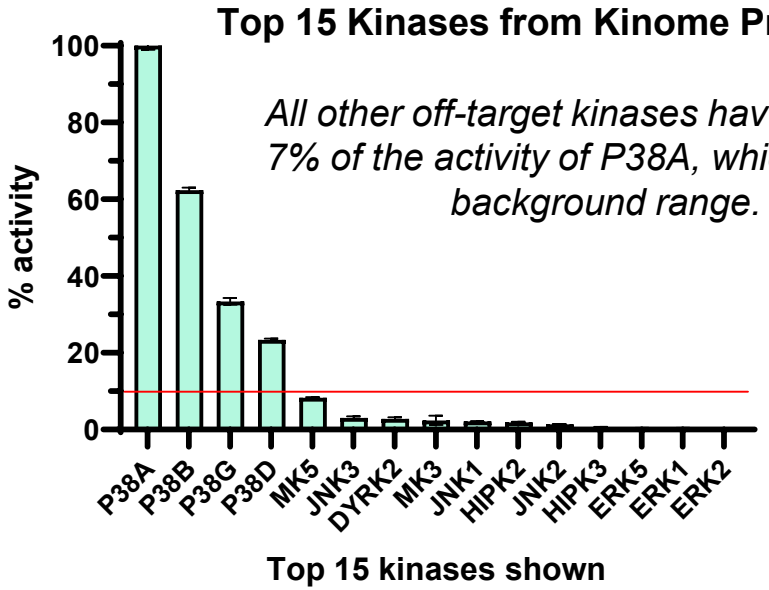
PhosphoSens® Cell Lysate Activity Assay Format

P38 Assay Validation Using the AQT1280 Selective Sensor Peptide Substrate

HGNC Name: MAPK14 (P38α), MAPK11 (P38β), MAPK13 (P38δ), and MAPK12 (P38γ)
Long Names: **M**itogen-**A**ctivated **P**rotein **K**inase- P38

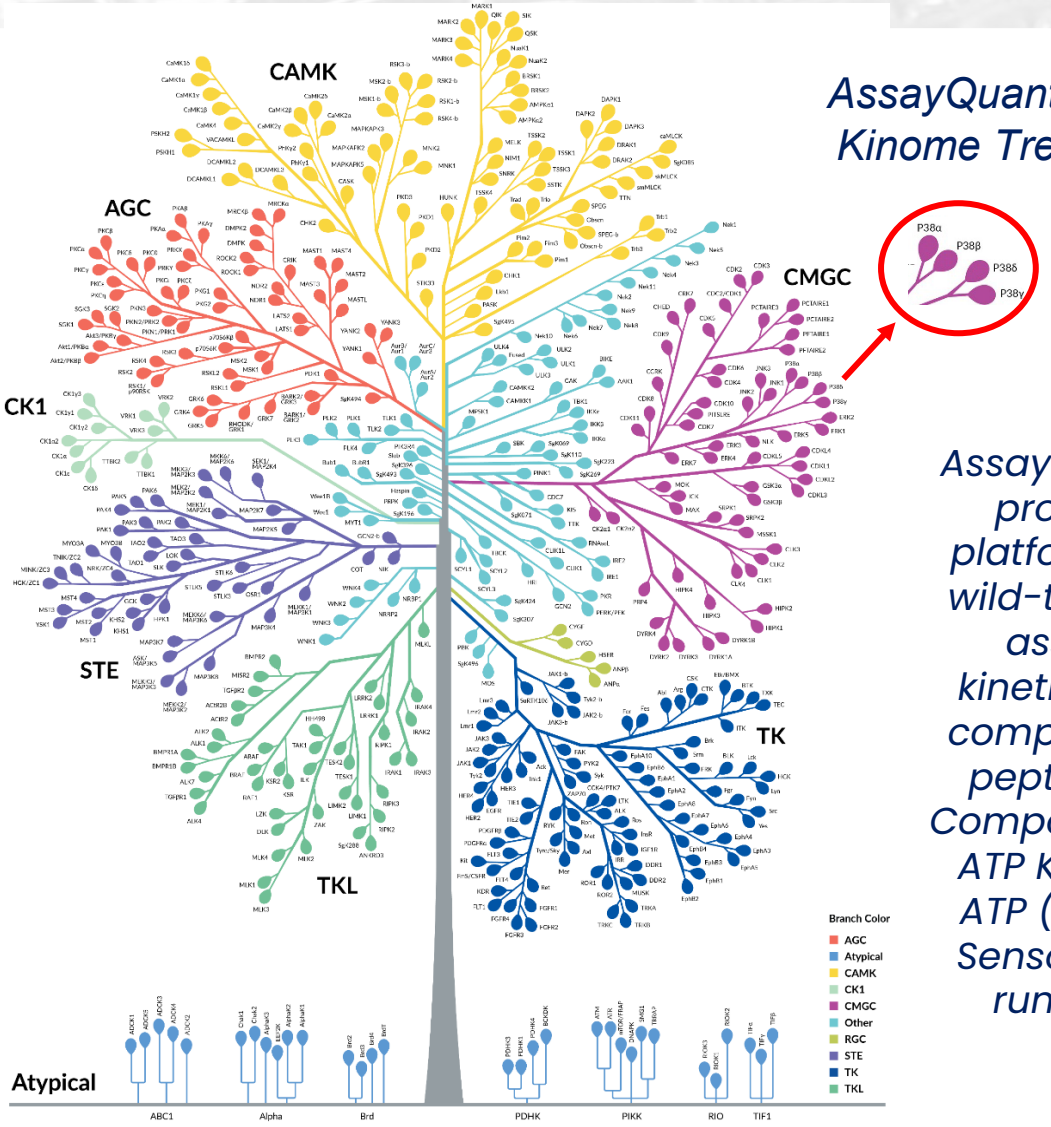
Selectivity of AQT1280 for P38α/β/δ/γ Determined with AQT's Kinome Profiling Service



All other off-target kinases have less than 7% of the activity of P38A, which is in the background range.

