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

PhosphoSens®–Lysate Assay Format

Outline for this Study

PhosphoSens-Lysate Assay Validation

AssayQuant TECHNOLOGIES INC.

Lysate Source:

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

See slides 11-13 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)

Experiments to be run:

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

AQT1076 substrate Km determination

DMSO Tolerance Test

Reference Compound IC_{50} Determinations

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 MgCl2

15 µM AQT1076

0, 0.020, 0.039, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, 5.0, 10, and 20 µg/well NIH-3T3 (activated with 25 ng/mL PDGF)

Reaction Set Up:

20 µL Reaction Mix with AQT1076, ATP, & DTT
5 µL Enzyme dilution buffer (EDB) with ERK1/2 Lysate Buffer (1x) or ERK1/2 Lysate in Lysate Buffer (5x in EDB)

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.

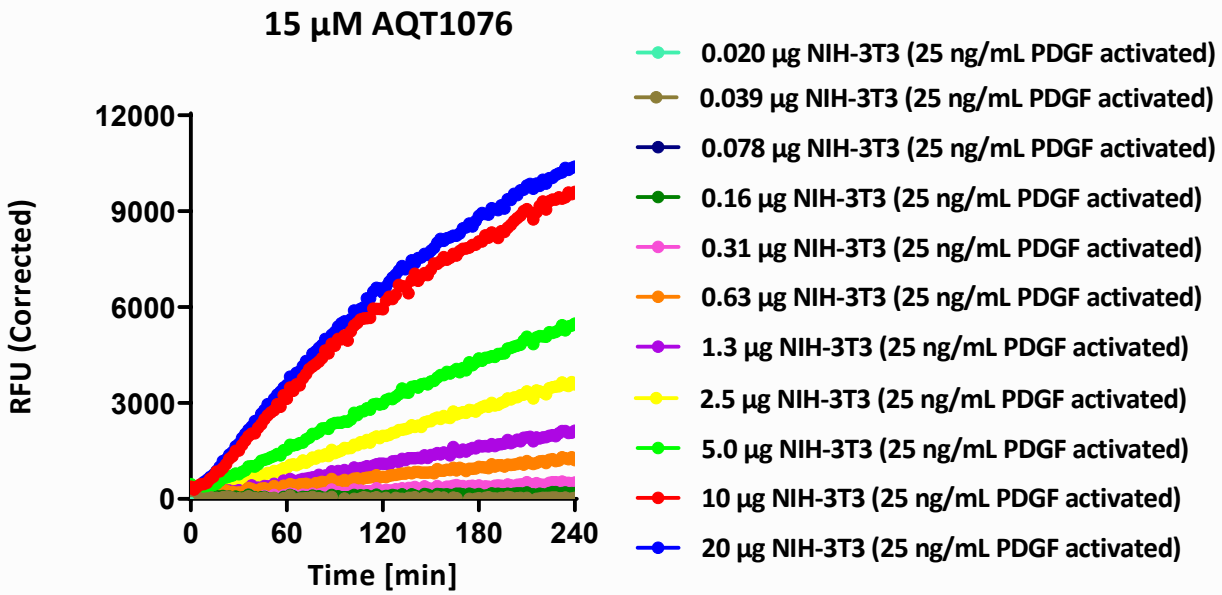
Notes:

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.

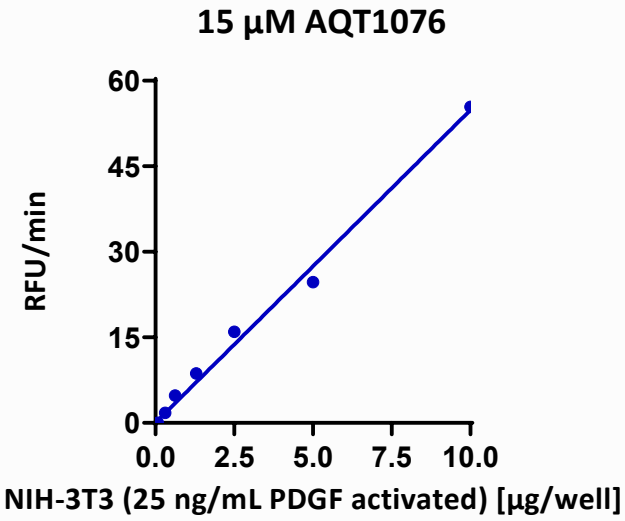
Lysate Titration

Progress Curves

Complete Progress Curves



Linear Region of Progress Curves



The ERK1/2 Lysate Assay is linear from 0.63 - 10 µg/well lysate

ERK1/2 Sensor Peptide K_m Determination

AssayQuant TECHNOLOGIES INC.

