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PhosphoSens® Cell Lysate Activity Assay Format

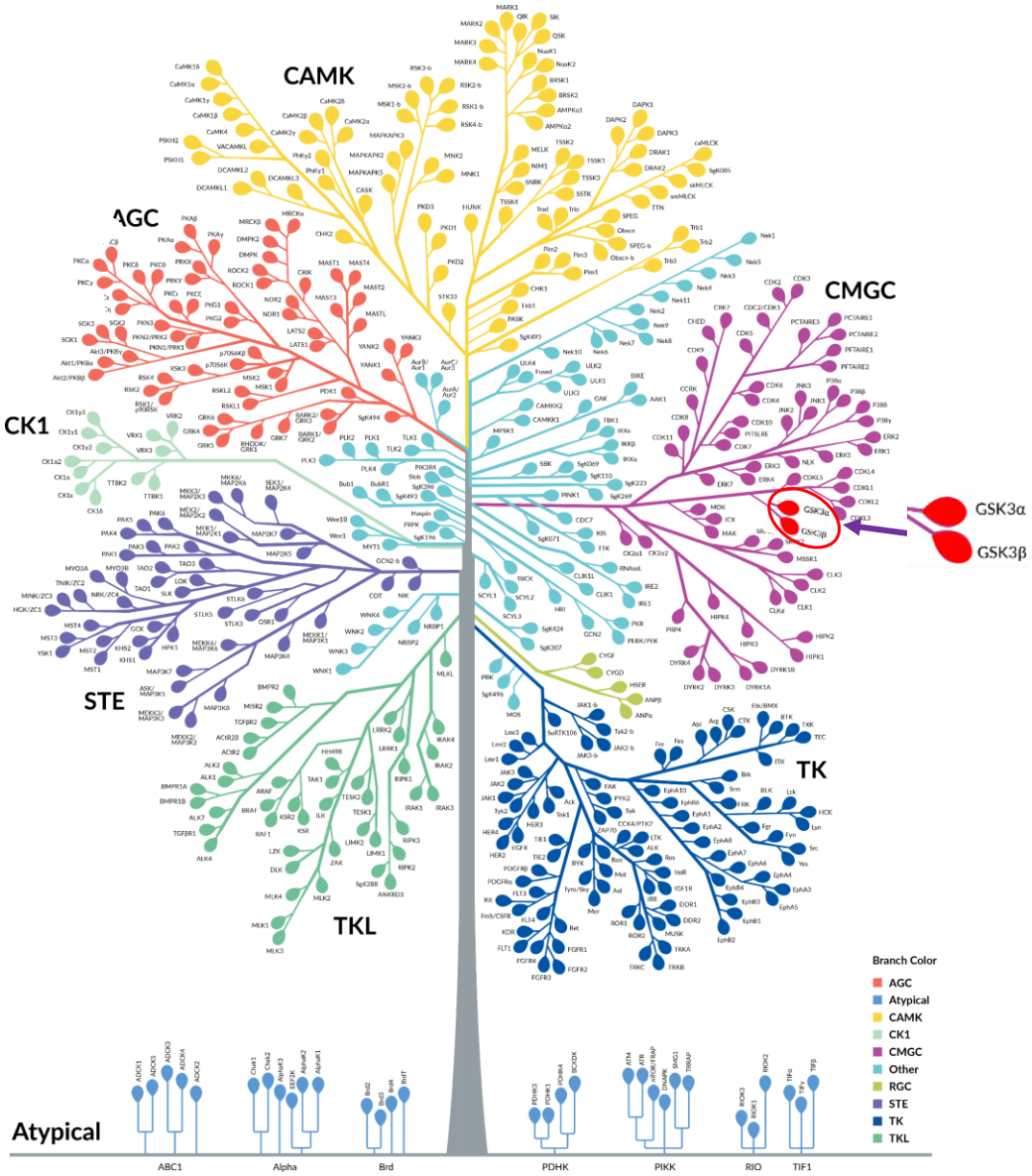
GSK3A/B Assay Validation Using the AQT1211 Sensor Peptide