#	Kinase target	Kinase group	AQT1280 (μM)	Enzyme, (nM)	RFU/pmol/min	% Activity
1	P38A	CMGC	10	1	10560.0	100.0
2	P38B	CMGC	10	1	6584.7	62.4
3	P38G	CMGC	10	1	3531.3	33.4
4	P38D	CMGC	10	1	2470.7	23.4
5	MK5	CAMK	10	7	880.4	8.3
6	JNK3	CMGC	10	5	313.7	3.0
7	DYRK2	CMGC	10	3	296.0	2.8
8	MK3	CAMK	10	1	256.7	2.4
9	JNK1	CMGC	10	4	216.8	2.1
10	HIPK2	CMGC	10	10	204.7	1.9
11	JNK2	CMGC	10	4	148.8	1.4
12	HIPK3	CMGC	10	9.1	66.0	0.6
13	ERK5	CMGC	10	5.3	38.5	0.4
14	ERK1	CMGC	10	0.4	29.5	0.3
15	ERK2	CMGC	10	0.4	26.0	0.2

AssayQuant's Kinome Tree



AssayQuant's Kinome profiling service platform features 411 wild-type kinases. All assays are run kinetically to assess compound or sensor peptide selectivity. Compounds are run at ATP K_m and/or 1 mM ATP (physiological). Sensor peptides are run at 1 mM ATP.

Outline for this Study

PhosphoSens-Lysate Assay Validation Using the AQT1280 Sensor Peptide Substrate

AssayQuant® TECHNOLOGIES INC.

Lysate Source:

HeLa cells (passage 10) +/- 1 µM Anisomycin (Resuspended in DMSO; MedchemExpress, HY-18982)

Reference Compound Information:

1. SCH772984 (ERK1/2 inhibitor) *This inhibitor is included in all reactions to eliminate any off-target signals with ERK1/2.*
2. Ralimetinib LY2228820 mesylate (p38 inhibitor)

Experimental Validation Performed:

- Lysate from HeLa cells (+/- 1 µM Anisomycin for 15 minutes, as a stress stimulus, to activate P38 isoforms)
- Phosphopeptide Control (AQT1251) and comparison to phosphorylation of AQT1280 sensor peptide with recombinant P38α (Cat: AQT-M39-10BG)
- AQT1280 sensor peptide substrate K_m determination
- DMSO Tolerance Test
- Reference Compound IC_{50} Determinations with Ralimetinib
- Assessment of P38 Activity in Multiple Cell Lines +/- Anisomycin
- Western blot comparison

Preparation of Cell Lysates from Multiple Cell Lines Treated +/- Anisomycin to Activate P38 Isoforms in 96-well Plates

1) Cells (*A549, Calu-6, HCT 116, HEK293,* HeLa [passage 10], *MCF7, MiaPaCa-2, U87MG*) were plated in separate 96-well tissue culture-treated plates and incubated for 48 hours at 37°C in EMEM (Calu-6, HEK293, HeLa, MCF7, U87MG), McCoy's 5A (HCT 116), F-12K (A549), or DMEDM (MiaPaCa-2) medium supplemented with 10% FBS (ThermoFisher, A56708-01) and 1% Penicillin-Streptomycin (ThermoFisher, 15140122) in a 5% CO2 atmosphere.
2) Cells were washed with PBS, then incubated with culture medium containing 0.1% FBS (serum-starved to make the cells quiescent) and 1% Penicillin-Streptomycin for 24 hours. Subsequently, buffer (negative control) or Anisomycin was added directly to the medium to a final concentration of 1 µM Anisomycin (MeChemExpress, HY-18982), and the cells were incubated for 15 minutes at 37°C.
3) After the treatment, the culture medium was removed, the cells were washed with cold 1X PBS,and then lysed with Cell Extraction Buffer (CEB) containing protease and phosphatase Inhibitors (see recipe below) for 15 minutes on ice. The assay reaction mix (see next slide) and AQT1280 were added to lysates in each well to assess P38 activity.

Cell Extraction Buffer (CEB) with Protease and Phosphatase Inhibitors:

- 50 mM HEPES, pH 7.4
- 150 mM NaCl
- 2 mM EGTA
- 1 mM DTT
- 1% Triton X-100
- PhosphoPreserve Phosphatase Inhibitor Cocktail - 1, 100X
- PhosphoPreserve Phosphatase Inhibitor Cocktail - 3, 60X
- PhosphoPreserve Protease Inhibitor Cocktail, 60X

Titration of Cell Number and Lysate from HeLa Cells +/-Anisomycin (1 µM) to Activate P38 isoforms

Growth of HeLa Cells, Treatment +/- Anisomycin, Cell Extraction, & Determination of P38 Activity with AQT1280 is Performed in the Same 96-well Plate

AssayQuant® TECHNOLOGIES INC.

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 AQT1280 sensor peptide
1 µM SCH772984 to eliminate any off-target ERK1/2 activity

P38 Enzyme:

- **Cell Lysate from titration of HeLa cells:**
  - See below
- **Recombinant P38α for AQT1280 phosphorylation:**
  - 1 nM full-length P38α

Reaction Set Up:

- To **20 µL of Cell Lysate** in the 96-well plate
- Add **78 µL Reaction Mix with AQT1280, ATP & DTT**
- Add **2 µL of 50X (Ralimetinib + SCH772984)** in DMSO or **50X SCH772984 alone** in DMSO
- To obtain **100 µL** Final reaction volume

Notes:

Reaction was run at 30 ˚C for 240 minutes in Falcon® 96-well White Flat Bottom TC-treated Microplate (Corning Cat. #353296) at 100 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.