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 MgCl2

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 (activated with 25 ng/mL PDGF)

Reaction Set Up:

5 µl 5X AQT1076 Substrate dilutions

15 µL Reaction Mix with ATP & DTT

5 µL Enzyme dilution buffer (EDB) with lysate buffer(1x) or Lysate in lysis buffer (5x in EDB)

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.

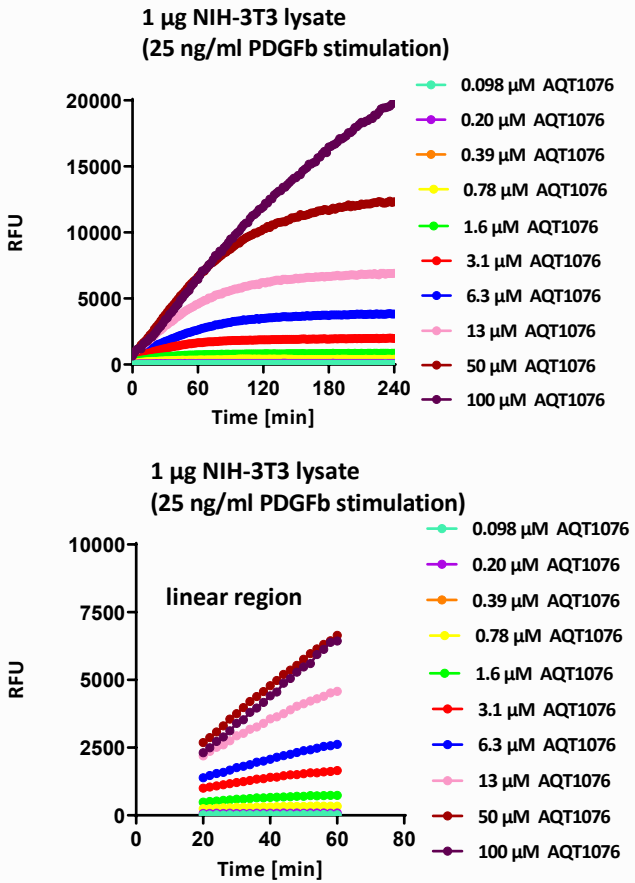
Notes:

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.

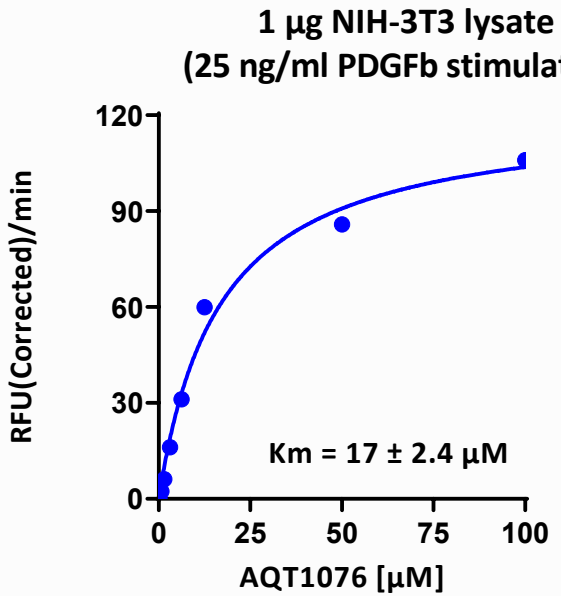
Sensor Peptide K_m Determination

Titration Curves and K_m Plot and Table

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 MgCl2

15 µM AQT1076

1.0 µg/well NIH-3T3 (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
5 µL Enzyme dilution buffer (EDB) (1x) or Kinase (5x in EDB)

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.