HGNC Name: GSK3A/B
Long Names: Glycogen Synthesis Kinase 3-alpha and beta

High selectivity for GSK3A/B with the AQT1211 Sensor Peptide

Top 25 Hits for AQT1211 with 407 kinases in Kinome Profiling

Kinase target	Enzyme conc. (nM)	Average (RFU/pmol/min)	Rank	Selectivity Ratio (RFU/pmol/min)	% Activity (RFU/pmol/min)
GSK3B	**2**	**2972.1**	**1**	**1.0**	**100.0**
GSK3A	**2.0**	**2661.5**	**2**	**1.1**	**89.5**
PKACA	1.5	300.1	**3**	9.9	10.1
p70S6KA	2.0	282.6	**4**	10.5	9.5
MST1R	0.75	259.6	**5**	11.4	8.7
CAMK1B	1.0	241.9	**6**	12.3	8.1
ERK2	0.40	241.5	**7**	12.3	8.1
AMPKα1β2γ3	0.50	231.8	**8**	12.8	7.8
MST3	2.0	228.5	**9**	13.0	7.7
AMPKα1β1γ2	0.30	220.2	**10**	13.5	7.4
MARK2	1.0	213.4	**11**	13.9	7.2
PIM3	1.0	212.0	**12**	14.0	7.1
PKCQ	0.75	208.2	**13**	14.3	7.0
CAMK1A	1.0	205.9	**14**	14.4	6.9
PKCB2	1.0	196.8	**15**	15.1	6.6
PKCD	1.0	191.5	**16**	15.5	6.4
SIK1	3.0	185.1	**17**	16.1	6.2
ABL1	0.50	185.0	**18**	16.1	6.2
MARK4	1.0	184.6	**19**	16.1	6.2
IKKE	2.0	182.9	**20**	16.2	6.2
ERK1	0.50	170.2	**21**	17.5	5.7
ROS	0.50	165.9	**22**	17.9	5.6
SIK2	3.0	161.9	**23**	18.4	5.4
IGF1R	1.0	158.4	**24**	18.8	5.3
AMPKα2β1γ2	0.40	154.0	**25**	19.3	5.2



The top off-target kinases were determined by comparing rates (RFU/pmole/min) for each of the 407 kinases in the AQT Kinome Panel. PKACA and p70S6KA were the kinases with the highest off-target rates at 10% and 9.5% of GSK3 activity, respectively, showing high selectivity for the target kinases, GSK3α/β.

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Outline for this Study

PhosphoSens-Lysate Assay Validation

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Lysate Source:

- A375, Malignant Melanoma

Reference Compound Information:

- LY2090314

Experimental Validation at AssayQuant:

- A375 cell lysate titration
- AQT1211 substrate K_m determination
- DMSO Tolerance Test
- Reference Compound IC_{50} Determination
- Full-length active HIS-GSK3beta

Preparation of Crude Cell Lysates from A375 Cells Using PhosphoPreserve Cell Extraction Buffer

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1) A375 Cells were plated in T-175 flasks and incubated for 48 hours at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/ Streptomycin (PenStrep) in an atmosphere of 5% CO_2. Cells were then serum-starved in culture medium with 0.1% FBS for 24 hours. Cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and lysed with 500 µL lysis buffer containing:

- 50 mM HEPES, pH 7.4
- 150 mM NaCl
- 2 mM EGTA
- 1 mM DTT
- 1% Triton X-100
- 50 mM β-glycerophosphate
- 0.2 mM Sodium Fluoride (NaF)
- 0.2 mM Sodium Pyrophosphate (Na4P2O7)
- 1 µM Sodium Orthovanadate (Na3VO4)
- PhosphoPreserve Protease Inhibitor Cocktail (AQT60XPTIC)
- PhosphoPreserve Phosphatase Inhibitor Cocktail (AQT60XPPIC)

2) The DNA strands were broken by briefly sonicating on ice for 2 seconds on low power or using an 18-gauge needle and syringe if necessary. Lysates were used immediately or aliquoted and frozen at –80 °C. Except for the lysate titration, analysis of kinase activity using the PhosphoSens Lysate Assay for GSK3beta used 1.1 µg/well.

Lysate Titration

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

1 mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.54 mM EGTA

10 mM $MgCl_2$

15 µM AQT1211

0, 0.020, 0.039, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, 5.0, 10, and 20 µg/well **crude cell lysate protein** from A375 cells

Reaction Set Up:

20 µL Reaction Mix with AQT1211, ATP, & DTT
5 µL Enzyme dilution buffer (EDB) with GSK3A/B Lysate Buffer (1x) or GSK3beta 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 for A375 Cells and GSK3A/B Activity Measured with the AQT1211 Sensor Peptide