1X Enzyme Dilution Buffer (EDB) is used to dilute enzyme and for the blank. Composition is 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.

Cell Plating and Protein Determination:

HeLa Cells were seeded in a 96-well plate (6 point, 2-fold serial dilution) at 5k, 10k, 20k, 40k, 80k, and 160k cells/well

Total protein was determined by the Bradford method: 2, 5, 11, 24, 27, and 32 µg of protein/well, respectively.

Recombinant P38α activity

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 AQT1280 sensor peptide or AQT1251 phosphopeptide control

1 µM SCH772984 to eliminate any off-target ERK1/2 activity

3 nM P38α

Reaction Set Up:

20 µL Reaction Mix with AQT1280, ATP, & DTT
15 minutes incubation at 30°C (in the reader)
5 µL of 5x enzyme 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 bottom polystyrene NBS microplates (Cat. #3824) at 20 or 25 µL final well volume or 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:

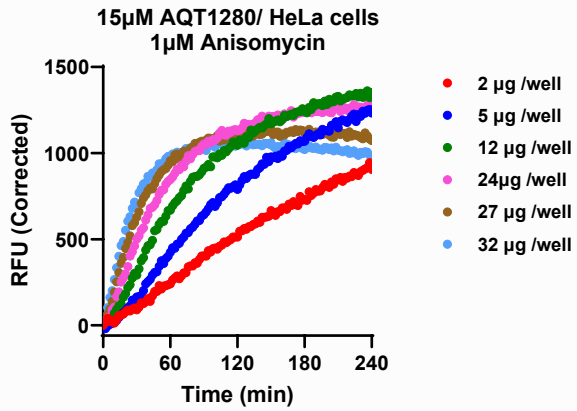
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

Titration of Cell Number and Lysate from HeLa Cells +Anisomycin (1 µM) to Activate P38 isoforms

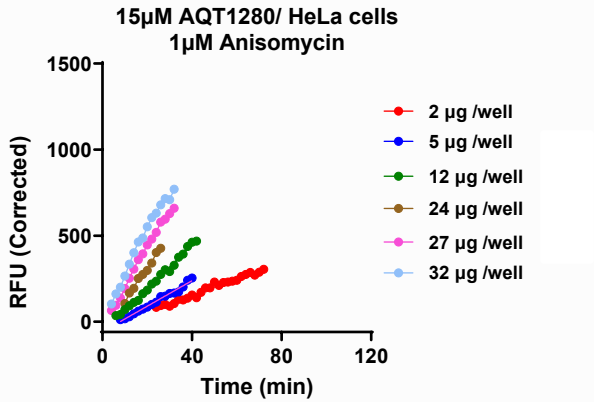
Progress Curves, Reaction Rates & Linearity for P38 Activity with AQT1280

Progress Curves

Complete Time Course

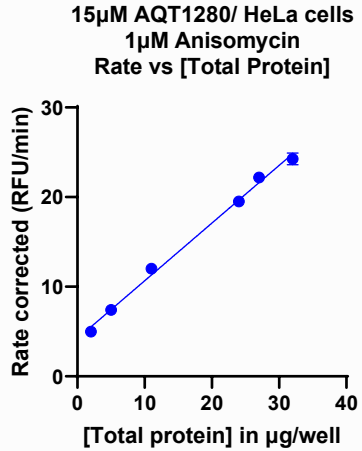


Linear Region



Assessment of Linearity

All Data & Linear Reaction



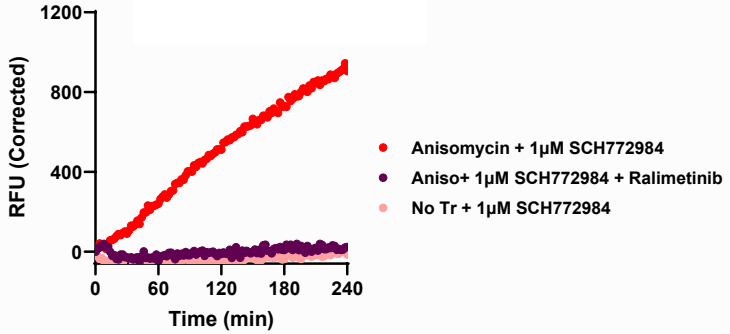
The P38 lysate activity assay is linear from 2 – 32 µg of total protein/well protein (16-fold range), corresponding to 5k – 160k HeLa cells seeded/well.

P38 Lysate Activity Assay with AQT1280

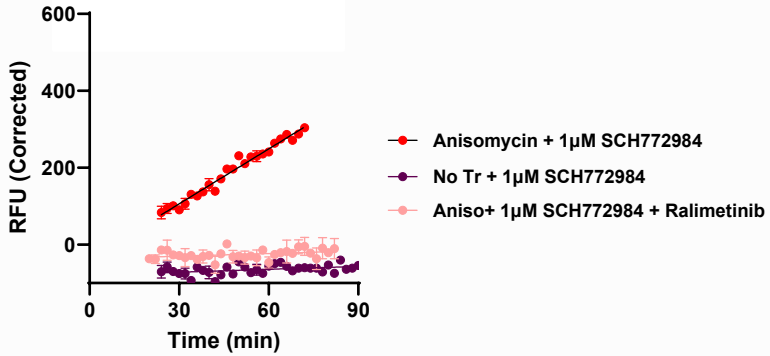
Activation of P38 in Lysates from HeLa Cells ± Anisomycin

A. Crude Lysate Samples: 5k HeLa cells (or 2 µg protein)/well with 15 µM AQT1280

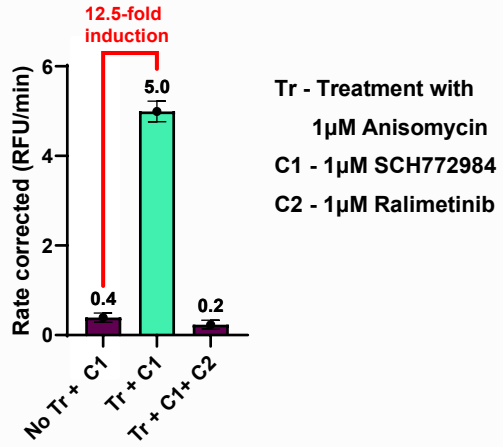
1) Full Time Course (0-240 min.)