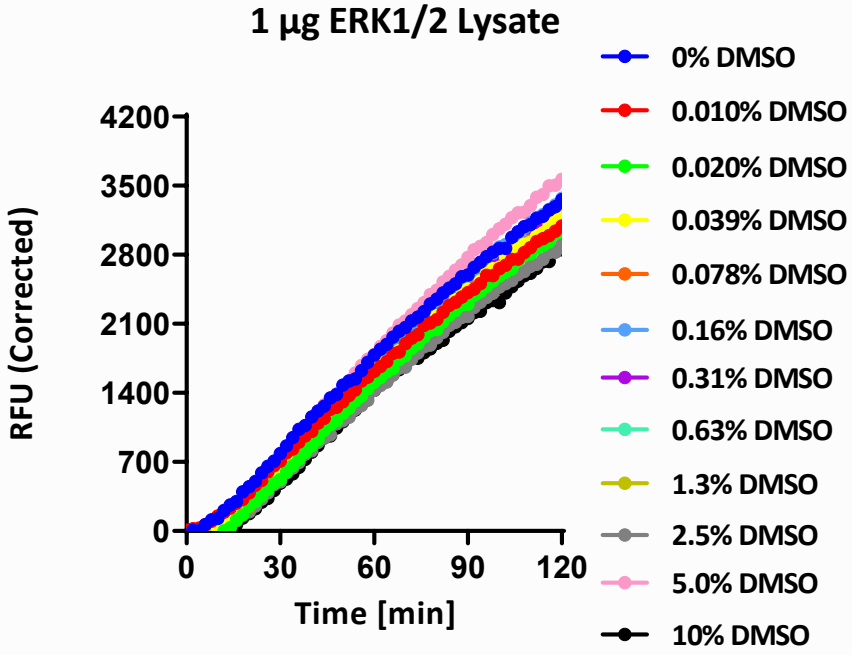
Notes:

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.

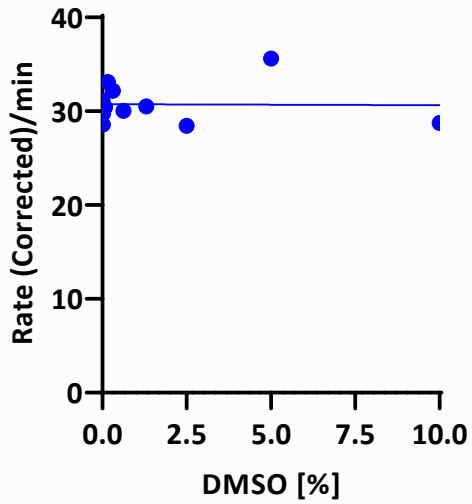
DMSO Tolerance Test

Titration Curves and Inhibition Plot

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 MgCl2
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 (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
5 µL Enzyme dilution buffer (EDB) (1x) or Kinase (5x in EDB)

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.

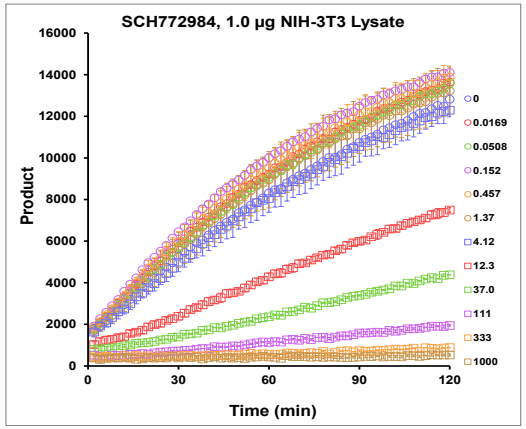
Notes:

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.

