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PhosphoSens® Cell Lysate Assay Protocol & Validation Data for P38 MAPK:

Determination of P38 MAPK Activity in Crude Lysates from HeLa Cells Using the Selective AQT1280 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

P38 PhosphoSens-Lysate Kinase Discovery Kit

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MATERIALS INCLUDED

Table 1

Component	Description	100 Assay Kit Volume
PhosphoSens Kinase-Selective Lysate Substrate, AQT1280, 1mM	p38-selective sensor peptide substrate for assaying kinase activity in complex biological samples	45 µL
ATP Solution, 100 mM	100 mM ATP in nuclease-free water	30 µL
DTT Solution, 1 M	1 M DTT in nuclease-free water	5 µL
Enzyme Reaction Buffer (ERB), 10X	500 mM HEPES, pH 7.5, 0.1% Brij-35, 100 mM MgCl2	300 µL
Enzyme Dilution Buffer (EDB), 5X Base	20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mg/mL Bovine Serum Albumin (BSA)	800 µL
EGTA Solution, 550 mM	550 mM EGTA in water (pH adjusted with NaOH)	30 µL
PhosphoPreserve Cell Extraction Buffer, 1X Base*	Please reach out to support@assayquant.com to inquire	1,000 µL
PhosphoPreserve Phosphatase Inhibitor Cocktail - 1, 100X	Please reach out to support@assayquant.com to inquire	20 µL
PhosphoPreserve Phosphatase Inhibitor Cocktail - 3, 60X	Please reach out to support@assayquant.com to inquire	20 µL
PhosphoPreserve Protease Inhibitor Cocktail, 60X	Please reach out to support@assayquant.com to inquire	20 µL
PhosphoSens PhosphoControl Peptide, AQT1251, 1 mM	Fully phosphorylated version of the p38-selective sensor peptide substrate, AQT1280.	10 µL
HeLa cells, No treatment – 2.7 µg/µL	Crude lysate of HeLa cells in CEB supplemented with DTT, protease and phosphatase inhibitors.	25 µL
HeLa cells, With 1µM anisomycin– 2.7 µg/µL	Crude lysate of HeLa cells (treated with 1µM Anisomycin) in CEB supplemented with DTT, protease and phosphatase inhibitors.	25 µL
SCH772984 (ERK1/2 inhibitor)	10 mM dissolved in 100% DMSO	5 µL
Ralimetinib [LY2228820 (mesylate)]	10 mM dissolved in 100% DMSO	5 µL

**PhosphoPreserve Cell Extraction Buffer, 1X Base, requires protease and phosphatase inhibitors to preserve native kinase activity (prevent protein degradation and dephosphorylation). See notes in point 7 on the right side of this slide*

MATERIALS NOT INCLUDED

1. Ultra-pure deionized water
2. Dimethyl Sulfoxide (DMSO), $(CH_3)_2SO$
3. Precision pipettes capable of dispensing down to 0.5 µL and pipette tips. Having both single and multichannel pipettes is helpful
4. Plasticware: Low Protein Binding Microcentrifuge Tubes (0.5 and 1.5 mL), and materials to make your own cell or tissue lysates for a titration.
5. Centrifuge capable of spinning plates at 200xg and microfuge tubes at 10,000xg (standard microcentrifuge)
6. Fluorescence microplate reader capable of reading kinetically with filter setup for excitation (360 nm) and emission (485 nm) wavelengths. Alternatively, an instrument with a monochromator can be used to set the excitation (360 nm) and emission (485 nm) wavelengths, although this can reduce the assay sensitivity.
7. 96-well plate: We have validated Falcon® 96-well White Flat Bottom TC-treated Microplate (Corning Cat. #353296) at 100 µL final well volume.
8. Plate Seal and Sealing Paddle: We have validated Dot Scientific, Seal Plate 50um Polyester Sealing Film, Non-Sterile (Cat. #T480).