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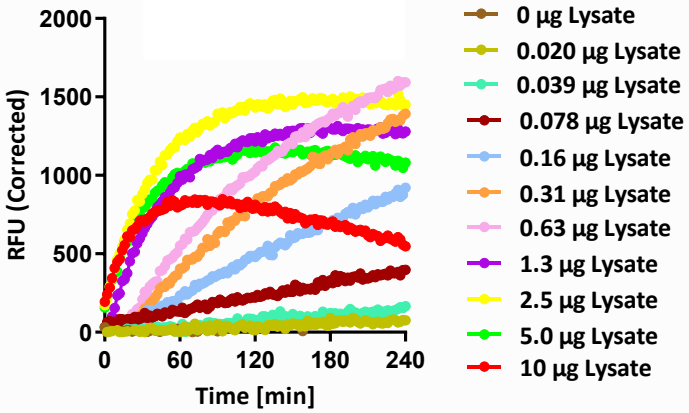
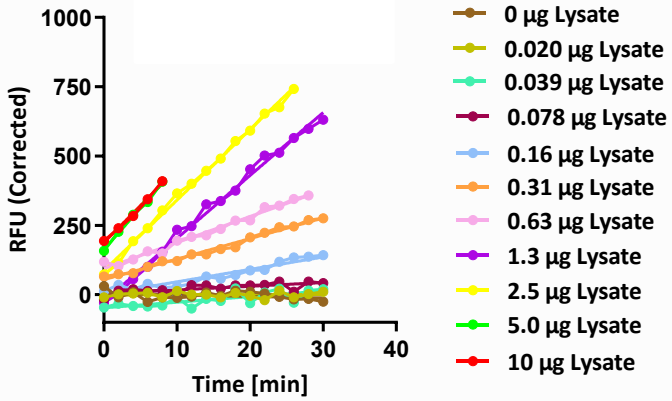
1. Full Time Course Progress Curves

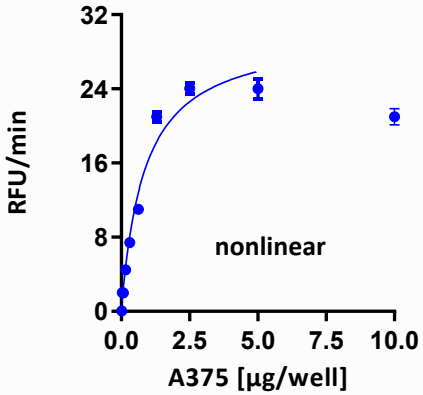


2. Linear Range of Progress Curves

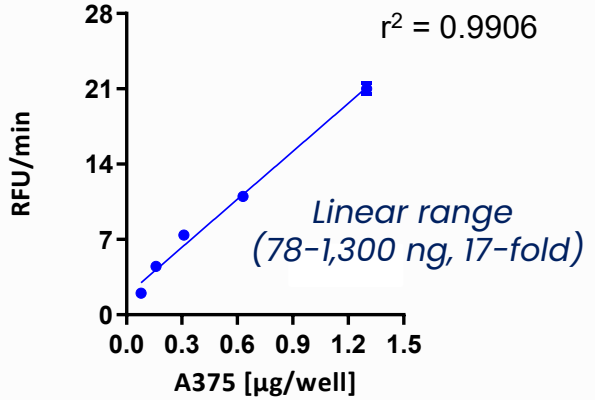


3. Reaction Rate vs Amount of Lysate/Well

A. All Lysate Amounts



B. Linear Range



The AQT1211 sensor peptide was used at 15 µM with an increasing amount of lysate from A375 cells treated. 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 > 0.99 **(3B)**. Having the concentration of crude lysate samples at 1 mg/mL or higher, ensures that the amount of CEB in the reaction is minimized, even at the highest concentrations to avoid any inhibition of the kinase activity that can reduce the linear range.

The PhosphoSens-Lysate kinase activity assay for GSK3A/B provides a selective, highly quantitative, and accurate measure of kinase activity in a complex sample.

GSK3A/B Sensor Peptide K_m Determination

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

1 mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.54 mM EGTA

10 mM $MgCl_2$

0, 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 µM **AQT1211 sensor peptide**

1.1 µg/well A375 crude cell lysate

Reaction Set Up:

2.5 µl 10X AQT1211 Substrate dilutions
17.5 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