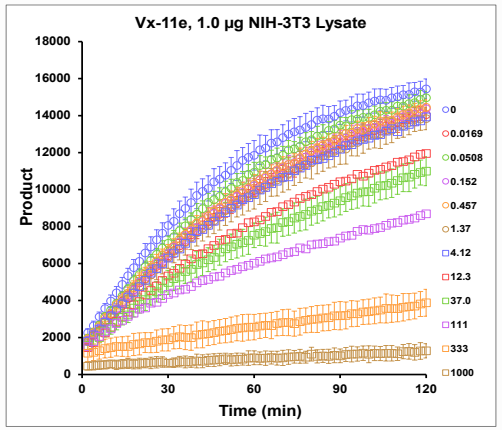
IC_{50} Determination

Progress Curves

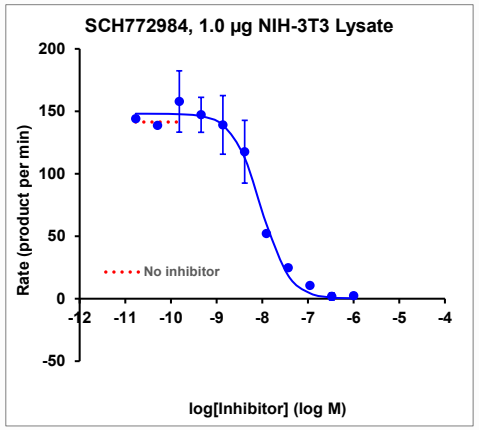
SCH772984

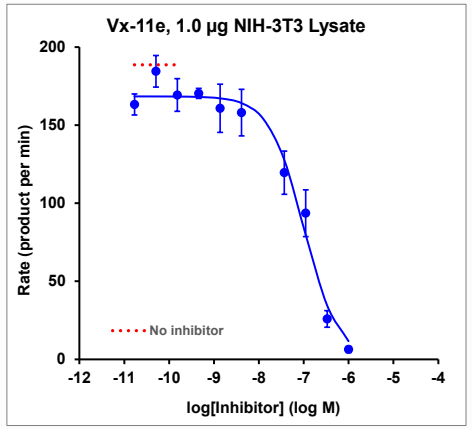


Vx-11e



IC_{50} Curve





IC_{50} Table

Compound ID	Highest conc. (mM)	Data points	IC_{50} (nM)	% Inh @ curve min	% inh at highest conc.	Min rate (product / min)	Max rate (product / min)	R squared
SCH772984	1.0	22	**9.3**	2.4	98	0	148	0.993
Vx-11e	1.0	20	**101**	6	96	0	169	0.980

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

AssayQuant TECHNOLOGIES INC.

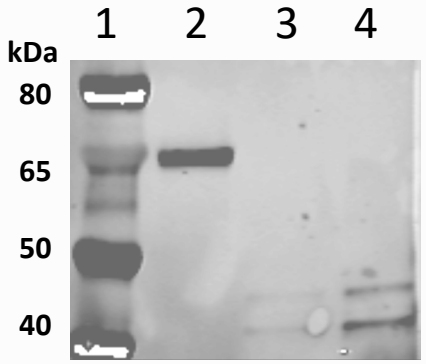
1) NIH-3T3 Cells (ATCC, CRL-1658.2) were plated in 6-well tissue culture-treated plates and incubated for 48 hours at 37°C in DMEM Medium with 10% FBS and PenStrep in an atmosphere of 5% CO2. Cells were then serum-starved in culture medium with 0.1% FBS (ThermoFisher, A56708-01) for 24 hours and incubated 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 Na3VO4
   - 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)

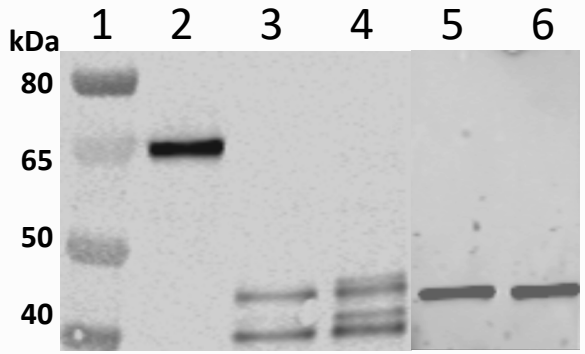
2) The DNA strands were broken by briefly sonicating on ice for 2 seconds on low power. Lysates were used immediately or aliquoted and frozen at -80°C. 1 µg of each Lysate was run on a gel, transferred to a nitrocellulose membrane, and the signal developed by Western blotting using antibodies to total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

