AQT0661 - JAK2 (JH1-JH2) Assay Validation

PhosphoSens®-Kinetic Assay Format

Outline for this Study

PhosphoSens-Kinetic Assay Validation

AssayQuant TECHNOLOGIES INC.

Enzyme Source, Construct, and Lot Information:

Carna JAK2(JH1JH2) (Cat/Lot #, 08-514/13CBS-0782F) amino acids 532-1132(end), N-term GST tag

Reference Compound Information:

Staurosporine MedChemExpress(Cat#/Lot#: HY-15141/125391)

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 µM AQT0661

0.01, 0.02, 0.04, 0.08, 0.16, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 nM JAK2 (JH1JH2)

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:

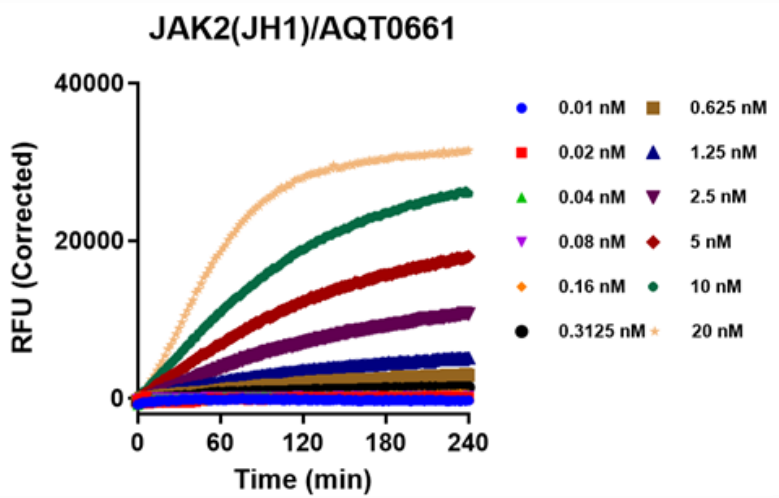
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

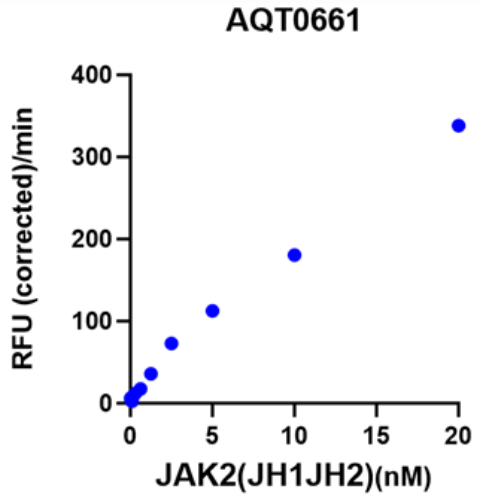
Enzyme Titration

Progress Curves

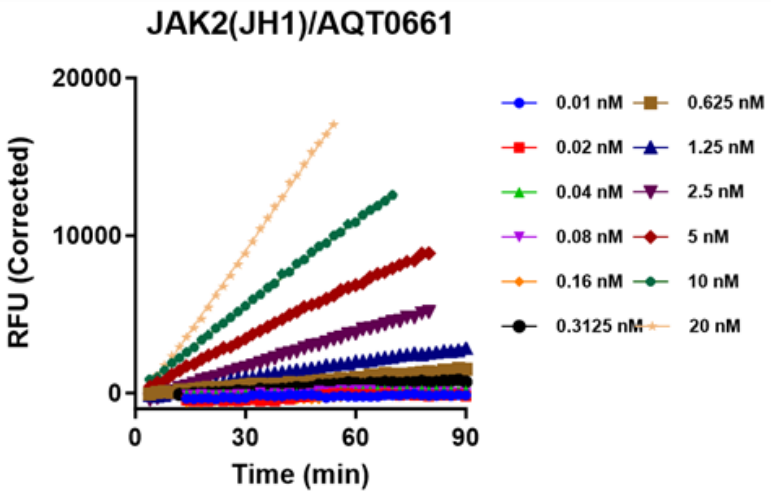
AssayQuant TECHNOLOGIES INC.