2) Linear Range (25-90 min.)

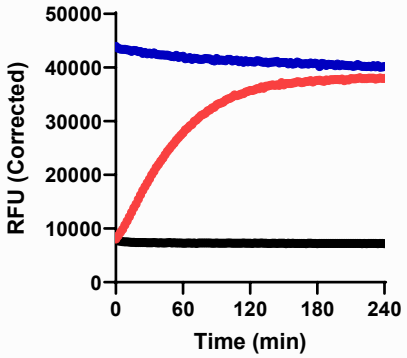


3) Histogram



B. Purified P38α & AQT 1251 Control

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



AQT1251 is a chemically synthesized phosphopeptide control

A. Crude lysate samples: The AQT1280 sensor peptide was used to generate RFU Corrected values (Total – Background) for **1)** Full progress curve time course (0-240 min.), and **2)** Linear range (25-90 min.), and to determine the slope for each condition, which is the Reaction rate (RFU Corrected/min. +/- standard deviations) shown as a histogram in **3)**, highlighting a 12.5-fold activation of P38 kinase activity in HeLa cell lysates treated with 1 µM Anisomycin for 15 minutes. The signal was eliminated by adding the selective P38α/β inhibitor Ralimetinib (1 µM). 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 activating stimulus's nature, concentration, and duration. These conditions can be varied to optimize P38 activity. The total amount of P38α/β/δ/γ protein can be determined by Western Blotting or an ELISA; however, with the short stimulation times typically used, these levels are not expected to change.

B.1. Purified recombinant P38α enzyme & the AQT1251 phosphopeptide Control: P38α enzyme (1 nM) fully phosphorylated the AQT1280 sensor peptide substrate by 240 min., as shown by convergence with the signal obtained with the AQT1251 phosphopeptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). The signal with AQT1251 is used to convert RFU (Corrected) values to nmoles of product.

Sensor Peptide K_m Determination for AQT1280 Using Lysate from HeLa Cells (5k/well) + Anisomycin

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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 $MgCl_2$

AQT1280 sensor peptide titration - 0, 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 µM.

1 µM SCH772984 to eliminate any off-target ERK1/2 activity

Reaction Set Up:

- To **20 µL Lysate** in the 96-well plate,
- Add **2 µL of 50X SCH772984 (ERK1/2 inhibitor)**
- Add **10 µL of 10X AQT1280**
- Add **68 µL 1.47X Reaction Mix with ATP, & DTT** to obtain **100 µL** Final reaction volume

Notes:

Reaction was run at 30 ˚C for 240 minutes in Falcon® 96-well White Flat Bottom TC-treated Microplate (Corning Cat. #353296) at 100 µL final well volume after sealing using optically-clea 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.

1X Enzyme Dilution Buffer (EDB) is used to dilute enzyme and for the blank. Composition is 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.

Cell Plating and Protein Determination:

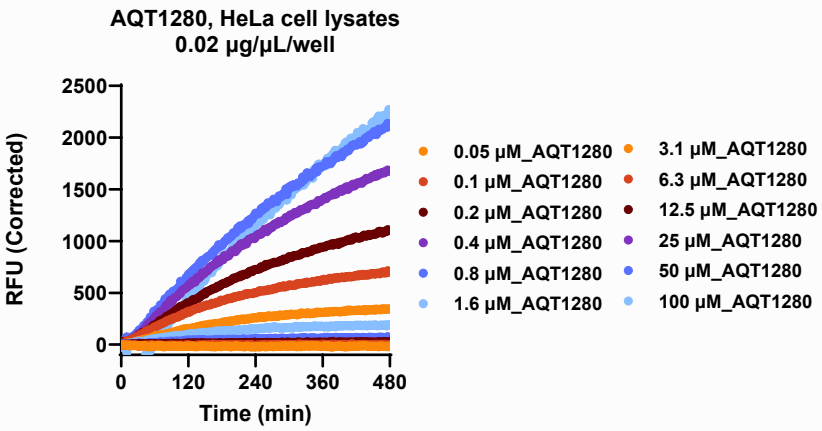
HeLa Cells were seeded at 5k cells/well in 24 wells (12 wells in duplicate) of the 96-well plate

Total protein was determined by the Bradford method: 2 µg protein/well

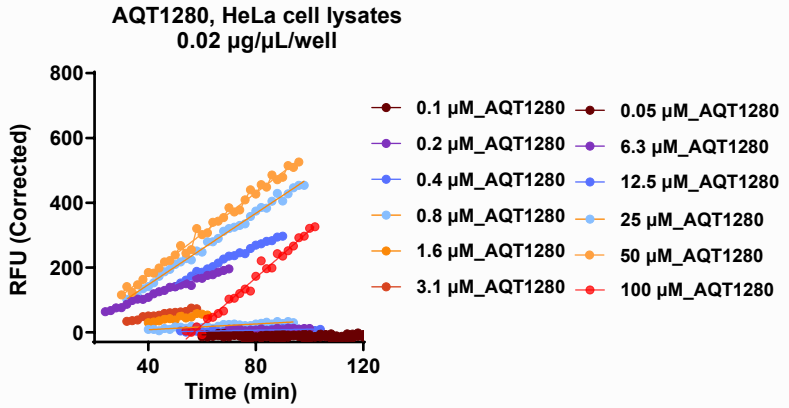
Sensor Peptide K_m Determination with AQT1280 Using Lysate from HeLa Cells (5k/well) + Anisomycin