Lysate Assay Conditions and Reaction Setup

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Assay 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 Sensor Peptide substrate, AQT1280 or Phosphopeptide control, AQT1251

Kinase Enzyme Source:

- 1 nM P38 Recombinant enzyme
- **For simple lysate assay** – 5 µg/well HeLa lysates (from cells treated ± 1 µM Anisomycin for 15 minutes) following overnight serum starvation to make cells quiescent)
- ***For Lysate titration** – 5k, 10k, 20k, 40k, 80k, and 160k cells/well HeLa lysates (from cells treated ± 1 µM Anisomycin for 15 minutes). Samples are prepared by serial dilution of the cells. See slide 7 for more details

**This test is performed with your cell lysates and not the samples provided. Lysates should be 1 mg/mL or higher total protein concentration.* In the example experiment we ran and detailed, we started at 5000 cells/well (2 µg of protein/well). This should be determined empirically and will vary depending on the cell line and the treatment conditions.*

Notes:

1. Anisomycin was obtained from MedchemExpress (HY-18982) and resuspended in DMSO.
2. Total protein concentration for cell lysates was determined using a modified Bradford assay (Cat # 5000006, BIO-RAD).
3. **Cell Extraction Buffer:** 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, and **supplemented with PhosphoPreserve Phosphatase Inhibitor Cocktail -1, PhosphoPreserve Phosphatase Inhibitor Cocktail – 3** ,and **PhosphoPreserve Protease Inhibitor Cocktail** as indicated in slide 3 and as prepared on slides 8 and 12 **just before use**.
4. **Final 1X Enzyme Dilution Buffer (EDB):** 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 1 mg/ml Bovine Serum Albumin (BSA), and supplemented with 0.2 mM EGTA and 1 mM DTT just before use.
5. Reactions were run in Falcon® 96-well White Flat Bottom TC-treated Microplate (Corning Cat. #353296) at 100 µL final well volume and after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185] or Dot Scientific [Cat. #T480]) in a Biotek Synergy Neo2 microplate reader with filter setup for excitation (360 nm) and emission (485 nm) wavelengths.

   Alternatively, reactions can be run in 25 µL final volume in Corning, low volume 384-well, white

Reagent Preparation for a Low Volume 96-well Format (100 µL final reaction volume)*

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The protocol is for cells grown in 96-well plates

1. Using the stock solutions provided with the kit (Table 1), prepare the reagents shown in Table 2 and Table 3 (final concentrations shown in parentheses).
   - **10 mM ATP :** Make 400 µL of 10 mM ATP by adding 40 µL of 100 mM ATP to 360 µL of ultrapure deionized water.
   - **10 mM DTT:** Make 400 µL of 10 mM DTT by adding 4 µL of 1M (1000 mM) DTT to 396 µL of ultrapure deionized water.
   - **5.5 mM EGTA:** Make 400 µL of 5.5 mM EGTA by adding 4 µL of 550 mM EGTA to 396 µL of ultrapure deionized water.
   - **150 µM Sensor Peptide:** Make 400 µL of 150 µM AQT1280 by adding 60 µL of 1 mM AQT1280 to 340 µL of ultrapure deionized water.
   - **50 µM P38 Inhibitor (Ralimetinib) and SCH772984 (ERK1/2 inhibitor):** Make 200 µL of 50 µM Ralimetinib and SCH772984 by adding 1 µL of 10 mM stocks of these compounds in 199 µL DMSO, respectively.
2. Prepare final 1X *PhosphoPreserve* Cell Extraction Buffer (CEB) using the volume of components listed in Table 2B
3. Prepare '1.28X Master Mix' by combining volumes of the components listed in Tables 2 The volumes for a single well and 38 wells (Table 2) are shown.
4. Prepare 2584 µL (sufficient for 38 wells but test requires only 36 wells and includes dead volume) of 1.28X Master mix per Table 2.
5. When adjusting the volume for a different number of wells, ensure that you include an additional 5% dead volume (~136 µL) above the actual volume required.