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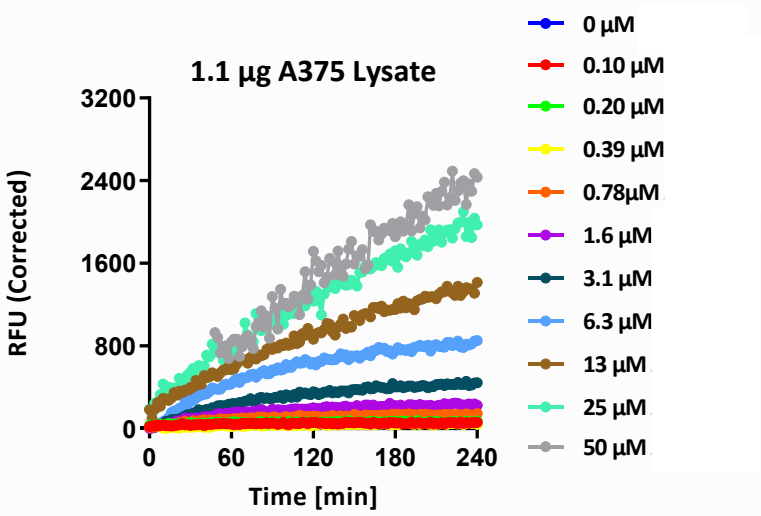
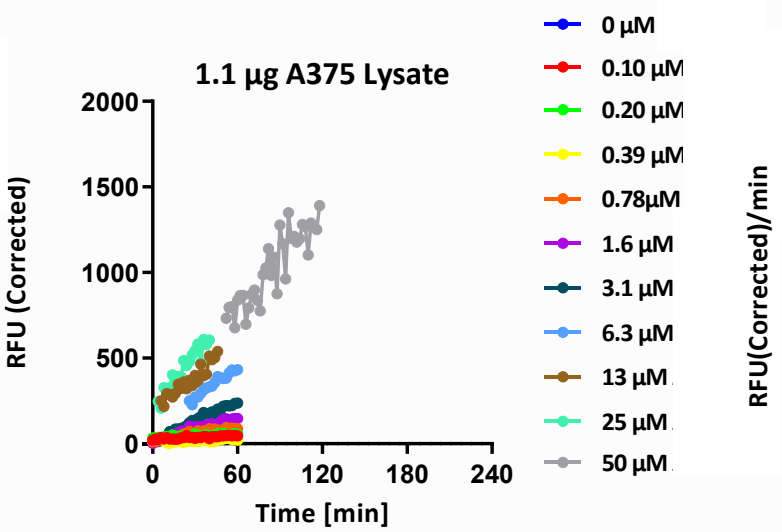
AQT1211 Sensor Peptide Titration Curves

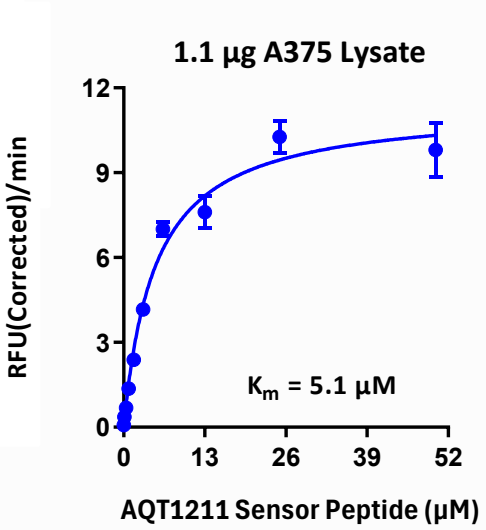
All Time Points



Linear Region



Sensor Peptide K_m Plot



Vmax	11.36
Km	5.061
95% CI (profile likelihood)	
Vmax	10.33 to 12.53
Km	3.699 to 6.930
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9875
Sum of Squares	1.964
Sy.x	0.4672
Constraints	
Km	Km > 0
Number of points	
# of X values	11
# Y values analyzed	11

The K_m value for AQT1211 with the A375 lysate is 5.1 µM. The K_m value observed for recombinant GSK3beta was 11 µM.

DMSO Tolerance Test

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

1 mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.54 mM EGTA

10 mM $MgCl_2$

15 µM AQT0982

1.1 µg/well A375 crude cell lysate

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 Lysate (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.

