dual phosphorylation of ERK1/2 by western blotting, commonly used as a measure of activation, was also demonstrated and consistent, although this method is only semi-quantitative. Moreover, there are more than 20 phosphorylation sites on ERK1/2, with both positive and negative regulation, that need to be taken into account.

The PhosphoSens-Lysate Assay for ERK1/2 using the AQT1076 selective sensor peptide provides a robust and more physiologically relevant assay that measures endogenous ERK1/2 activity with all the cellular components and functional signaling complexes. Results demonstrated include:

- The ERK1/2 Lysate titration linearity from 0.31 to 10 µg/well (32-fold). This linear range is much wider than the 4-fold obtained with recombinant ERK1 or 2 enzymes.
- Sensor peptide substrate AQT1076 has a K_m of 17 µM.
- The IC_{50} values for SCH772984 and Vx-11e were 9.3 nM and 101 nM, respectively.