**** The protocol is primarily for a 96-well plate assay. If you have active lysates to perform the assay in a 384-plate, please follow the protocols in Slide 7 and 8.***

Table 2A - Selective Sensor Peptide Substrate AQT1280

Components for 1.28X Master Mix:	For 1 Well:	For 38 Wells:
Enzyme Reaction Buffer (**10X**)	10 µL	380 µL
ATP (**10 mM**)	10 µL	380 µL
DTT solution (**10 mM**)	10 µL	380 µL
EGTA Solution (**5.5 mM**)	10 µL	380 µL
Selective Sensor Peptide Substrate *AQT1280* (**150 µM**)	10 µL	380 µL
Enzyme dilution buffer	20 µL	380 µL
Ultrapure deionized water	8.0 µL	304 µL
Total volume	**78 µL**	**2584 µL**

Table 2B - 1X *PhosphoPreserve* Cell Extraction Buffer

Components	Con.	Volume
PhosphoPreserve Cell Extraction Buffer (CEB)	1X	956.6 µL
PhosphoPreserve Phosphatase Inhibitor Cocktail -1	100X	10 µL
PhosphoPreserve Phosphatase Inhibitor Cocktail - 3	60X	16.7 µL
PhosphoPreserve Protease Inhibitor Cocktail	60X	16.7 µL
Total volume		**1000 µL**

Reagent Preparation for a Low Volume 384-well Format (25 µL final reaction volume)*

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*If you are working in a Corning 96-well plate (Cat. # 3642), multiply the volume of components by 2 for a final reaction volume of 50 µL per well

1. Using the stock solutions provided with the kit (Table 1), prepare the reagents shown in Table 2 and Table 3 (final concentrations shown in parentheses).
   - **10 mM ATP :** Make 90 µL of 10 mM ATP by adding 9 µL of 100 mM ATP to 81 µL of ultrapure deionized water.
   - **10 mM DTT:** Make 300 µL of 10 mM DTT by adding 3 µL of 1M (1000 mM) DTT to 297 µL of ultrapure deionized water.
   - **5.5 mM EGTA:** Make 300 µL of 5.5 mM EGTA by adding 3 µL of 550 mM EGTA to 297 µL of ultrapure deionized water.
   - **150 µM Sensor Peptide:** Make 90 µL of 150 µM AQT1280 by adding 13.5 µL of 1 mM AQT1280 to 76.5 µL of ultrapure deionized water. Make 14 µL of 150 µM AQT1251 by adding 2.1 µL of 1 mM AQT1251 to 11.9 µL of ultrapure deionized water.
   - **50 µM P38 tool compound mix (Ralimetinib + SCH772984) and SCH772984 (ERK1/2 inhibitor):** Make 200 µL of 50 µM Ralimetinib + SCH772984 by adding 1 µL of 10 mM Ralimetinib and 1 µL of 10 mM SCH772984 in 198 µL DMSO, respectively. Make 200 µL of 50 µM SCH772984 separately by adding 1 µL of 10 mM SCH772984 in 199 µL DMSO
2. Prepare final 1X *PhosphoPreserve* Cell Extraction Buffer (CEB) using the volume of components listed in Table 2B (slide 6)
3. Prepare '1.28X Master Mix' by combining volumes of the components listed in Tables 2C and 3. The volumes for a single well and 32 wells (Table 2C) or 5 wells (Table 3) are shown.
4. Prepare 624 µL (sufficient for 32 wells but test requires only 28 wells and includes dead volume) of 1.28X Master mix per Table 2C and 97.5 µL of 1.28X Master mix per Table 3.
5. When adjusting the volume for a different number of wells, ensure that you include an additional 8% dead volume above the actual volume required.

Table 2C - Selective Sensor Peptide Substrate AQT1280

Components for 1.28X Master Mix:	For 1 Well:	For 32 Wells:
Enzyme Reaction Buffer (**10X**)	2.5 µL	80 µL
ATP (**10 mM**)	2.5 µL	80 µL
DTT solution (**10 mM**)	2.5 µL	80 µL
EGTA Solution (**5.5 mM**)	2.5 µL	80 µL
Selective Sensor Peptide Substrate *AQT1280* (**150 µM**)	2.5 µL	80 µL
Ultrapure deionized water	7.0 µL	224 µL
Total volume	**19.5 µL**	**624 µL**

Table 3 - Phospho-Peptide Control AQT1251

Components for 1.28X Master Mix:	For 1 Well:	For 5 Wells:
Enzyme Reaction Buffer (**10X**)	2.5 µL	12.5 µL
ATP (**10 mM**)	2.5 µL	12.5 µL
DTT solution (**10 mM**)	2.5 µL	12.5 µL
EGTA Solution (**5.5 mM**)	2.5 µL	12.5 µL
Sensor Phosphopeptide Control *AQT1251* (**150 µM**)	2.5 µL	12.5 µL
Ultrapure deionized water	7.0 µL	35.0 µL
Total volume	**19.5 µL**	**97.5 µL**