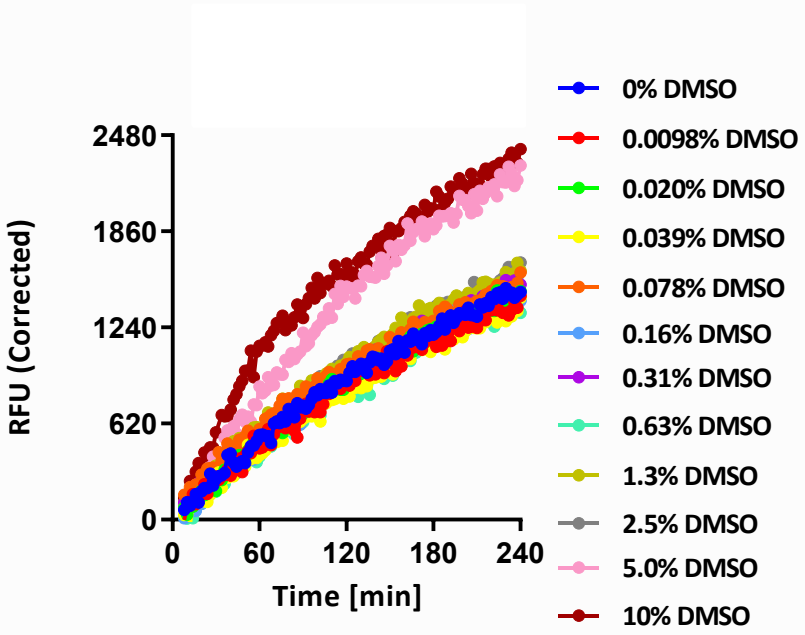
Notes:

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.

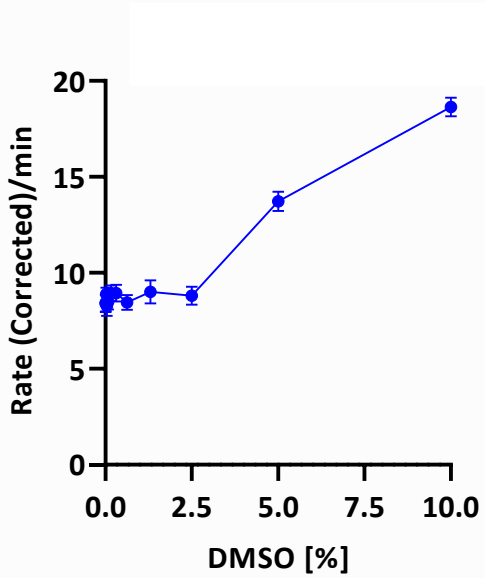
DMSO Tolerance Test for A375 Lysate with the AQT1211 Sensor Peptide

Titration Curves and Inhibition Plot using 1.1 µg A375 cell lysate/well

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No significant loss in enzyme activity was observed up to 2.5% DMSO. There was an increase in rate above 2.5% DMSO. We assessed compound potency using 2% DMSO final.

IC_{50} Determination

Reaction Conditions and Set Up

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Reaction Conditions:

54 mM HEPES, pH 7.5
1.0 mM ATP
1.2 mM DTT
0.012% Brij-35
1% glycerol
0.2 mg/ml BSA
0.54 mM EGTA
10 mM $MgCl_2$
15 µM AQT1211 sensor peptide substrate

Compounds:

- 0-1.0 µM LY2090314 was diluted in 100% DMSO at 50X the final concentrations and diluted 50-fold into the assay for a final concentration of 2% DMSO.

GSK3A/B Enzyme Source:

- Lysate: 1.1 µg/well A375 crude cell lysate

Reaction Set Up:

0.5 µL 50X LY2090314 dilution in 100% DMSO
19 µL Reaction Mix with CSx Substrate, ATP & DTT
5 µL Enzyme dilution buffer (EDB) (1x), A375 Lysate (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 with A375 Lysate using LY2090314 Using the AQT1211 Sensor Peptide

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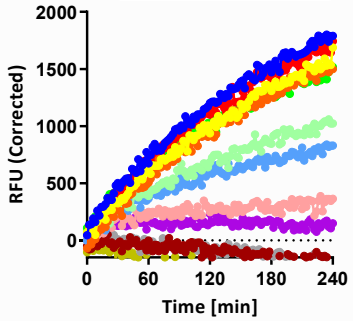
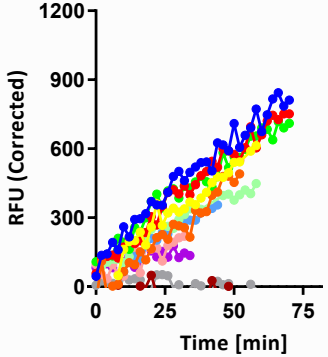
Full Progress Curves

