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PhosphoSens® Cell Lysate Assay Protocol & Validation Data for JNK1/2/3 MAPK:

Determination of JNK1/2/3 MAPK Activity in Crude Lysates from HEK293 Cells Using the Selective AQT1196 Sensor Peptide Substrate

HGNC Name: MAPK8 (JNK1), MAPK9 (JNK2), and MAPK10 (JNK3)
Long Names: c-**J**un **N**-terminal **K**inase, **M**itogen-**A**ctivated **P**rotein **K**inase (MAPK)

INTRODUCING: PhosphoSens-Lysate Assays

Simple, Powerful & Flexible

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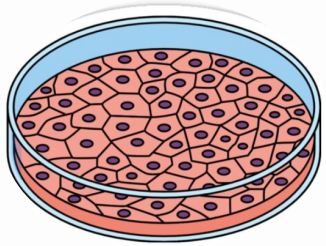
1. Grow Cells

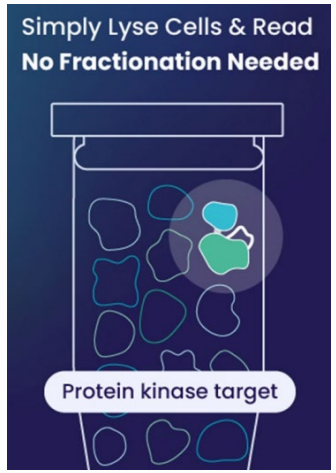
(+/- Pathway Activation)

or Access Tissues



2. Harvest

ADD *PhosphoPreserve*
Lysis Buffer + Protease & Phosphatase Inhibitors



Study Kinase Enzyme Activity Where Biology Happens

We have combined innovative PhosphoSens detection technology, invented at MIT, with high-throughput peptide synthesis methods to design sensor peptide substrates that are highly selective for the target of interest. This development integrates the advantages of the PhosphoSens platform (direct, homogeneous, continuous/kinetic, quantitative) with the enabling capability of measuring endogenous target kinase activity in unfractionated cell or tissue lysates.

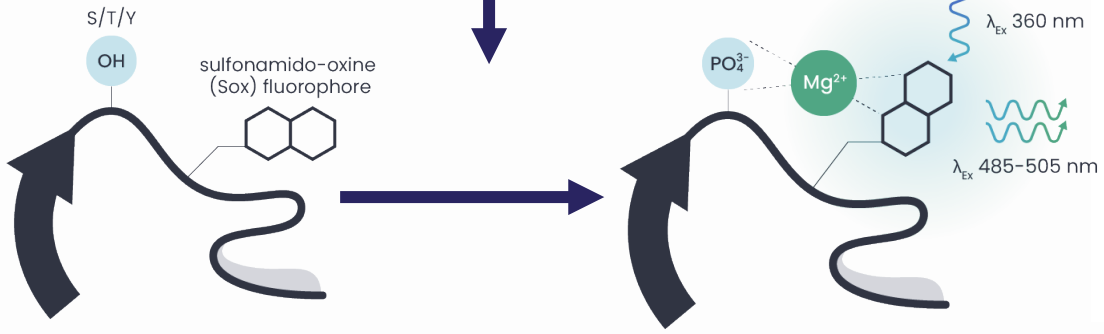
Easy-to-use Kits & Bulk Sensor Peptide

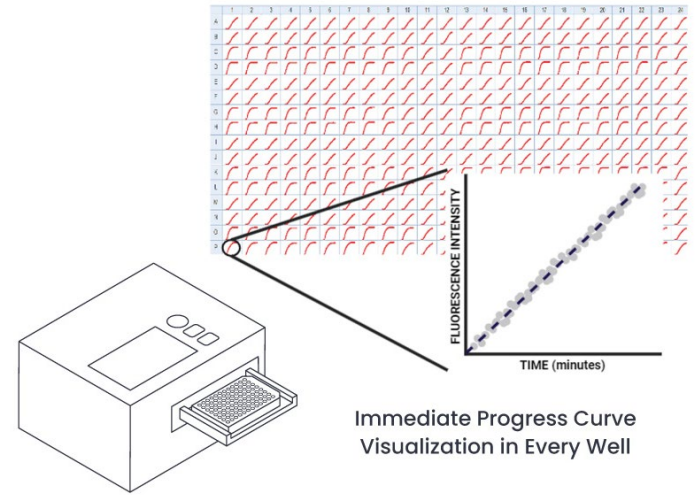
(Native substrates, stable, scalable, excellent lot consistency)