Step-by-Step Guide to growing HeLa cells in a 96-well Plate for lysate titration

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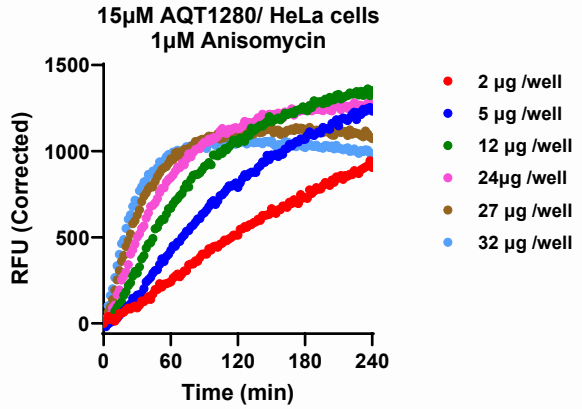
- Perform this experiment if you are plan to grow cells in a 96-well plate format.

1. Day1: Seed HeLa cells (1 x 10^6 cells) in a T75 flask (12 mL EMEM medium with 10% FBS + 1% Pen-strep) and incubate for 48 h at 37 °C, 5% CO_2 to allow cells to attach and recover.
2. Day 3: Determine the cell count and prepare 1000 µL of 1,600,000 cells/mL in a sterile tube. Seed 100 µL cells (1,600,000 cells/well) in column 6 (Rows A – F) of a 96-well plate [Falcon® 96-well White Flat Bottom TC-treated Microplate (Corning Cat. #353296)]. Perform 4-point, 2-fold serial dilution from column 5 to column 1 (5k, 10k, 20k, 40k, 80k, and 160k cells/well HeLa lysates) and incubate for 48 h at 37°C, 5% CO_2 to allow cells to attach and recover. Mix at each step.
3. Day 4: Remove media and add 100 µL of low-serum medium (EMEM/0.1% FBS/1% Pen-strep). Incubate for 4 h at 37°C, 5% CO_2 as a serum-starvation step to make the cells quiescent.
4. Day 5: Cell Lysis and assay:
    1. Add 1 µM Anisomycin (1 µL of 303 µM stock to Rows D, E, F, and G –columns 1 – 6) and incubate at 37°C, 5% CO_2 for 15 minutes at 37 °C (See the plate map on the right for guidance).
    2. Remove the medium and wash the cells with 100 µL ice-cold PBS (1X) in each well. Keep the plate on ice and add 20 µL of ice-cold CEB containing protease and phosphatase inhibitors. Incubate on ice for 15 minutes.
    3. Add 2 µL of the ERK1/2 tool compound (50 µM SCH772984) to the wells labeled "***HeLa_S.S 1µM_Anisomycin_15 min incubation + 1µM SCH772984***" and DMSO to all the other wells
    4. Add 2 µL of the tool compound mix (50 µM Ralimetinib + SCH772984) to the wells labeled "***With inhibitor: 1µM Ralimetinib + 1µM SCH772984***" and DMSO to all the other wells.
    5. Prewarm the 1.28X Master mix (Table 2A) at 30 ° C and add 78 µL to all the wells shown in the plate map.
    6. Re-seal the plate, centrifuge at 200xg for one minute, and place the plate in the microplate reader set at 30 °C.
    7. Read the plate in kinetic mode with continuous fluorescence intensity detection (Ex/Em 360/485 nm) every 2 minutes for 1-4 hours. The frequency of the readings and the overall duration can be adjusted as needed.
    8. Determine the total protein concentration of the cells in row F using the Bradford assay (Cat # 5000006, BIO-RAD).

Plate map for 96-well lysate titration

	1	2	3	4	5	6	
	15µM AQT1280						
A	No cells	5K/well	10K/well	20K/well	40K/well	80K/well	HeLa_S.S No Treatment
B							
C	No cells	5K/well	10K/well	20K/well	40K/well	80K/well	HeLa_S.S 1µM_Anisomycin_15 min incubation + 1µM SCH772984
D							
E	No cells	5K/well	10K/well	20K/well	40K/well	80K/well	With inhibitor: 1µM Ralimetinib + 1µM SCH772984
F							
F	No cells	5K/well	10K/well	20K/well	40K/well	80K/well	For protein conc.