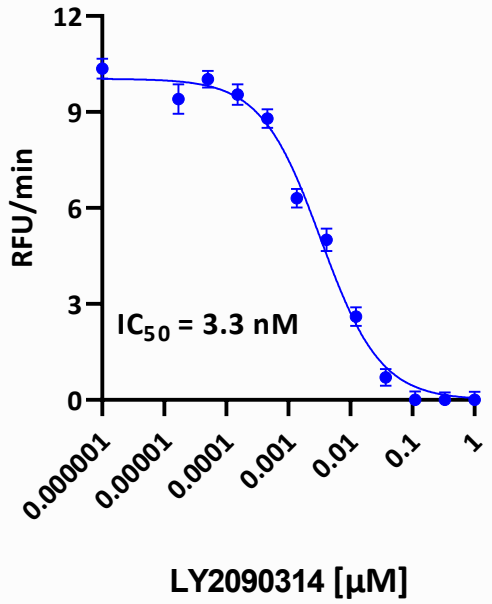


Linear Region



0 µM LY2090314
0.000017 µM LY2090314
0.000051 µM LY2090314
0.00015 µM LY2090314
0.00046 µM LY2090314
0.0014 µM LY2090314
0.0041 µM LY2090314
0.012 µM LY2090314
0.037 µM LY2090314
0.11 µM LY2090314
0.33 µM LY2090314
1.0 µM LY2090314

IC_{50} Curve



IC_{50} Table

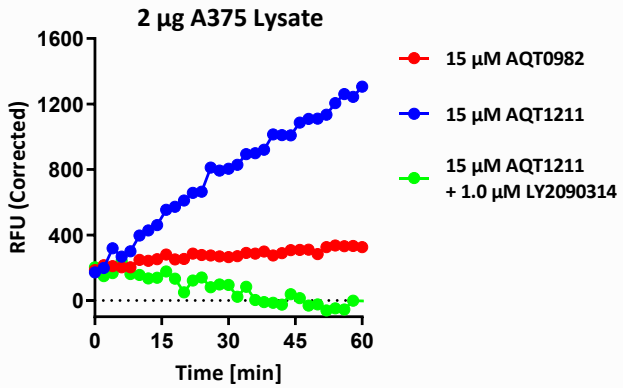
[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	10.04
IC50	0.003343
HillSlope	-0.9009
logIC50	-2.476
Span	10.04
95% CI (profile likelihood)	
Top	9.512 to 10.61
IC50	0.002430 to 0.004543
HillSlope	-1.125 to -0.7313
logIC50	-2.614 to -2.343
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9930
Sum of Squares	1.462
Sy.x	0.4030
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

The LY2090314 IC_{50} value in lysate is 3.3 nM at 1 mM ATP. The IC_{50} value with recombinant GSK3beta was 0.87 nM at 18 µM ATP (ATP Km).

GSK3A/B Lysate Activity Assay Using the AQT1211 Sensor Peptide (15 µM)

A. Crude Lysate Sample

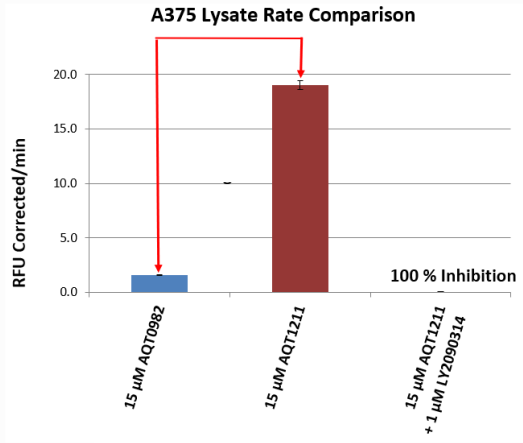
1) Linear Time Course (0-60 min.)



2) Reaction Rates to Assess Changes

	Reaction Rate (RFU/min)	Change
2.0 µg A375, 15 µM AQT0982 (AKT1/2/3 Substrate)	1.6 ± 0.045	
2.0 µg A375, 15 µM AQT1211 (GSK3A/B Substrate)	19 ± 0.41	12-fold difference in rates
2.0 µg A375, 15 µM AQT1211 (GSK3A/B Substrate) + 1 µM LY2090314 (GSK3A/B Inhibitor)	0 ± 0.074	100% inhibition of GSK3A/B Activity