3. Prepare Reaction Mix

(PhosphoSens Sensor Peptide Substrate, ATP, Mg^{2+})

ADD Lysate & Read





Immediate Progress Curve Visualization in Every Well

JNK PhosphoSens-Lysate Kinase Discovery Kit

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MATERIALS INCLUDED

TABLE 1		
Components	**Description**	**100 Assay Kit Volumes**
Selective Sensor Peptide Substrate, AQT1196, 1 mM	PhosphoSens Selective Sensor Peptide Substrate for JNK	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
AQT1232 PhosphoControl, 1 mM	PhosphoSens Selective Sensor Peptide Substrate PhosphoControl that provides the fluorescence intensity maximum of AQT1196	10 µL
HEK293 Lysate Control	Crude lysate of HEK293 cells (treated with 3µM Anisomycin) in CEB supplemented with DTT and protease and phosphatase inhibitors.	26 µL
JNK Inhibitor: JNK-IN-8	10 mM JNK-IN-8 dissolved in 100% DMSO	5 µL

MATERIALS NOT INCLUDED

1. Ultra-pure deionized water
2. Dimethyl Sulfoxide (DMSO), (CH3)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).

**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.*

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, AQT1196 or Phosphopeptide control, AQT1232

Kinase Enzyme Source:
- 5 nM JNK1 Recombinant enzyme
- **Simple lysate assay** – 5 µg/well HEK293 lysates (from cells treated ± 3 µM Anisomycin for 40 minutes) following overnight serum starvation to make cells quiescent)
- ***Lysate titration performed with increasing number of cells seeded in a 96-well plate** – 2.5k, 5.0k, 10k, 20k, 40k, and 80k cells seeded/well HEK293 lysates using a serial dilution. Cells are treated ± 3 µM Anisomycin for 40 minutes, then solubilized with CEB per point #3 in the Notes. See slide 7 for more details

****This test is performed with your cell lysates and not the sample provided. Lysates should be 1 mg/mL or higher total protein concentration.** *In the example experiment we ran and detailed, we started at 2500 cells/well (0.1 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 flat bottom polystyrene NBS microplates (Cat. #3824)

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 AQT1196 by adding 60 µL of 1 mM AQT1196 to 340 µL of ultrapure deionized water.
   - **50 µM JNK Inhibitor (JNK-IN-8):** Make 200 µL of 50 µM JNK-IN-8 by adding 1 µL of 10 mM JNK-IN-8 in 199 µL DMSO.
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 Table 2A The volumes for a single well and 38 wells (Table 2A) 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 2A.
5. When adjusting the volume for a different number of wells, ensure that you include an additional 5% dead volume (~136 µL in this example) above the actual volume required.

** The protocol is designed 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.*

Table 2A - Selective Sensor Peptide Substrate AQT1196

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 *AQT1196* (**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

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)*

*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 indicated 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 AQT1196 by adding 13.5 µL of 1 mM AQT1196 to 76.5 µL of ultrapure deionized water. Make 14 µL of 150 µM AQT1232 by adding 2.1 µL of 1 mM AQT1232 to 11.9 µL of ultrapure deionized water.
   - **50 µM JNK1/2/3 Inhibitor (JNK-IN-8):** Make 200 µL of 50 µM SCH772984 by adding 1 µL of 10 mM JNK-IN-8 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, 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, include an additional 5-8% dead volume above the required volume.

Table 2C - Selective Sensor Peptide Substrate AQT1196

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 *AQT1196* (**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 AQT1232

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 *AQT1232* (**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 HEK293 Cells in a 96-well Plate for Lysate Titration

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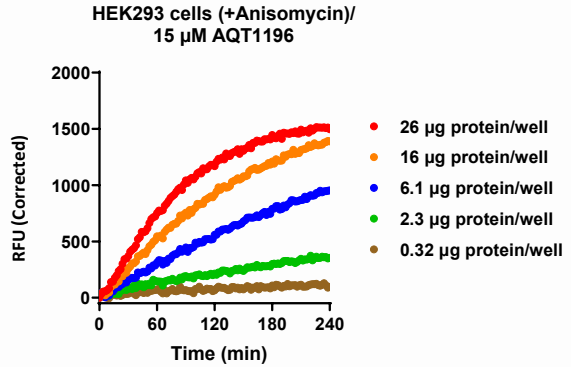
- Perform this experiment if you are plan to grow cells in a 96-well plate format.