Titration Curves and K_m Plot and Table

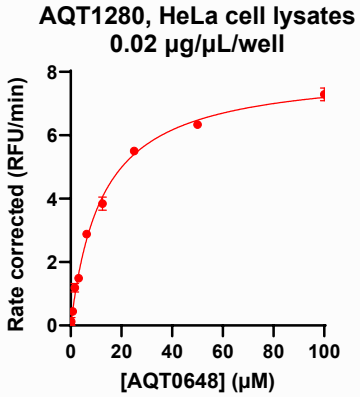
Sensor Peptide progress Curves



Sensor Peptide Linear range



Sensor Peptide K_m Plot



Best-fit values	
Vmax	8.091
Km	12.47
Std. Error	
Vmax	0.1719
Km	0.8253
95% CI (asymptotic)	
Vmax	7.708 to 8.474
Km	10.63 to 14.31
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9973
Sum of Squares	0.2121
Sy.x	0.1456

The K_m value for AQT1280 is 12.5 µM.

DMSO Tolerance Test Using Lysate from HeLa Cells (5k/well) + Anisomycin and AQT1280

AssayQuant® TECHNOLOGIES INC.

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 AQT1280 sensor peptide

1 µM SCH772984 to eliminate any off-target ERK1/2 activity

Cell Plating and Protein Determination:

HeLa Cells were seeded at 5k cells/well in 24 wells (12 wells in duplicate) of the 96-well plate

Total protein was determined by the Bradford method: 2 µg protein/well

Reaction Set Up:

- To **20 µL Lysate** in the 96-well plate,
- Add **2 µL of 50X SCH772984 (ERK1/2 inhibitor)**
- Add **10 µL of 10X AQT1280**
- Add **68 µL 1.47X Reaction Mix with ATP, & DTT** to obtain **100 µL** Final reaction volume

Notes:

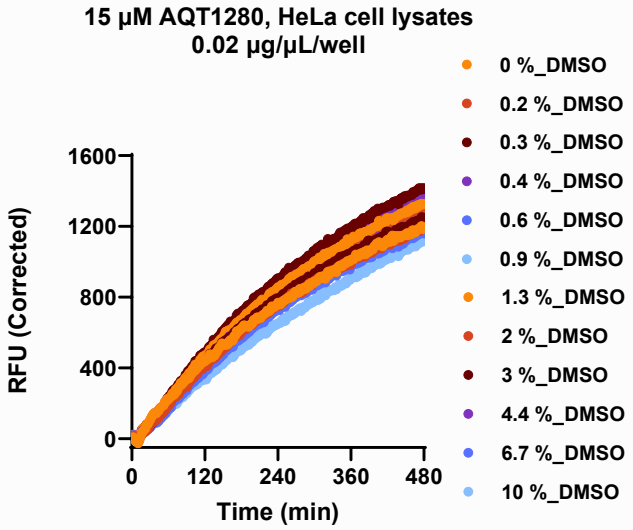
Reaction was run at 30 ˚C for 240 minutes in Falcon® 96-well White Flat Bottom TC-treated Microplate (Corning Cat. #353296) at 100 µL final well volume after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

1X Enzyme Dilution Buffer (EDB) is used to dilute enzyme and for the blank. Composition is 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.

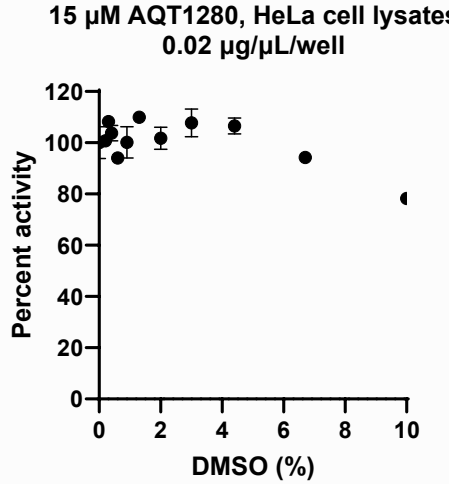
DMSO Tolerance Test Using Lysate from HeLa Cells (5k/well) + Anisomycin and AQT1280

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No significant loss in P38 activity was observed up to 4% DMSO.
A final concentration of 2% DMSO will be used to assess inhibitor potency.

IC_{50} Determination using AQT1280 in 96-well plates with Lysates from HeLa Cells (5k/well)

AssayQuant® TECHNOLOGIES INC.

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 AQT1280 sensor peptide

1 µM SCH772984 to eliminate any off-target ERK1/2 activity

Tool Compound - Ralimetinib was titrated with 3-fold dilutions in 100% DMSO at 50X the final concentrations and then diluted 50-fold into the assay for final concentrations from 0-10 µM in 2% DMSO.

Reaction Set Up:

- To **20 µL Lysate** in the 96-well plate,
- Add **2 µL of 50X Ralimetinib + 1µM SCH772984 (ERK1/2 inhibitor)**
- Add **10 µL of 10X AQT1280**
- Add **68 µL 1.47X Reaction Mix with ATP, & DTT** to obtain **100 µL** Final reaction volume

Notes:

Reaction was run at 30 ˚C for 240 minutes in Falcon® 96-well White Flat Bottom TC-treated Microplate (Corning Cat. #353296) at 100 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.

1X Enzyme Dilution Buffer (EDB) is used to dilute enzyme and for the blank. Composition is 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.

