AQT0688 – NEK6 Assay Validation

PhosphoSens®-Kinetic Assay Format

Outline for this Study

PhosphoSens®-Kinetic Assay Validation

AssayQuant TECHNOLOGIES INC.

Enzyme Source, Construct, and Lot Information:

Carna NEK6 (05-130/08CBS-0105J) amino acid full length; N-terminal GST tag

Reference Compound Information:

Staurosporine MedChemExpress (Cat. HY-15141)

Experiments to be run:

- **Enzyme Titration**
- **Sensor Peptide K_M Determination**
- **ATP K_M Determination**
- **DMSO Tolerance Test**
- **Reference Compound IC_{50} Determination at ATP K_M**

Enzyme 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

0.55 mM EGTA

10 mM $MgCl_2$

15 uM AQT0688

0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 5, 10, 15, and 20 nM NEK6

Reaction Set Up:

2 or 2.5 µL	10x Sensor Peptide
14 or 17.5 µL	Reaction Mix with ATP & DTT
4 or 5 µL	1x EDB or Kinase dilutions (5x in EDB)
20 or 25 µL	Final reaction volume

Reactions were 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.

Enzyme Titration

Progress Curves

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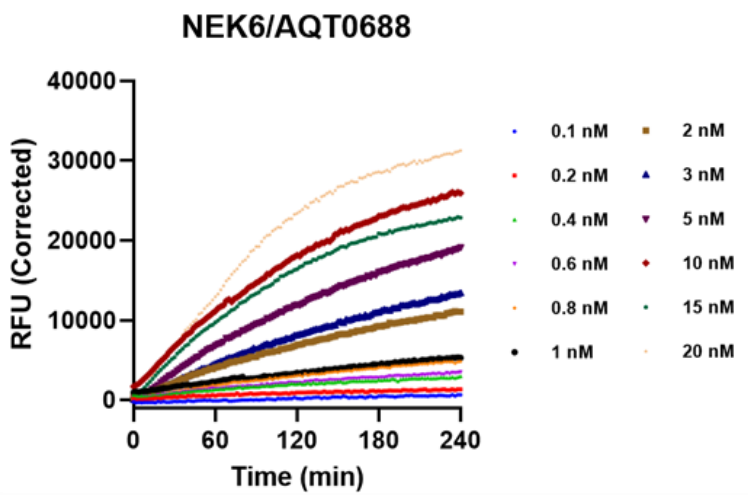
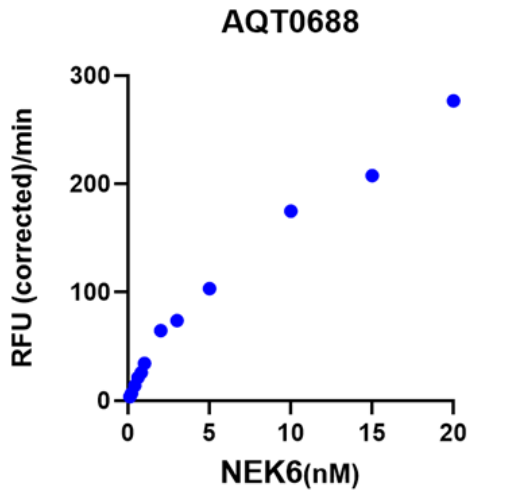
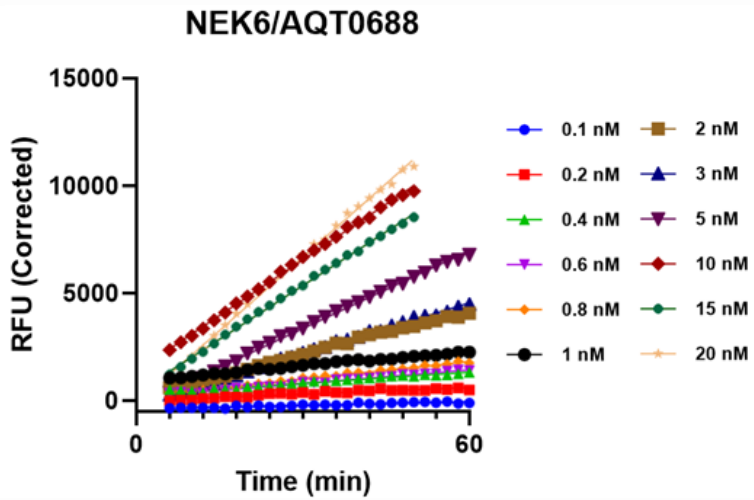
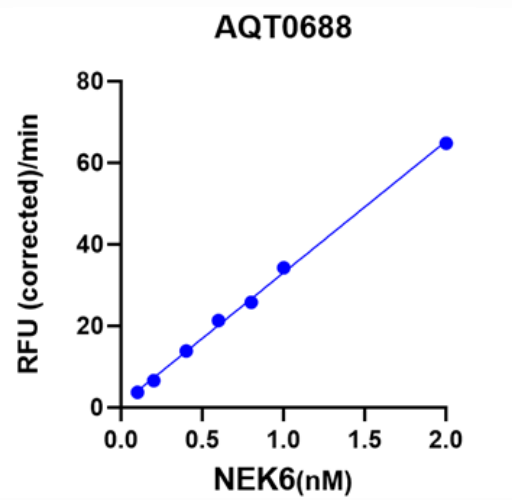
Complete Progress Curves