1. **Day1:** Seed HEK293 cells (1 × 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 800,000 cells/mL in a sterile tube. Seed 100 µL cells (80,000 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 (2.5k, 5.0k, 10k, 20k, 40k, and 80k cells/well HEK293 lysates) and incubate for 48 h at 37 °C, 5% CO_2 to allow cells to attach and recover.
3. **Day 4:** Remove media and add 100 µL low-serum media (EMEM/**0.1% FBS**/1% Pen-strep) and incubate for 24 h at 37 °C, 5% CO_2.
4. **Day 5:** Cell Lysis and Assay:
    1. Add 3 µM Anisomycin (1 µL of 303 µM to Rows D, E, F, and G –columns 1 to 6) and incubate at 37 °C, 5% CO_2 for 40 minutes (See the plate map on the right for guidance).
    2. Remove the media and wash the cells with 100 µL ice-cold PBS (1X) in each well. Keep the plate on ice and add 20 µL ice-cold CEB with phosphatase and protease inhibitors and incubate on ice for 15 minutes.
    3. Add 2 µL of the tool compound (50 µM JNK-IN-8) to the wells labeled “with inhibitor JNK-IN-8” or DMSO to all the other wells.
    4. Prewarm the 1.28X Master mix (Table 2A) at 30 °C and add 78 µL to all the wells shown in the plate map.
    5. Re-seal the plate, centrifuge at 200xg for one minute, and place the plate in the microplate reader set at 30 °C.
    6. 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.
    7. 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 AQT1196						
A	No cells	5K/well	10K/well	20K/well	40K/well	80K/well	HEK293_S.S No Treatment
B							
C	No cells	5K/well	10K/well	20K/well	40K/well	80K/well	HEK293_S.S 3µM_Anismoycin_40 min incubation
D							
E	No cells	5K/well	10K/well	20K/well	40K/well	80K/well	With inhibitor JNK-IN-8
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
5000	0.3	0.36
10000	2.3	1.76
20000	6.1	4.9
40000	16.4	9.9
80000	26.1	13.8

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 JNK1 (5X) by adding 1.1 µL of the 4410 nM JNK1 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 (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 JNK-IN-8 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 AQT1196 from Table 2 to all the wells except those labeled 'AQT1232'. For the wells labeled 'AQT1232', add 19.5 µL of 1.28X Master Mix for AQT1232 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: The additions in step 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 AQT1232 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 'AQT1232'
    - Add 5 µL of the stimulated (+Anisomycin) lysates to the corresponding wells labeled '2.5 µg Lysate'
    - Add 5 µL of 25 nM JNK1 (5X) to the wells labeled '5 nM JNK1'.
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.

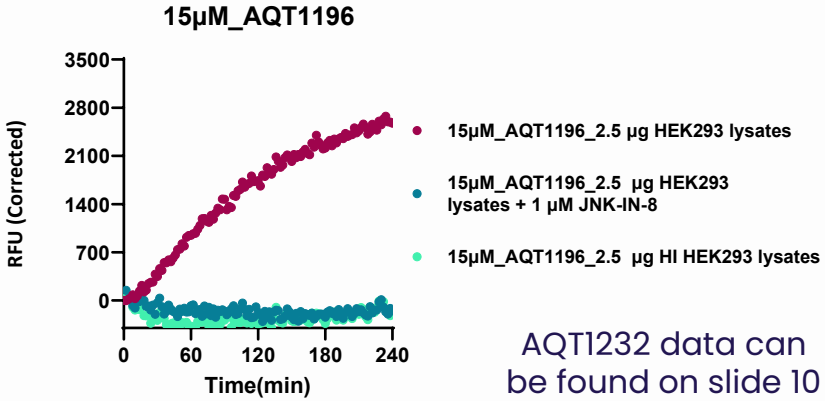
Plate Additions and Plate Map For a Simple Lysate Activity Assay

Component	Volume to add to wells
Tool Compound or 100% DMSO	0.5 µL
Master mix	19.5 µL
Lysate, JNK1, BLANK, or 1X EDB	5 µL