ERK1/2 Lysate Western Blots

Phospho-ERK1/2



Total ERK1/2 and Actin Loading Control

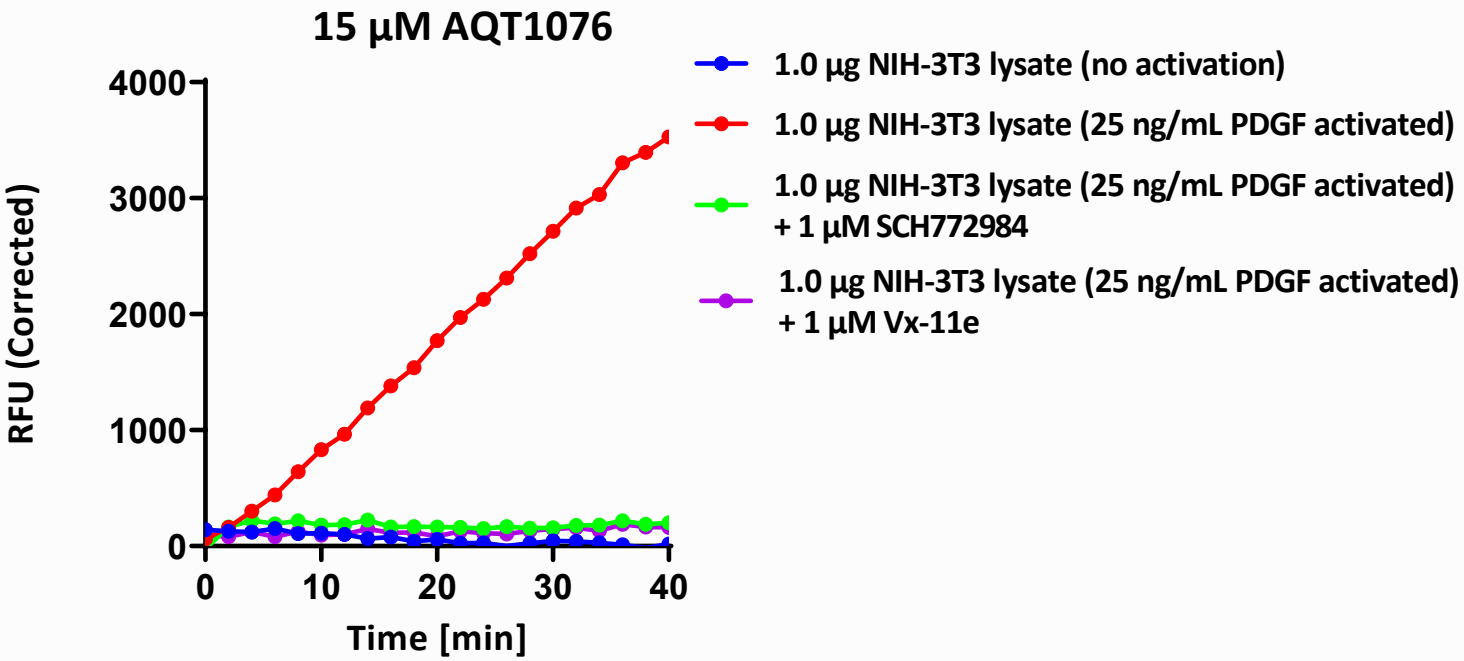


Lane Description:

1. MW Markers
2. Recombinant ERK2
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 2000-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 20000-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.

ERK1/2 Lysate Activity Assay



NIH-3T3 PDGF activation significantly increases ERK1/2 lysate activity which is inhibited by 1.0 µM of both SCH772984 and Vx-11e.

	Reaction Rate (RFU/min)	Change
1.0 µg NIH-3T3 lysate (no activation)	0	-
1.0 µg NIH-3T3 lysate (+25 ng/mL PDGF activated)	92	92-fold increase above control
1.0 µg NIH-3T3 lysate (+25 ng/mL PDGF activated), + 1 µM SCH772984	1.1	99% inhibition of PDGF activation
1.0 µg NIH-3T3 lysate (+25 ng/mL PDGF activated), + 1 µM Vx-11e	1.8	98% inhibition of PDGF activation

Summary

NIH-3T3 cell activation with 25 ng/mL PDGF-bb increases ERK1/2 phosphorylation and subsequent activity which is inhibited fully by 1 µM of both SCH772984 and Vx-11e

The ERK1/2 Lysate Assay with the AQT1076 selective sensor peptide demonstrates a robust and more physiologically relevant assay using the PhosphoSens Platform:

- The ERK1/2 Lysate titration was linear from 0.31 to 10 µg/well.
- 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.