Linear Region of Progress Curves





Linear Range

Enzyme Titration

Reaction Rate Table

Enzyme Conc. (nM)	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate Stnd Error (RFU/pmole/min)
0.1	2,826	222
0.2	2,595	171
0.4	2,159	76
0.6	1,787	40
0.8	1,711	33
1	1,130	24
2	1,672	19
3	1,321	13
5	1,208	10
10	863	8
15	555	5
20	570	6

The reaction is linear from 0.6 - 2.0 nM

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

0.55 mM EGTA

10 mM $MgCl_2$

1, 2, 3, 5, 7, 10, 15, 20, 30, 50, 70, & 100 uM AQT0688

10 nM NEK6

Reaction Set Up:

2 or 2.5 µL	10x Sensor Peptide
14 or 17.5 µL	Reaction Mix with ATP & DTT
4 or 5 µL	1x EDB or Kinase dilutions (5x in EDB)
20 or 25 µL	Final reaction volume

Reactions were run at 30˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 25 µL final well volume or in in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 µL final well volume after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

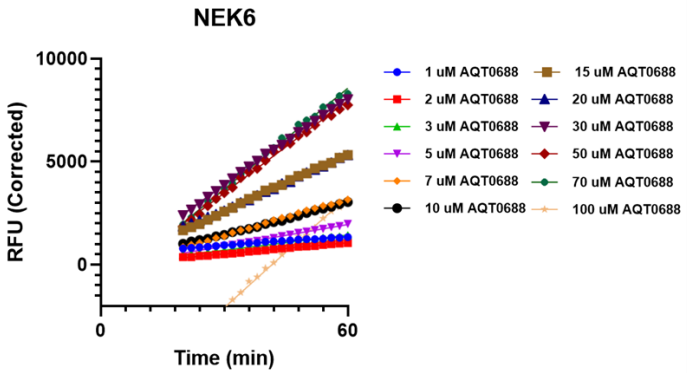
Notes:

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

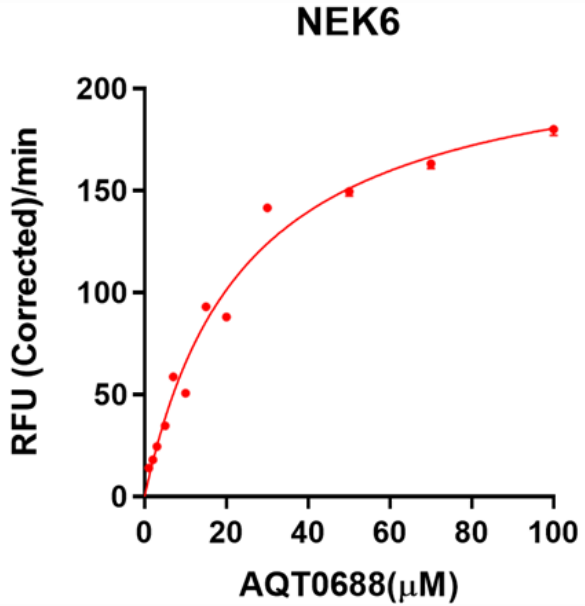
Sensor Peptide K_M Determination

Titration Curves and K_M Plot and Table

Sensor Peptide Titration Curves



Sensor Peptide K_M Plot



Sensor Peptide K_M Table

Michaelis-Menten	
Best-fit values	
Vmax	224.4
Km	24.27
Std. Error	
Vmax	13.74
Km	3.725
95% CI (profile likelihood)	
Vmax	197.1 to 258.8
Km	17.38 to 34.27
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9780

Sensor Peptide K_M is 24 uM

ATP K_M Determination

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

0, 2.0, 3.9, 7.8, 16, 31, 63, 125, 250, 500, 1000, and 2000 µM ATP

1.2 mM DTT

0.012% Brij-35

1%glycerol

0.2 mg/ml BSA

0.55 mM EGTA

10 mM $MgCl_2$
15 uM AQT0688

10 nM NEK6

Reaction Set Up:

2 or 2.5 µL	10x Sensor Peptide
14 or 17.5 µL	Reaction Mix with ATP & DTT
4 or 5 µL	1x EDB or Kinase dilutions (5x in EDB)
20 or 25 µL	Final reaction volume

Reactions were run at 30˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 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.