Total cells/ well	[Total protein] in each well (in µg)	Rate in RFU/ min
5k	2	4.9
10k	5	7.4
20k	12	12
40k	24	19.5
80k	27	22.2
160k	32	24.2

Step-by-Step Guide to Performing a Lysate Activity Assay in a 384-well Plate

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A plate map for a simple lysate assay is shown on the next slide, which serves as a guide for making additions to the plate as outlined below.

1. Prepare 1X EDB using the 5X stock of EDB, Base provided, and supplement with DTT and EGTA. For example, to make 5000 µL of 1X EDB, add 1000 µL 5X EDB Base along with 5 µL of 1M DTT, 5 µL of 550 mM EGTA, and 3990 µL of ultrapure deionized water to create the final composition shown on slide 5. **Keep on ice.**
2. Prepare 250 µL BLANK by adding 167 µL 1X EDB and 83 µL of 1X Final PhosphoPreserve Cell Extraction Buffer .
3. Prepare 200 µL of 25 nM recombinant P38 (5X) by adding 1.1 µL of the 4410 nM P38 stock to 248.9 µL of 1X EDB. **Keep on ice until needed.**
4. Prepare 80 µL of 0.5 mg/mL lysate by adding 10.3 µL of the 3.9 mg/mL stock to 69.7 µL of 1X EDB. **Keep on ice until needed**
    1. ***Freeze-dried lysates:*** *Add 26 µL of ddH_2O to the freeze-dried sample and mix gently until the lysates are resuspended. To prevent bubble formation, centrifuge on a tabletop centrifuge (2 min@ @ 13k RPM). Keep on ice for 15 minutes, mix gently and centrifuge again on a tabletop centrifuge (for 5 min @ 13k RPM).*
5. Transfer 20 µL of stimulated lysate to a new tube and heat at 95 °C for 15 minutes to serve as a Heat Inactivated (HI) negative control. Remove the tube and cool to room temperature.
6. Add 0.5 µL of 50 µM Ralimetinib Inhibitor to all wells in columns 2 and 5 (as shown on the plate map on the next slide). Add 0.5 µL of DMSO to all wells without the tool compound in columns 1, 3, 4, and 8 (as shown on the plate map on the next slide).
7. Add 19.5 µL of 1.28X Master Mix for AQT1280 from Table 2 to all the wells except the wells labeled ‘AQT1251’. For the wells labeled ‘AQT1251’, add 19.5 µL of 1.28X Master Mix for AQT1251 from Table 3.
8. Seal the plate using the plate seal supplied and press down with the supplied paddle. Incubate at 30 °C for 15 minutes to equilibrate the plate and Master Mix. This can be done by placing the plate inside a plate reader set at 30 °C. This step is important to prevent temperature changes that can create anomalies in the data at the beginning of the reaction.

Note: Steps 9 and 10 should be performed quickly since the reaction will start with these additions.

9. Source of Kinase Enzyme or control (BLANK or EDB, for background determination and the AQT1251 sensor phosphopeptide positive control):
    - Add 5 µL BLANK (prepared in step 2 above) to the wells labeled ‘BLANK’ or 1X EDB to wells labeled ‘1X EDB’
    - Add 5 µL 1X EDB to wells labeled ‘AQT1251’
    - Add 5 µL of the stimulated (+Anisomycin) lysates to the corresponding wells labeled ‘2.5 µg Lysate’
    - Add 5 µL of 25 nM P38 (5X) to the wells labeled ‘5 nM P38’.
10. Re-seal the plate, centrifuge at 200xg for one minute, and place the plate in the microplate reader set at 30 °C.
11. Read the plate in kinetic mode with continuous fluorescence intensity detection (Ex/Em 360/485 nm) every 2 minutes for 1-4 hours. The frequency of the readings and the overall duration can be adjusted as needed.