3) Histogram of Results

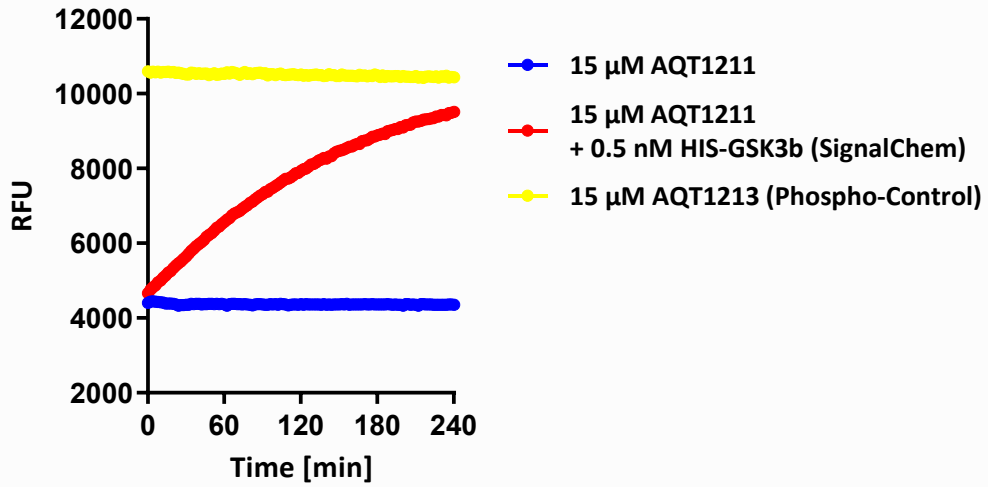


A. A375 Crude Lysate Sample: The AQT1211 and AQT0982 sensor peptides were used to generate **1)** A linear progress curve time course (0-60 min). The reaction rates (RFU Corrected values [Total – Background]/min. +/- standard deviations) are the slope of the linear region of each progress curve, which are presented in the table in **2)** and as a histogram in **3)**, highlighting a 12-fold difference in rates comparing the activity for AKT1/2/3 with the AQT0982 substrate to the activity for GSK3A/B with the AQT1211 substrate since phosphorylation of GSK3 by AKT is inhibitory. The GSK3A/B signal was eliminated by adding the selective GSK3A/B inhibitor LY2090314. **Note:** It is unclear why there is a slight downward slope with the Ly2090314 compound (this also occurs with heat-inactivated lysate), but these rates are close to zero. The amount of GSK3 activation depends on several factors, including cell type, the serum deprivation pretreatment used to make cells quiescent, as well as the nature, concentration, and duration of the activating stimulus, if applicable. These conditions can be varied to determine the effect on GSK3A/B activity. The total amount of GSK3beta 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.

GSK3B Lysate Activity Assay Using the AQT1211 Sensor Peptide (15 µM) & AQT1148 Phospho-Control (15 µM)