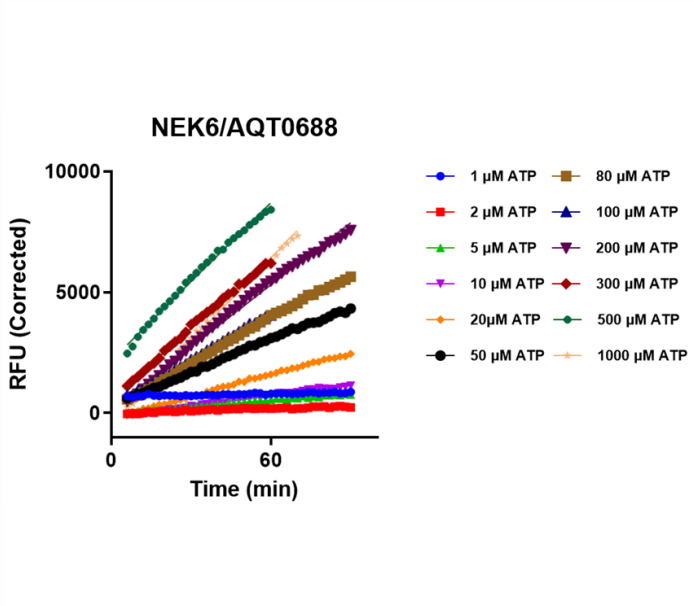
Notes:

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.

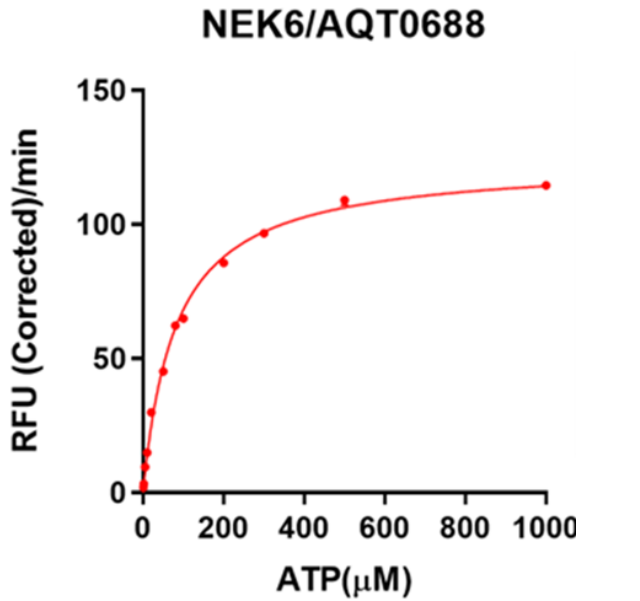
ATP K_M Determination

Titration Curves and K_M Plot and Table

ATP Titration Curves



ATP K_M Plot



ATP K_M Table

Michaelis-Menten	
Best-fit values	
Vmax	123.6
Km	81.30
Std. Error	
Vmax	2.392
Km	5.288
95% CI (profile likelihood)	
Vmax	118.3 to 129.2
Km	69.92 to 94.29
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9965

ATP K_M is 81 uM

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

0.55 mM EGTA

10 mM $MgCl_2$

0, .01, .02, .04, .08, .16, .31, .63, 1.3, 2.5, 5.0, and 10% DMSO
15 uM AQT0688

10 nM NEK6

Reaction Set Up:

2 or 2.5 µL	10x DMSO dilutions
14 or 17.5 µL	Reaction Mix with Sensor Peptide, ATP & DTT
4 or 5 µL	1x EDB or Kinase dilutions (5x in EDB)
20 or 25 µL	Final reaction volume

Reactions were run at 30˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 25 µL final well volume or in in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 µL final well volume after sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