Plate Additions and Plate Map For a Simple Lysate Activity Assay

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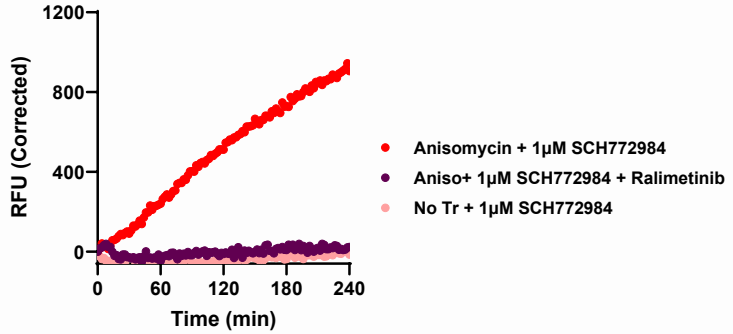
Component	Volume to add to wells
Tool Compound or 100% DMSO	0.5 µL
Master mix	19.5 µL
Lysate, P38, BLANK, or 1X EDB	5 µL

Total volume 25 µL

	Plate map for Lysate activity assay					
	Stimulated lysates Anisomycin			With recombinant enzyme		
	With 1µM SCH772984	With 1µM (Ralimetinib+ SCH772984)	Heat inactivated lysate	With 1µM SCH772984	With 1µM (Ralimetinib+ SCH772984)	Phosphopeptide control
	1	2	3	4	5	8
A	BLANK	BLANK	BLANK	1X EDB	1X EDB	AQT1251
B	BLANK	BLANK	BLANK	1X EDB	1X EDB	AQT1251
C	0.5 µg Lysate	0.5 µg Lysate	0.5 µg Lysate	1 nM P38	1 nM P38	AQT1251
D	0.5 µg Lysate	0.5 µg Lysate	0.5 µg Lysate	1 nM P38	1nM P38	AQT1251

Progress Curves for Total Fluorescence with AQT1280

AQT1251 data can be found on slide 11



This test uses 32 wells (28 wells for the AQT1280 sensor peptide substrate and 4 wells for the AQT1251 phosphopeptide control). All conditions are tested in duplicate.

Data Analysis

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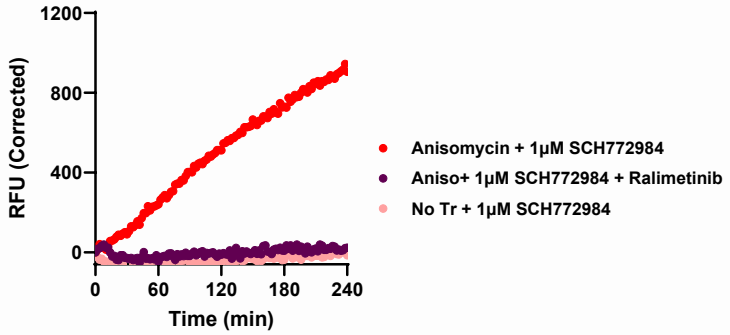
1. Collect the data from the microplate reader. This is a complete time course (Progress Curve) for every well with values in Relative Fluorescence Units (RFU) for each time point for Total (for each experimental condition) and “EDB/blank” wells.
2. Take the average of duplicate “EDB/blank” wells for each condition at each time point. Subtract the average EDB/blank values from the corresponding Total RFU of individual wells for each condition at each time point to obtain the background corrected RFU values. For example, take the average of A1 and B1, and subtract the value from the total RFU determined for individual wells C1 and D1 at each time point. You can then either plot these RFU (Corrected) values separately to assess individual wells or take the average of the RFU (Corrected) values at each time point and plot this data.
3. It is highly recommended to run the “EDB/blank" wells at each compound concentration to correct for tool compound autofluorescence, if any. Since this is a kinetic assay format, the background with compounds will not change over time and can be subtracted from the total.
4. From the plot of the RFU (Corrected) values, determine the slope from the points in the linear region. This is the “initial reaction rate” in RFU (Corrected)/min. We recommend using ~30 minutes of the linear region of the progress curve to determine the rate. This can be performed in Excel, Excel-Fit, GraphPad Prism, the software provided with your microplate reader, or any other suitable software package, such as DynaFit, GeneData Screener, KinTek, Mathematica, MATLAB, or SigmaPlot.
5. Compare the RFU (Corrected)/min values for the samples to evaluate the activity of the P38 in each sample.
6. Refer to slide 11 for representative validation data for this simple lysate assay.