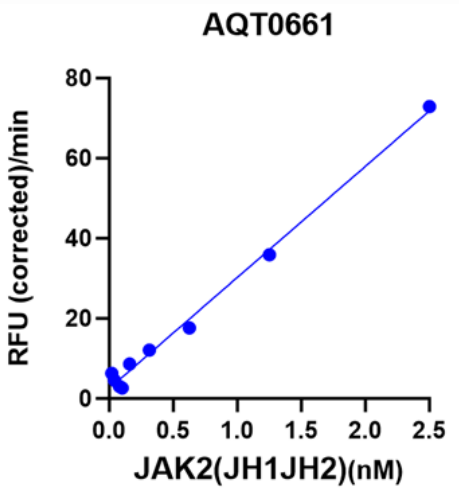
Complete Progress Curves





Linear Region of Progress Curves





Linear Range

Enzyme Titration

Reaction Rate Table

Enzyme Conc. (nM)	Reaction Rate (RFU/min)	Normalized Reaction Rate (RFU/pmol/min)
0.01	2.6	13005
0.02	6.3	15805
0.04	9.1	11433
0.08	6.1	3785
0.16	17	5376
0.31	24	3788
0.63	35	2821
1.25	72	2874
2.5	141	2817
5	225	2248
10	361	1805
20	677	1692

The reaction is linear from 0.31 - 2.5 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 µM AQT0661

5 nM JAK2(JH1JH2)

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.

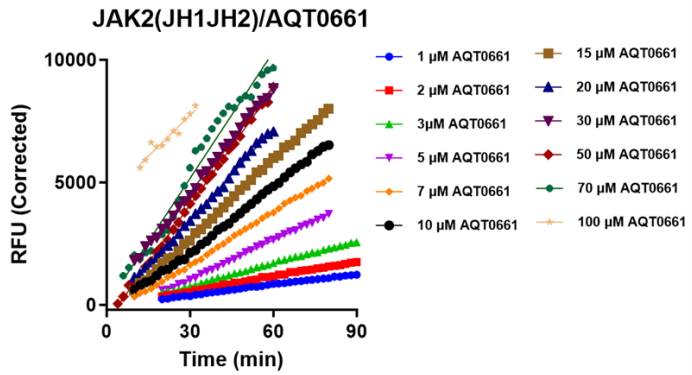
Notes:

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.

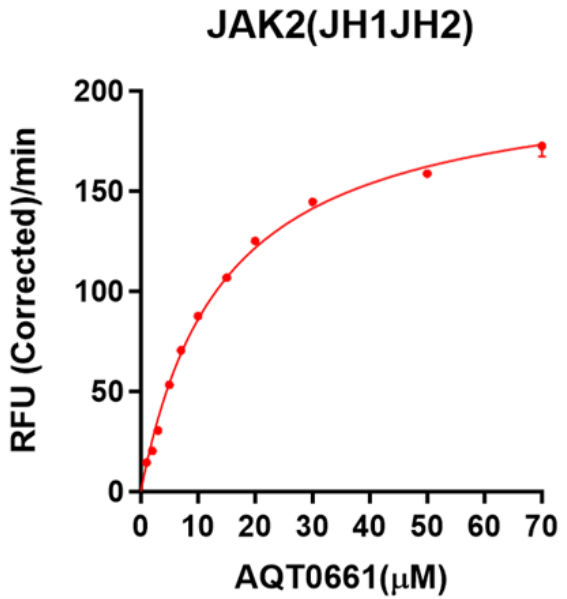
Sensor Peptide K_m Determination

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	208.4
Km	14.25
Std. Error	
Vmax	4.534
Km	0.8145
95% CI (profile likelihood)	
Vmax	198.7 to 218.9
Km	12.56 to 16.18
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9967

Sensor Peptide K_m is 14.3 µM

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 µM AQT0661

5 nM JAK2(JH1JH2)

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.

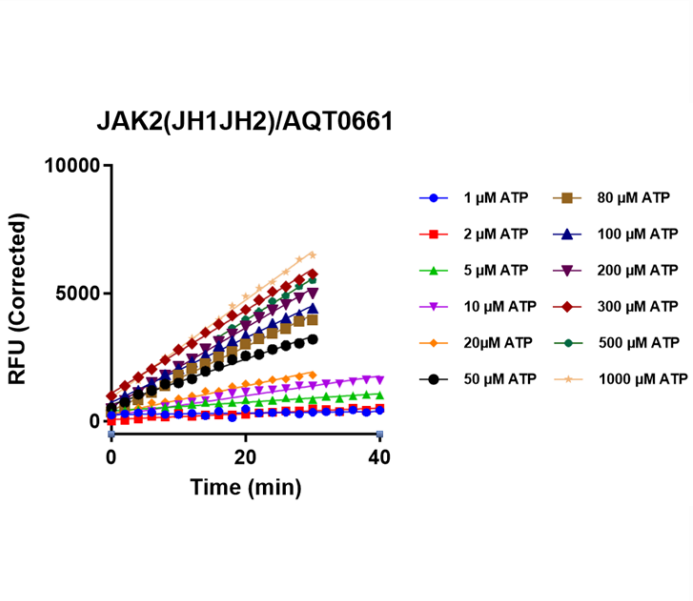
Notes:

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.