Total volume 25 µL

	Plate map for Lysate activity assay					
	Stimulated lysates Anisomycin			With recombinant enzyme		
	No tool compound	With tool compound JNK-IN-8	Heat inactivated lysate	No tool compound	With tool compound JNK-IN-8	Phosphopeptide control
	1	2	3	4	5	8
A	BLANK	BLANK	BLANK	1X EDB	1X EDB	AQT1232
B	BLANK	BLANK	BLANK	1X EDB	1X EDB	AQT1232
C	2.5 ug Lysate	2.5 ug Lysate	2.5 ug Lysate	5 nM JNK1	5 nM JNK1	AQT1232
D	2.5 ug Lysate	2.5 ug Lysate	2.5 ug Lysate	5 nM JNK1	5 nM JNK1	AQT1232

Progress Curves for Total Fluorescence with AQT1196



AQT1232 data can be found on slide 10

This test uses 32 wells (28 wells for the AQT1196 sensor peptide substrate and 4 wells for the AQT1232 phosphopeptide control). All conditions are tested in duplicate.

Data Analysis

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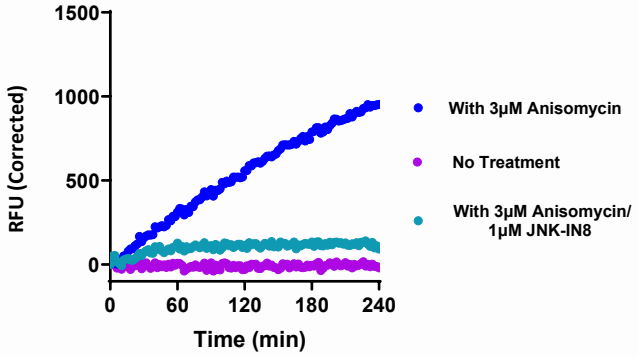
1. Collect the data from the microplate reader. This is a complete time course (Progress Curve) for every well with values in Relative Fluorescence Units (RFU) for each time point for Total (for each experimental condition) and “EDB/blank” wells.
2. Take the average of duplicate “EDB/blank” wells for 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 kinase in each sample.
6. Refer to slide 10 for representative validation data for this simple lysate assay.

JNK Lysate Activity Assay data from 96-well Plate assay

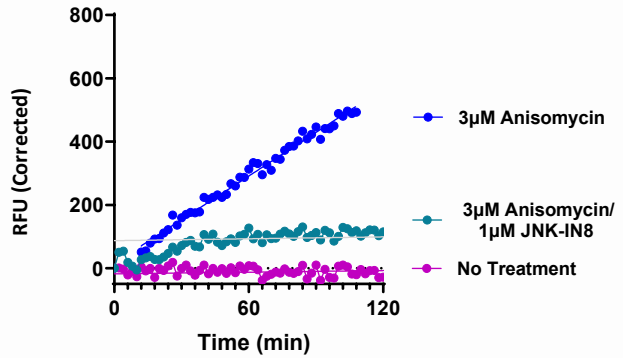
Activation of JNK in Lysates from HEK293 Cells + Anisomycin

A. Crude Lysate Samples: 20k HEK293 cells (or 6.1 µg protein)/well with 15 µM AQT1196

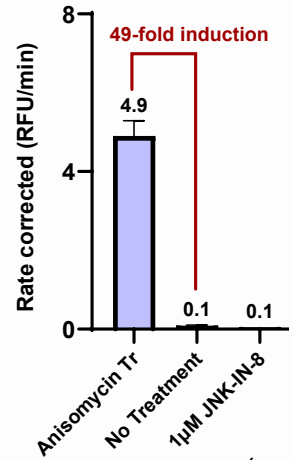
1) Full Time Course (0-240 min.)



2) Linear Range (4-120 min.)