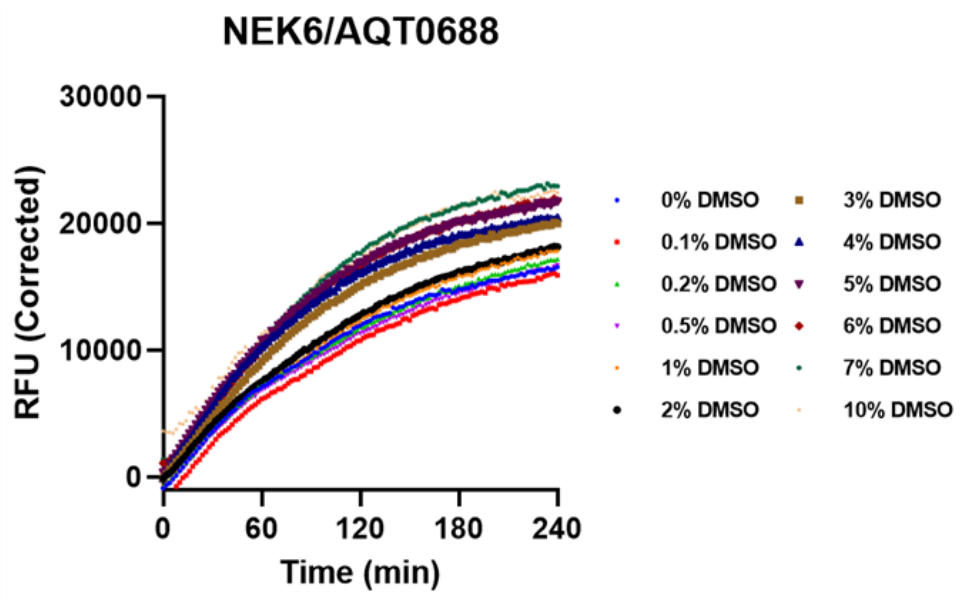
Notes:

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

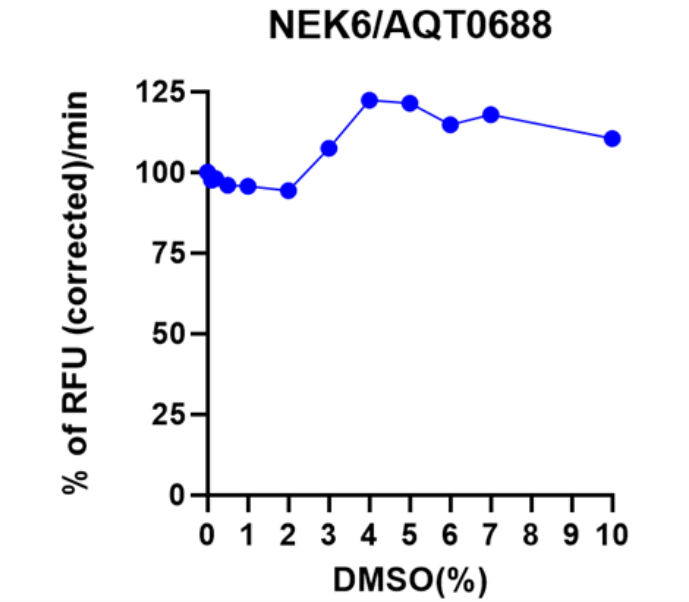
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



There is no significant inhibitory effect up to 2% DMSO

IC_{50} Determination

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

ATP at K_M

1.2 mM DTT

0.012% Brij-35

1%glycerol

0.2 mg/ml BSA

0.55 mM EGTA

10 mM $MgCl_2$

1% DMSO

15 uM AQT0688

10 nM NEK6

5 mM Staurosporine with 3-fold titration in 100% DMSO then diluted 10-fold into BSA (with a final concentration of 0.2 mg/ml) for a DMSO concentration of 10% before diluted 10-fold into reaction mixture with a final DMSO concentration of 1%

Reaction Set Up:

16 µL	Reaction Mix with Sensor Peptide and Inhibitor
4 µL	1x EDB or Kinase dilutions (5x in EDB)
20 µL	Final reaction volume

Reactions were 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 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.

Inhibitors are added via direct (0.4 µL of 50X stock in 100% DMSO) or intermediate dilutions (2.0 µL of 10X stock in 10% DMSO).