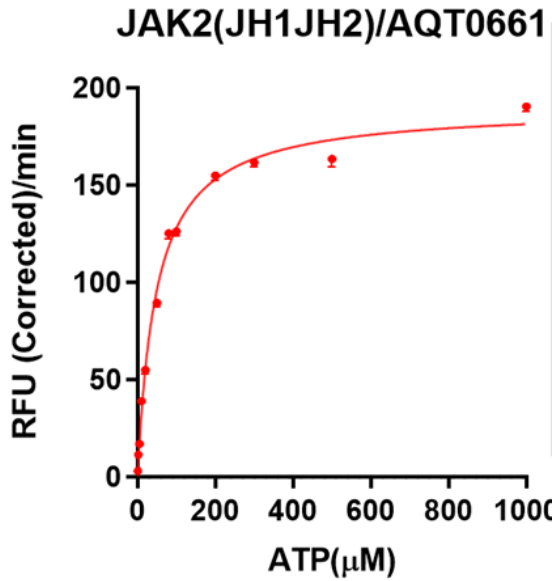
ATP K_m Determination

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	190.1
Km	48.64
Std. Error	
Vmax	4.394
Km	4.388
95% CI (profile likelihood)	
Vmax	180.5 to 200.3
Km	39.56 to 59.51
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9932

ATP K_m is 49 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

0.55 mM EGTA

10 mM $MgCl_2$

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

15 µM AQT0661

5 nM JAK2 (JH1JH2)

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.

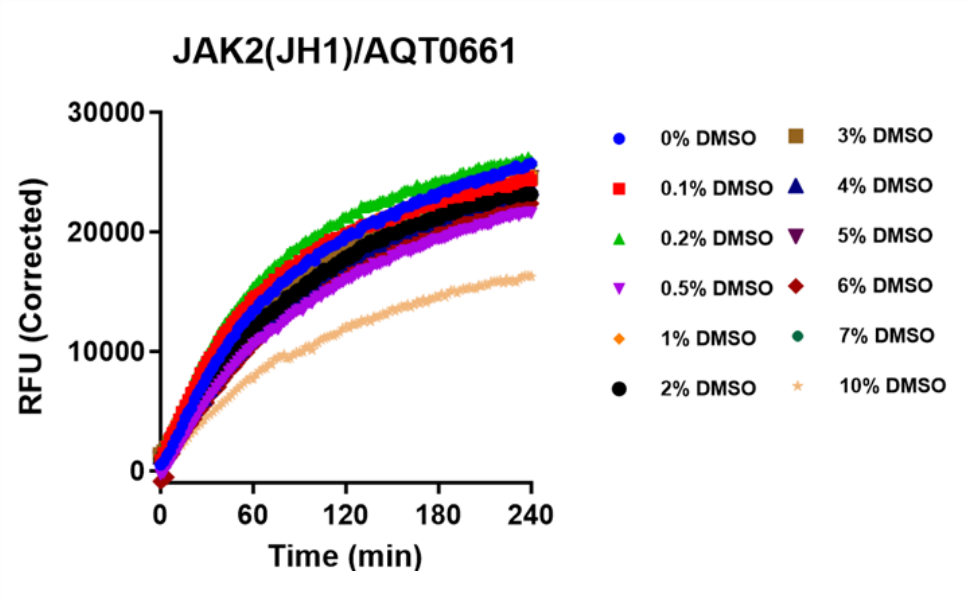
Notes:

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.

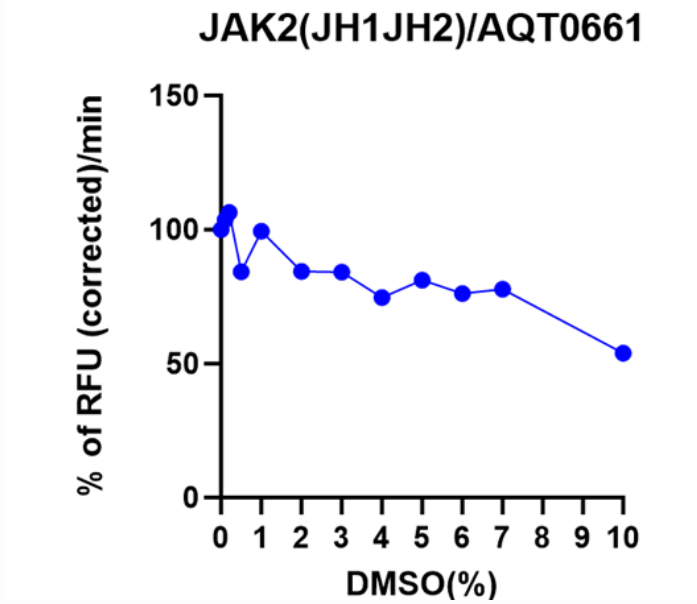
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No change in enzyme activity out 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 µM AQT0661

5 nM JAK2(JH1JH2)

0.1 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).