P38 Lysate Activity Assay data from 96-well Plate assay

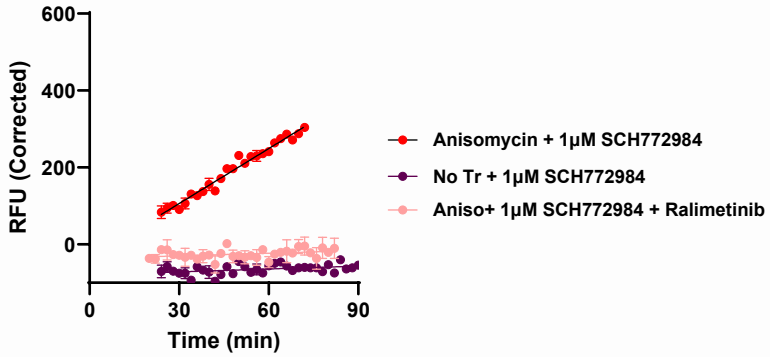
Activation of P38 in Lysates from HeLa Cells + Anisomycin

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

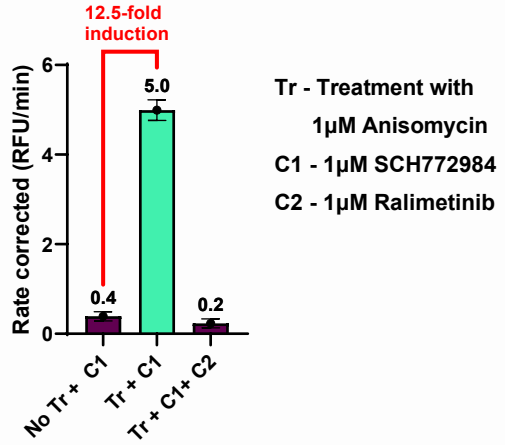
1) Full Time Course (0-240 min.)



2) Linear Range (25-90 min.)

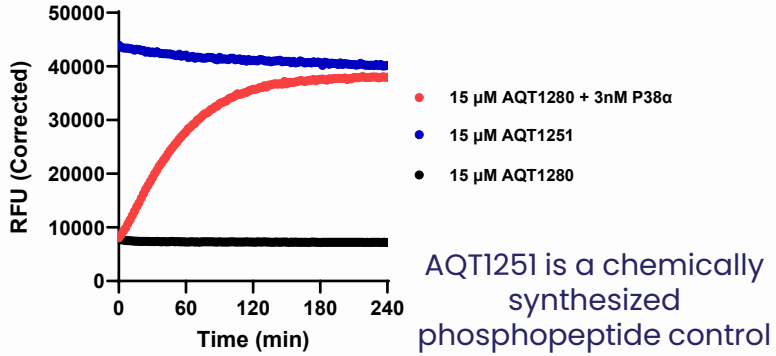


3) Histogram



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.

Lysate Titration for HeLa Cells Treated with Anisomycin and P38 Activity Measured Using AQT1280

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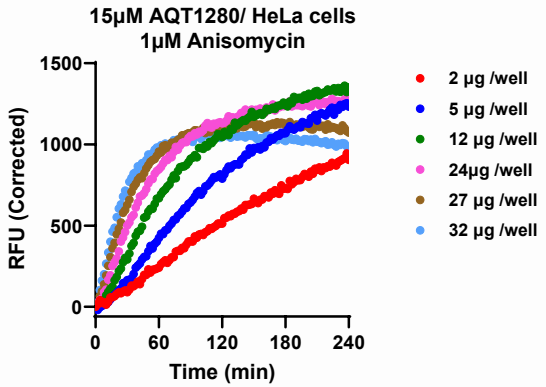
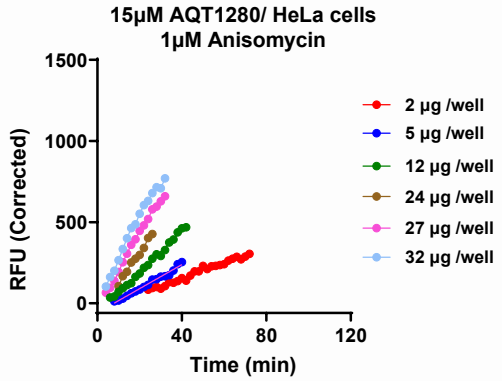
Progress Curves