Notes:

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

IC_{50} Determination

Progress Curves and IC_{50} Curves and Table

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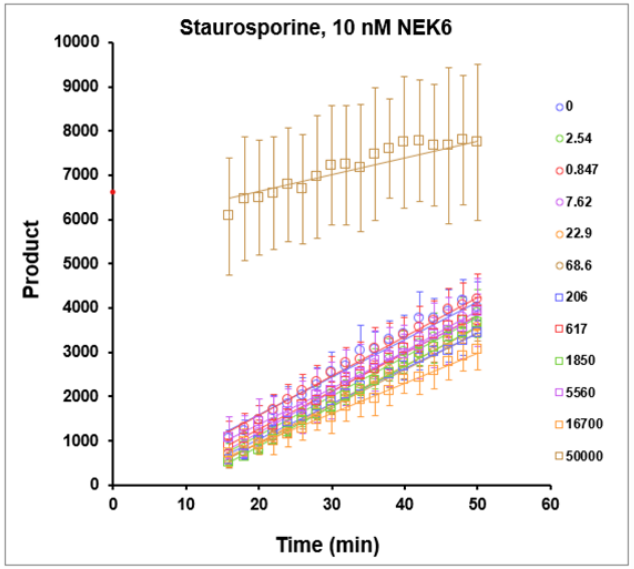
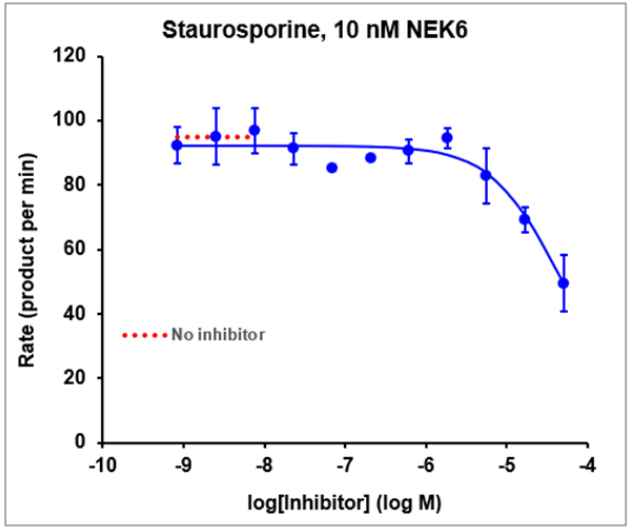
Linear Region of Progress Curves



IC_{50} Curve



IC_{50} Table

Parameter	Value
Bottom	15.0
Top	92.3
log IC50	-4.39
IC50 (nM)	40648.66
Ki (nM)	20324.33
Slope	-1.052
R squared	0.952
IC50 approx SE (nM)	893.71
50% inhibition (nM)	59022.51

The Y-axis label is RFU/min.

Staurosporine IC_{50} at ATP K_M is 40649 nM

Summary

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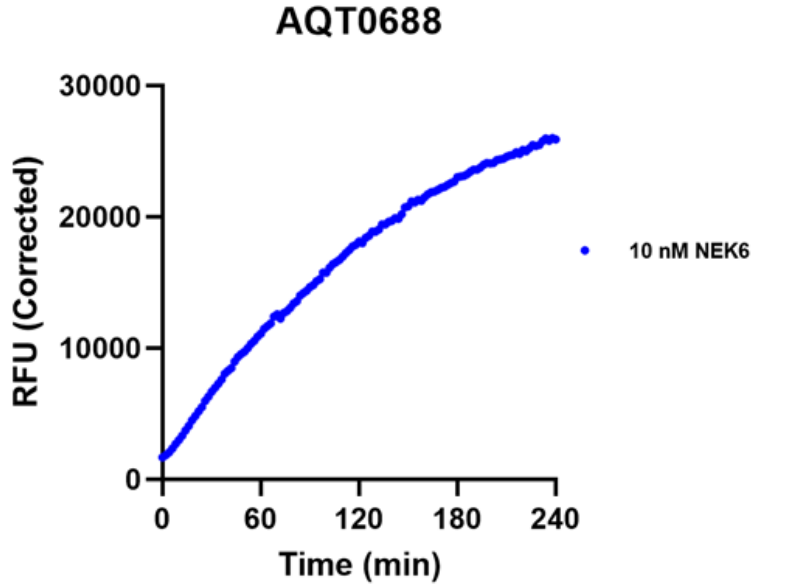
Assay Validation Results and Progress Curve and Assay Strength at 1 mM ATP

Experiment	Result
Enzyme Titration Linear From	0.6 - 2.0 nM
Sensor Peptide K_M Determination	24 uM
ATP K_M Determination	81 uM
DMSO Tolerance Test	2%
Staurosporine IC50 Determination	40649 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate StndError (RFU/pmole/min)
NEK6	10	AQT0688	863	8

Assay Strength Key	
Very Strong	>1,000 (RFU/pmole/min)
Strong	300 to 999 (RFU/pmole/min)
Moderate	100 to 299 (RFU/pmole/min)
Weak	30 to 99 (RFU/pmole/min)

Under the conditions utilized for this experiment, the assay is Strong