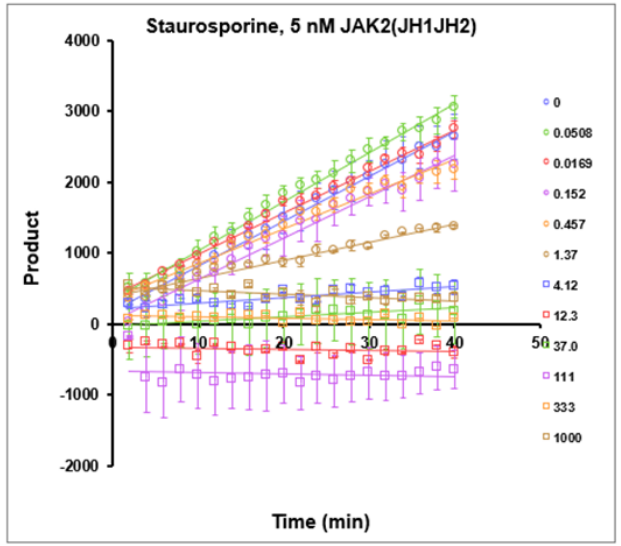
Notes:

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.

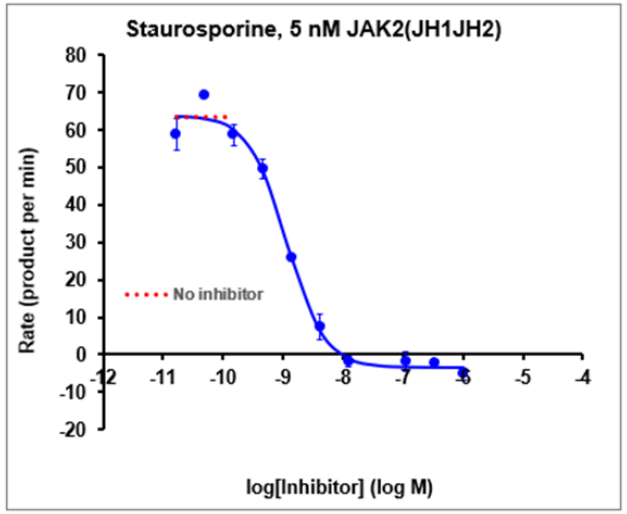
IC_{50} Determination

Progress Curves and IC_{50} Curves and Table

Linear Region of Progress Curves



IC_{50} Curve



IC_{50} Table

Parameter	Value
Bottom	-3.4
Top	63.9
log IC50	-8.93
IC50 (nM)	1.16
Ki (nM)	0.58
Slope	-1.388
R squared	0.991
IC50 approx SE (nM)	0.06
50% inhibition (nM)	1.08

The Y-axis label is RFU/min.

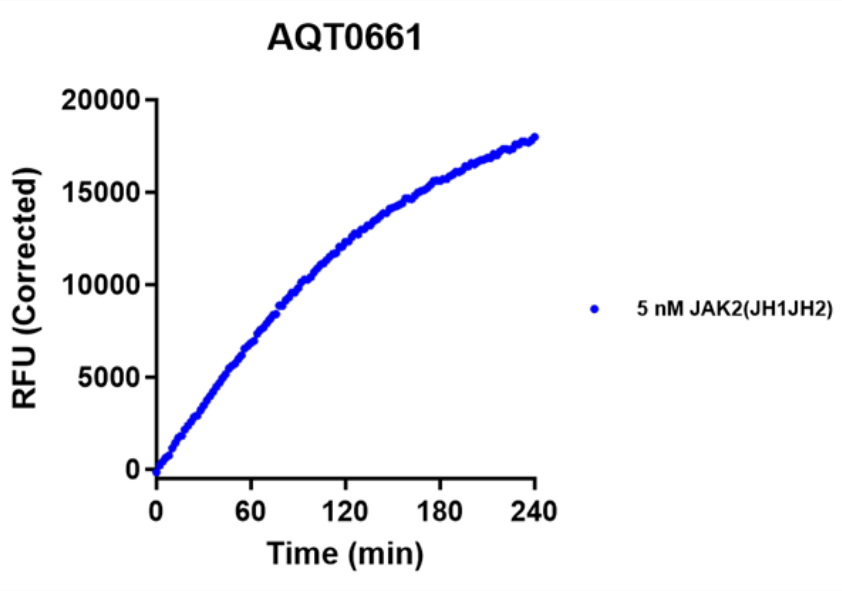
Staurosporine IC_{50} at ATP K_m is 1.2 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.31 - 2.5 nM
Sensor Peptide K_m Value	14.3 µM
ATP K_m Value	49 µM
DMSO Tolerance (highest % recommended)	2
Staurosporine IC_{50} Determination at ATP K_m	1.2 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate StndError (RFU/pmole/min)
JAK2(JH1JH2)	5	AQT0661	2,248	15

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 Very Strong