Cell Plating and Protein Determination:

HeLa Cells were seeded at 5k cells/well in 24 wells (12 wells in duplicate) of the 96-well plate

Total protein was determined by the Bradford method: 2 µg protein/well

IC_{50} Determination using AQT1280 in 96-well plates with Lysates from HeLa Cells (5k/well)

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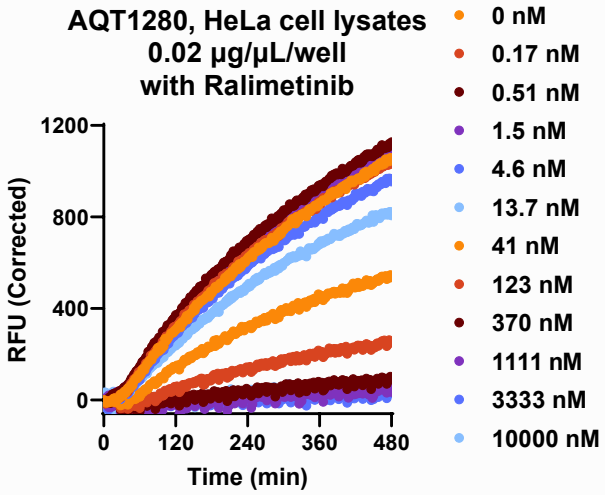
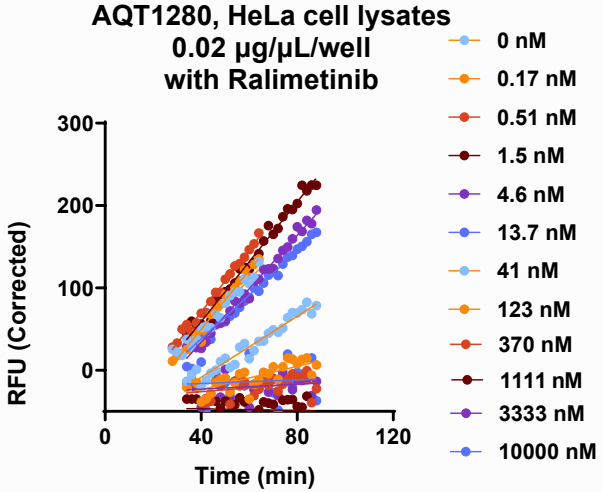
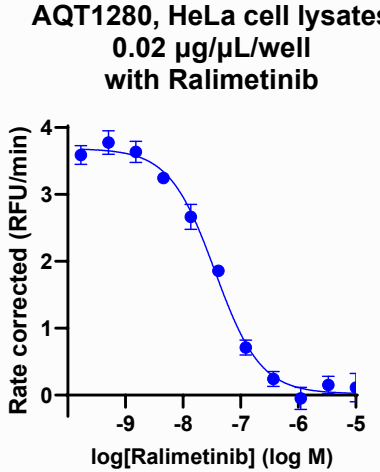
Full Progress Curves



Progress Curves (Linear Region)



IC_{50} Curve



Best-fit values	
Bottom	0.01852
Top	3.682
LogIC50	-7.446
HillSlope	-1.098
IC50	3.585e-008
Span	3.663
Std. Error	
Bottom	0.07835
Top	0.08218
LogIC50	0.04869
HillSlope	0.1227
Span	0.1247

The IC_{50} value for Ralimetinib in HeLa Lysates is 36 nM. The Value is close to the reported value in Anisomycin-induced mouse RAW264.7 macrophages (IC_{50} = 35 nM).

Reference: ***Campbell et al. Mol Cancer Ther (2014) 13 (2): 364–374.***

Effect of multiple tool compounds on AQT1280 phosphorylation in 384-well plates with Lysates from HeLa Cells (5k/well)

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 AQT1280 sensor peptide

Tool Compounds - 1 µM SCH772984 (ERK1/2 inhibitor)] 1 µM Ralimetinib, 1 µM staurosporine, and 1 µM MK2-inhibitor III at 50X the final concentrations and then diluted 50-fold into the assay for final concentrations from 0-10 µM in 2% DMSO.

HeLa cell lysates – 0.5µg final concentration

Reaction Set Up:

- To **5 µL Lysate** in the 384-well plate,
- Add **0.5 µL of 50X tool compounds**
- Add **3 µL of 5X AQT1280**
- Add **16.5 µL 1.51X Reaction Mix with ATP, & DTT** to obtain **25 µL** Final reaction volume

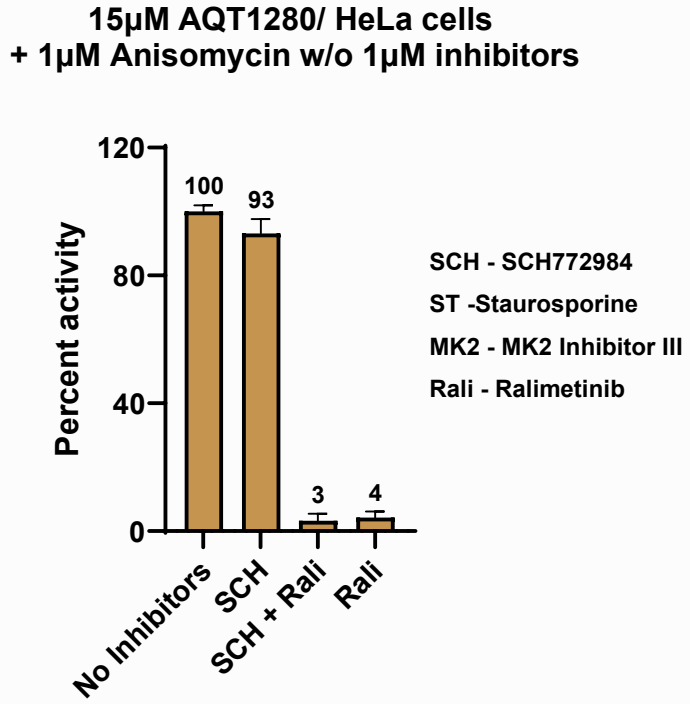
Notes:

Reaction was run at 30 ˚C for 240 minutes in either Corning, low volume 384-well, white flat 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.