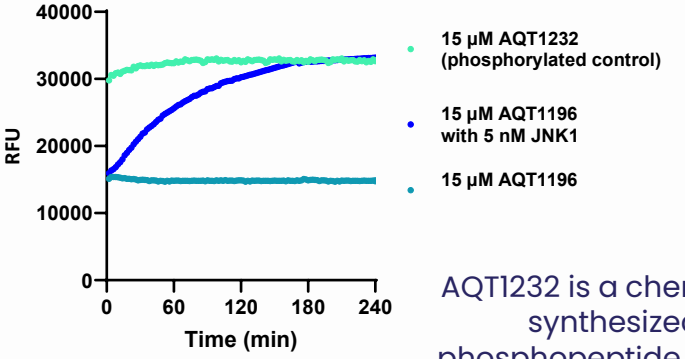


3) Histogram



B. Purified JNK1 & AQT 1232 Control

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



AQT1232 is a chemically synthesized phosphopeptide control

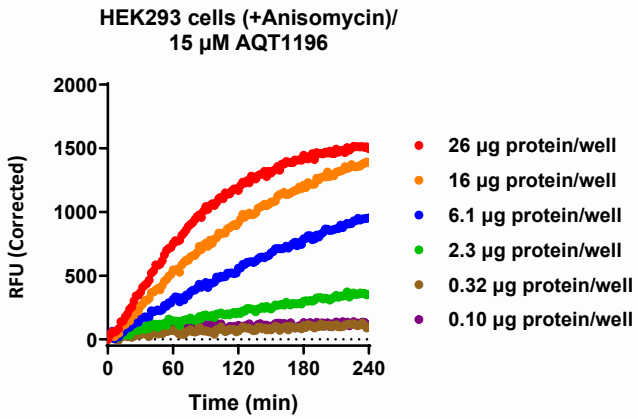
A. Crude lysate samples: The AQT1196 sensor peptide was used to generate RFU Corrected values (Total – Background) for **1)** Full progress curve time course (0-240 min.), and **2)** Linear range (4-120 min.), and to determine the slope for each condition, which is the Reaction rate (RFU Corrected/min. +/- standard deviations) shown as a histogram in **3)**, highlighting a 49-fold activation of JNK kinase activity in HEK293 cell lysates treated with 3 µM Anisomycin for 40 minutes. The signal was eliminated by adding the selective JNK1/2/3 inhibitor JNK-IN-8 (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 JNK activity. Western Blotting or an ELISA can determine the total amount of JNK1/2/3 protein; however, with the short stimulation times typically used, these levels are not expected to change.

B.1. Purified recombinant JNK1 enzyme with AQT1196 & AQT1232 Phosphopeptide Control: JNK1 enzyme (5 nM) fully phosphorylated the AQT1196 sensor peptide substrate by 240 min., as shown by convergence with the signal obtained with the AQT1232 phosphopeptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). The signal with JNK1 enzyme was eliminated by adding the JNK-IN-8 inhibitor (1 µM). The signal with AQT1232 is used to convert RFU (Corrected) values to nmoles of product.

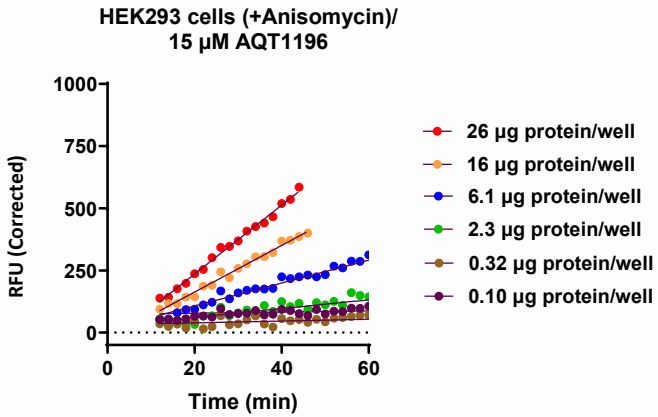
Lysate Titration for HEK293 Cells Treated +Anisomycin and JNK1/2/3 Activity Measured with AQT1196

AssayQuant TECHNOLOGIES INC.