B. AQT1211 ± recombinant GSK3beta & AQT1148 Phosphopeptide Control

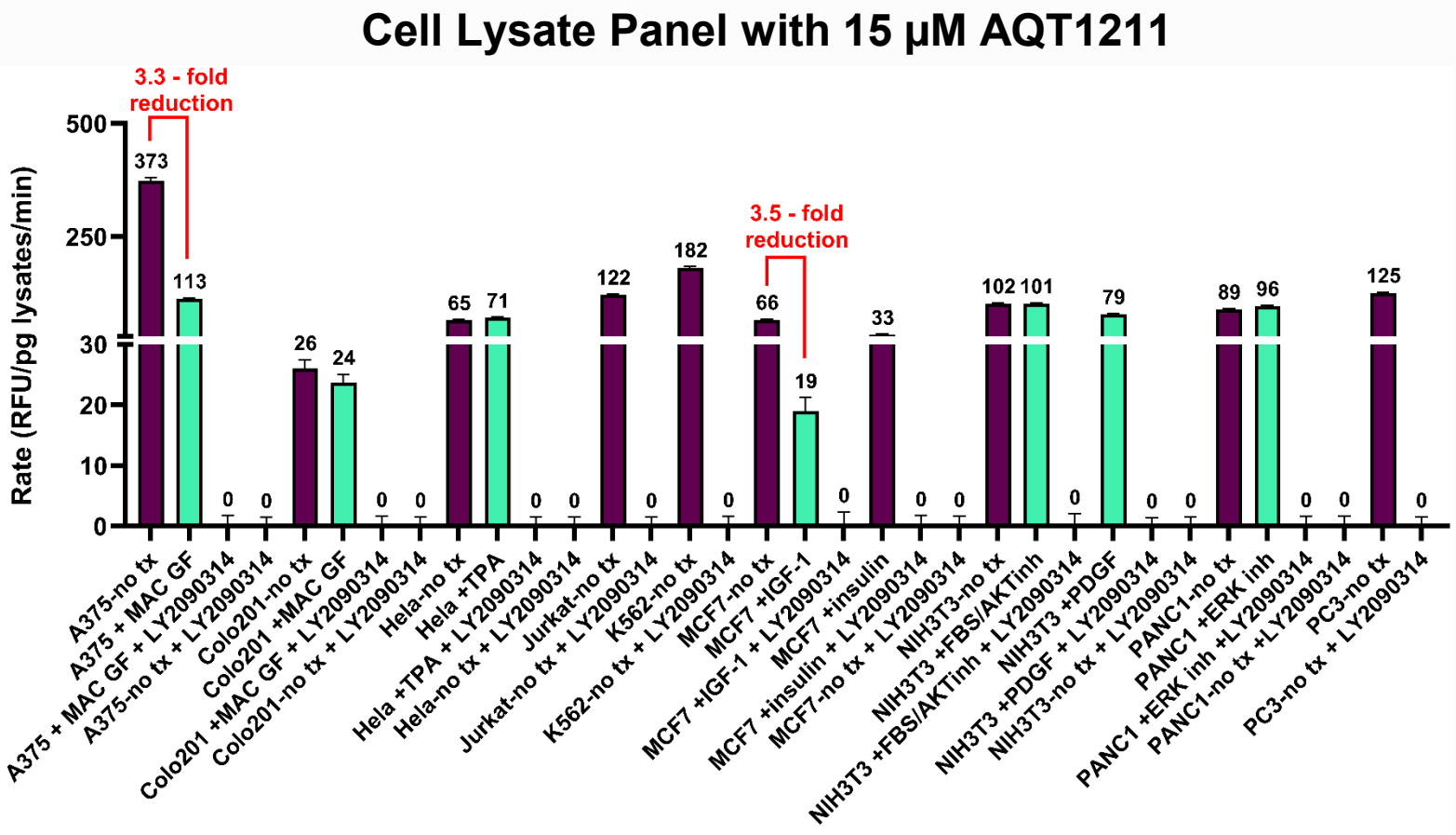
1) Full Time Course (0-4 hours)



AQT1213 is a chemically synthesized phosphopeptide control for AQT1211

B.1. 15 µM AQT1211 ± 0.50 nM GSK3beta & 15 µM AQT1213 Phospho Control: The GSK3beta protein (0.5 nM) fully phosphorylated the AQT1211 sensor peptide substrate by 240 min., as shown by convergence with the signal obtained with the AQT1213 phosphopeptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). The signal with the GSK3beta enzyme was eliminated by adding 1 µM LY2090314 inhibitor. The signal with AQT1213 is used to convert RFU (Corrected) values to nmoles of phosphopeptide.

GSK3A/B Lysate Activity Assay Using the AQT1211 Sensor Peptide Across a Variety of Cell Types



The method described on slide 6 was used to prepare cell lysates. Standard lysate assay conditions on slide 13-14 were used to run the assay with 2.0 µg total protein for each lysate. Reaction rates (RFU Corrected/pg of total lysate protein/min) were determined from the slopes using the linear portion of each progress curve. Values are the average of duplicate reactions +/- standard deviation. In two cell lines, GSK3 activity was reduced by more than 3-fold with treatment (Green bars), consistent with activation of AKT. Incorporation of the GSK3-selective inhibitor, LY2090314 @ 1 µM, blocked the signal with AQT1211, highlighting the selectivity of the sensor peptide for evaluating GSK3 activity in these complex samples.

Demonstrates selective detection of endogenous GSK3A/B activity in multiple cell lines

Summary

- The PhosphoSens-Lysate Assay for GSK3A/B using the AQT1211 selective sensor peptide demonstrates a robust and physiologically relevant assay that measures endogenous GSK3beta activity with all the cellular components and signaling complexes.
- Results include:
  - A375 cell kinase activity was 19 RFU/min. This can likely be improved further by varying conditions for the treatment of cells.
  - GSK3A/B activity with lysates was linear from 0.078 to 1.3 µg/well, a 17-fold linear range.
  - The GSK3A/B lysate activity is completely inhibited by 1 µM of LY2090314 reference compound, demonstrating the selectivity of the assay.
  - The IC_{50} value for LY2090314 with lysate from A375 cells was 3.3 nM.
  - Sensor peptide substrate AQT1211 has a K_m of 5.1 µM with A375 cell lysate.
- These measurements are direct and highly quantitative, and in an easy-to-use format.