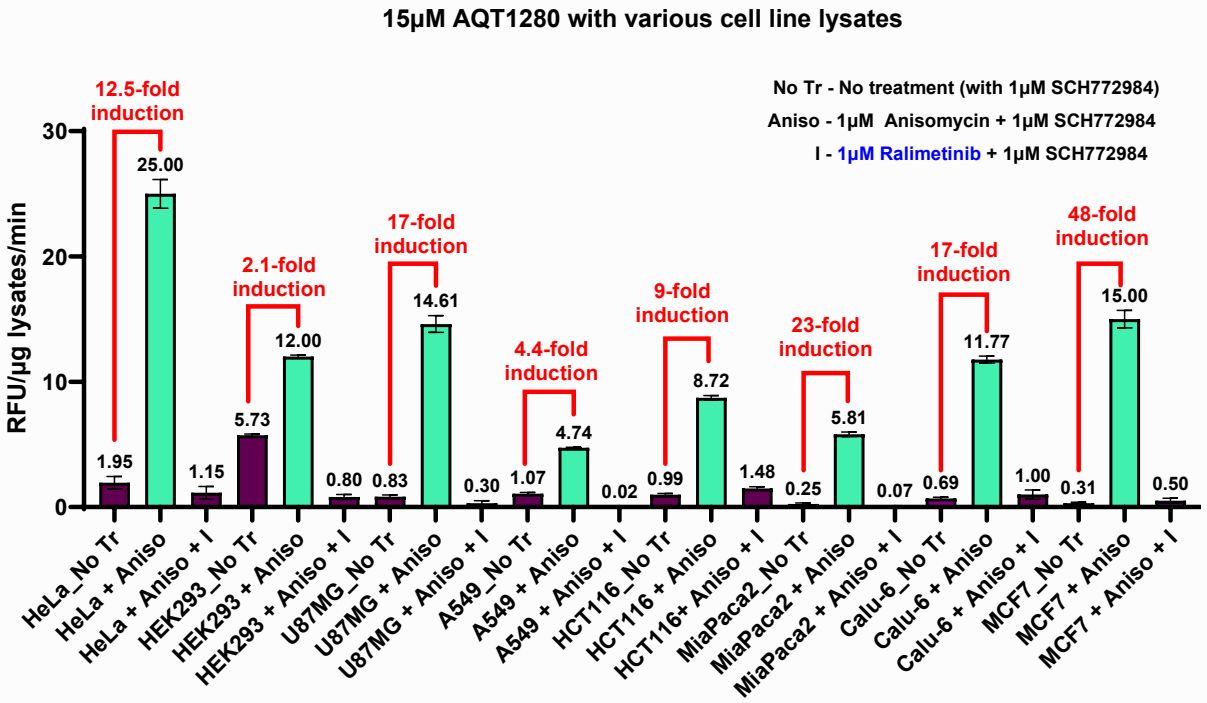
Effect of Tool Compounds on p38 Activity in Lysates from HeLa Cells (5k/well) Using AQT1280



Effect of tool compounds with 0.5 µg (0.02 µg/µL) HeLa cell lysates:

1. SCH772984 does not inhibit P38 isoforms.
2. Ralimetinib alone inhibits 96% of P38 activity.

P38 Lysate Activity Assay using AQT1280 Across a Variety of Cell Types at 5k cells/well



Description of cell line lysate with the highest P38 activity

HeLa cells are an immortal cell line with epithelial morphology derived from cervical cancer cells originally isolated from a 31-year-old African American female.

HEK293 cells are an immortal cell line with epithelial morphology isolated from the kidney of a human embryo.

U87MG cells have an epithelial morphology and were isolated from a male patient with malignant glioblastoma.

A549 cells are human alveolar basal epithelial cells isolated from the lung tissue of a white, 58-year-old male with lung cancer (adenocarcinoma).

HCT116 cells are an adherent cell line isolated from a patient with colon cancer. There is a mutation in codon 13 of the ras-proto-oncogene. This cell line is near-diploid and has a relatively stable genetic profile.

MiaPaCa-2 cells are an adherent epithelial cell line isolated from a pancreatic carcinoma of a 65-year-old male.

Calu-6 cells are a cell line exhibiting epithelial morphology that was derived from a 61-year-old, white female patient with anaplastic carcinoma.

MCF7 cells are a breast cancer cell line with epithelial morphology isolated in 1970 from a 69-year-old woman.

Method – Cell growth, treatment, and preparation of crude cell lysates are described on slide 6, and standard lysate assay conditions on slide 7 were used to run the assay. Reaction rates (RFU Corrected/min) were determined from the slopes using the linear portion of each progress curve. Values are the average of duplicate reactions +/- standard error.

Results – HeLa cells treated with 1 µM Anisomycin showed the highest P38 activity among the cell lines tested. While Anisomycin-treated HeLa cells had ~12.5-fold induction of P38 activity, MCF7, U87MG, MiaPaca-2, and Calu-6 cells had 48, 17, 23, and 17–fold induction of P38 activity.

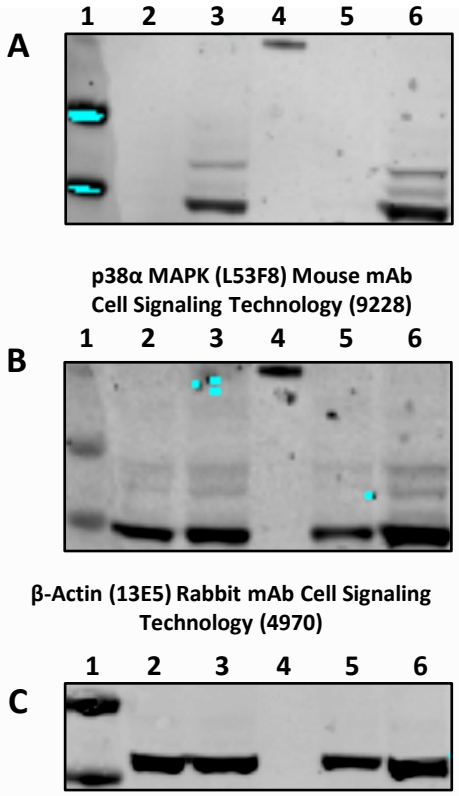
Demonstrates detection of high P38 activity in multiple cell lines with a wide range of induction with anisomycin (2-48-fold) as a stress stimulus

P38 Lysate Western Blots

A Commonly Used Method to Assess P38 Activation

AssayQuant TECHNOLOGIES INC.

Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP®
Rabbit mAb Cell Signaling Technology (4511)



Lane Description:

1. **Ladder**
2. **HeLa lysate (No treatment)**
3. **HeLa lysate (1µM Anisomycin)**
4. **Recombinant P38A (Sino, Cat: M39-10BG)**
5. **HEK293 lysate (No treatment)**
6. **HeK293 lysate (3µM Anisomycin)**

Method: Western blots were developed with antibodies from Cell Signaling Technology diluted 1000-fold for total P38α (92128) or Phospho-p38 MAPK (Thr180/Tyr182) (4511), and then LI-COR, IR Dye goat anti-mouse (800CW 926-32210) or donkey anti-rabbit antibody (680RD 926-68073) antibodies diluted 20,000-fold, followed by imaging in a LI-COR Odyssey.

- **Figure A** shows the presence of total phosphorylated P38 (P38α/β/δ/γ) in lysates from HeLa (Lanes 2 and 3) and HEK293 (Lanes 5 and 6) cells. The phosphorylation of recombinant full-length P38α expressed by baculovirus in Sf9 insect cells using an N-terminal GST tag is shown in Lane 4, as a reference for the Phospho-p38 antibody. HeLa and HEK293 lysates without Anisomycin treatment (Lanes 2 and 5) show no phosphorylated P38 in the western blot. With Anisomycin treatment, there is a strong induction of the phospho-p38 signal, however, the selectivity of the antibody to individual isoform is unknown.
- **Figure B** depicts a qualitative description of the total P38α present in lysates from HeLa (Lanes 2 and 3) and HEK293 (Lanes 5 and 6) cells. Recombinant full-length P38α was loaded as a reference in lane 4 for the antibody. P38α is present at similar levels in both HeLa and HEK293 lysates.
- **Figure C** shows the levels of β-Actin in HeLa and HEK293 lysates detected with the β-Actin antibody to show loading of samples across different lanes.

A phospho-specific antibody to the $pT^{180}GpY^{182}$ epitope is used as a routine measure of the active form of P38 isoforms. However, there are 34 predicted phosphorylation sites on P38, some activating while others inhibiting. Kinexus | PhosphoNET

The solution: *Measure P38 activity directly using the PhosphoSens-Lysate assay!*

Summary

- The PhosphoSens-Lysate Activity Assay for P38α/β/δ/γ using the sensor peptide AQT1280 demonstrates a robust, selective, and physiologically relevant assay that provides a functional assessment of endogenous P38 activity with all the cellular components and signaling complexes. This P38 activity assay is direct, highly quantitative, and in an easy-to-use format. To ensure selectivity for p38 across different stimuli and cell types, we have included the ERK1/2 inhibitor in this assay, given the high levels of ERK1/2 that can be observed in certain cell types and some overlap in substrate sequence recognition.
- Results include:
  - Anisomycin treatment resulted in vigorous activation of P38 activity across multiple cell lines, including MCF7 (48-fold), MiaPaca2 (23-fold), CALU6 (17-fold), U87MG (17-fold), HeLa (12.5-fold), and HCT116 (9-fold). Anisomycin treatment resulted in lower but significant induction of p38 activity in lysates from A549 (4.4-fold) and HEK293 (2.1-fold).
  - P38 activity with lysates from Anisomycin-treated HeLa cells was linear from 2 – 32 µg of total protein/well (16-fold range), corresponding to 5k – 160k HeLa cells seeded/well.
  - The Sensor peptide substrate AQT1280 has a K_m of 12.5 µM when tested with Anisomycin-treated HeLa cell lysates.
  - 96% of AQT1280 phosphorylation in lysates from Anisomycin-treated HeLa cells is inhibited by the reference compound for P38 (Ralimetinib, 1 µM).
  - The IC_{50} value for Ralimetinib in lysates from Anisomycin-treated HeLa cells with AQT1280 was 36 nM.

AQT1280 enables selective and precise quantitation of P38 isoform activity across cell types, providing a powerful tool for evaluating pathway activation or inhibition in complex samples from normal or disease states

Troubleshooting Tips

- P38 induction by Anisomycin (1 µM for 15 min) is evident from multiple cell line data. If a new cell line needs to be tested, we recommend growing the cells in 96-well plates and performing a cell titration by seeding varying numbers of cells per well to identify the optimal density that provides P38 induction (+/- Anisomycin treatment) using AQT1280. Seeding cells at low density (~5K cells/well) is recommended to determine the induction of P38 activity. Indeed, although P38 activity with lysates from Anisomycin-treated HeLa cells was linear from 2 – 32 µg of total protein/well (16-fold range), corresponding to 5k – 160k HeLa cells seeded/well, the p38 signal became increasingly constitutive with increasing cell density.

- If cells are grown in a T-75 flask, do not scrape when making lysates since these sheer forces will activate p38 and other stress pathways. Add ice-cold CEB (with protease and phosphatase inhibitors) directly to the flask, then detach cells by gently tapping until they release. Minimum scraping on ice may be OK.

- Add 1 µM of the ERK1/2 inhibitor in all the experiments to eliminate any off-target ERK1/2 activity. This is important given the high levels of ERK1/2 that can be observed in certain cell types and some overlap in substrate sequence recognition.