1. Full Time Course Progress Curves

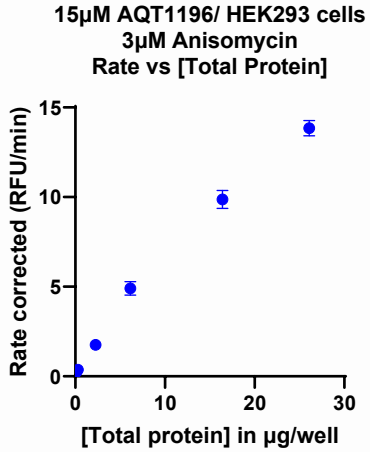


2. Linear Range of Progress Curves

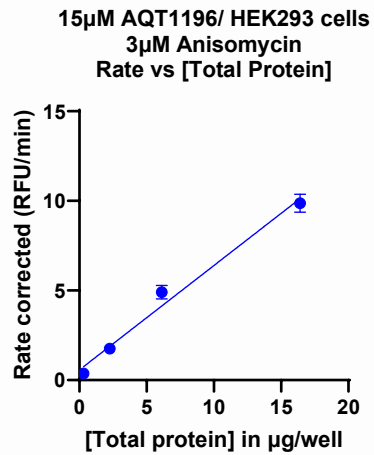


3. Reaction Rate vs Amount of Lysate per Well

A. All Lysate Amounts



B. Lysate Amounts in Linear Range

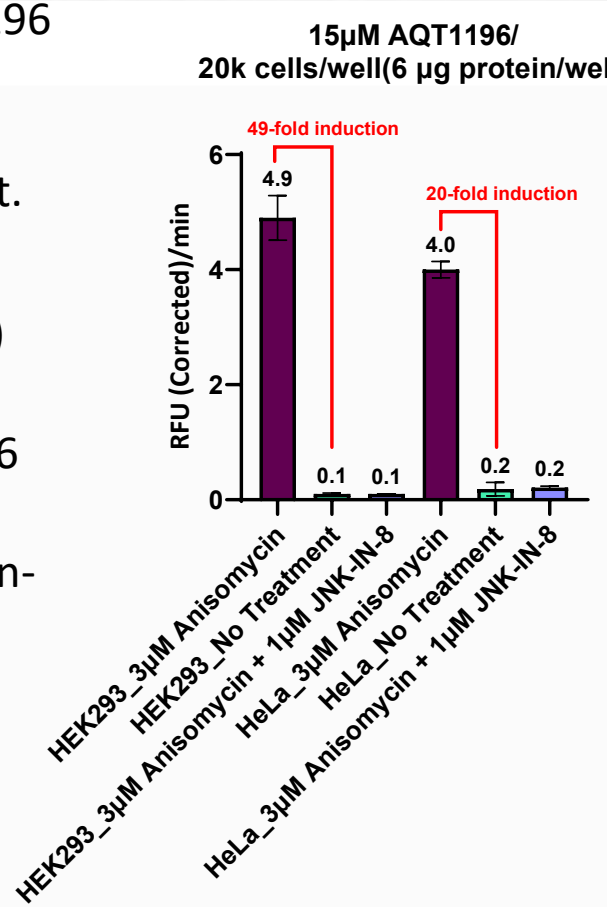


The AQT1196 sensor peptide was used at 15 µM with an increasing amount of lysate from HEK293 cells treated with Anisomycin to activate the JNK1/2/3 MAPK enzymes. RFU Corrected values (Total – Background) were determined for each condition. The results are presented for each amount of lysate for **1)** 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 inhibition of the kinase activity that can reduce the linear range.

The PhosphoSens-Lysate kinase activity assay for JNK1/2/3 provides a selective, highly quantitative, and accurate measure of kinase activity in a complex sample.

Summary

- The PhosphoSens-Lysate Activity Assay for JNK1-3 using the selective sensor peptide AQT1196 demonstrates a robust and physiologically relevant assay that provides a functional assessment of endogenous JNK activity with all the cellular components and signaling complexes. This JNK activity assay is direct, highly quantitative, and in an easy-to-use format.
- Results include:
  - Anisomycin treatment resulted in vigorous activation of JNK activity in HEK293 (49-fold) and also HeLa (20-fold) cells (see figure at right).
  - JNK activity with lysates from Anisomycin-treated HEK293 cells was linear from 0.3 to 16 µg protein/well, a 53-fold linear range.
  - The Sensor peptide substrate AQT1196 has a K_m of 6.5 µM when tested with Anisomycin-treated HEK293 cell lysates (see the Lysate Validation Report for JNK1/2/3).
  - 97% of AQT1196 phosphorylation in lysates from Anisomycin-treated HEK293 cells is inhibited by the reference compound for JNK (JNK-IN-8, 1 µM).
  - The IC_{50} value for JNK-IN-8 in lysates from anisomycin-treated HEK293 cells with AQT1196 was 533 nM (see the Lysate Validation Report for JNK1/2/3).



AQT1196 enables selective and precise quantitation of JNK1/2/3 activity with different cell types (demonstrated for HEK293 and HeLa cells), providing a powerful tool for evaluating pathway activation and inhibition in complex samples from normal and disease states