Complete Time Course

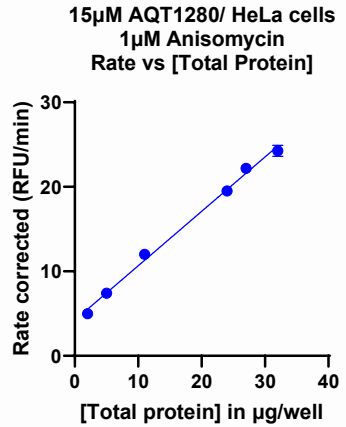


Linear Region



Assessment of Linearity

All Data & Linear Reaction



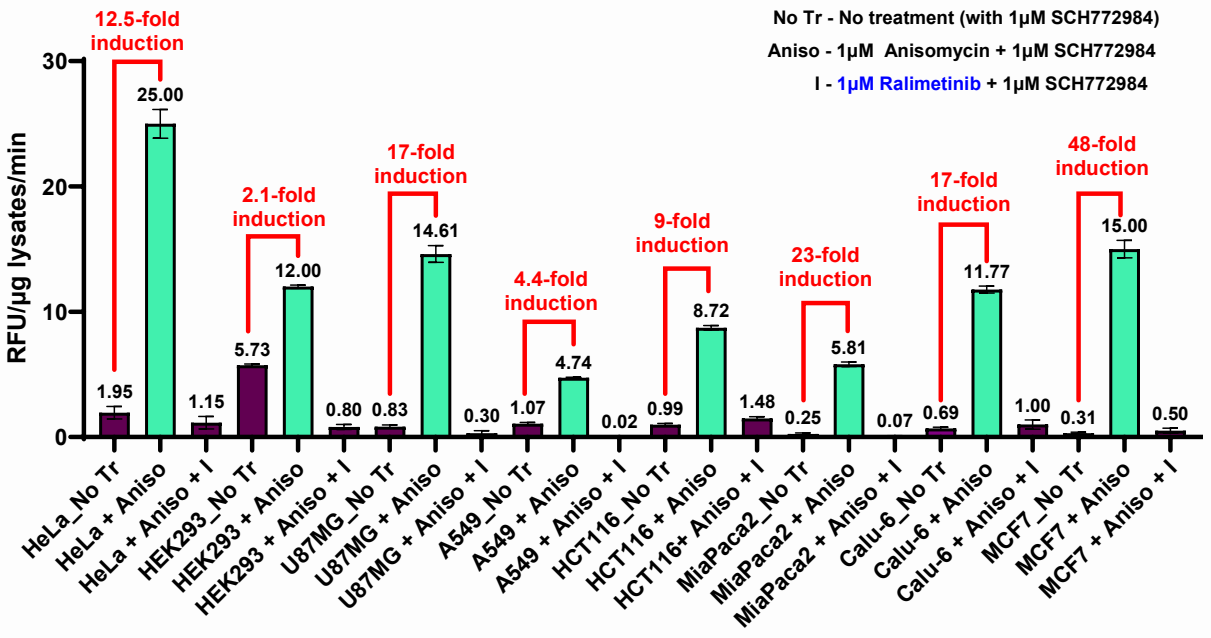
The AQT1280 sensor peptide was used at 15 µM with an increasing amount of lysate from HeLa cells treated with Anisomycin to activate the P38 MAPK enzymes. RFU Corrected values (Total – Background) were determined for each condition. The results are presented for each amount of lysate for **1)** Full 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 + phosphatase and protease inhibitors 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 P38 provides a selective, highly quantitative, and accurate measure of kinase activity in a complex sample.

P38 Lysate Activity Assay Across a Variety of Cell Types ± Anisomycin treatment and ± Ralimetinib in the Reaction using AQT1280

AssayQuant TECHNOLOGIES INC.

All cell lines seeded at 5k cells/well and p38 activity measured using 15 uM AQT1280 with various cell line lysates



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

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.