AQT1030 – VRK2 Assay Validation

PhosphoSens®-Kinetic Assay Format

Outline for this Study

PhosphoSens-Kinetic Assay Validation

AssayQuant TECHNOLOGIES INC.

Enzyme Source, Construct, and Lot Information:

Invitrogen VRK2 (Cat#/Lot#: A30986/248990) amino acids 1-375, N-terminal 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 AQT1030

0, 0.08, 0.16, 0.31, 0.63, 1.3, 2.5, 5, 10, 20, 40, and 80 nM VRK2

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:

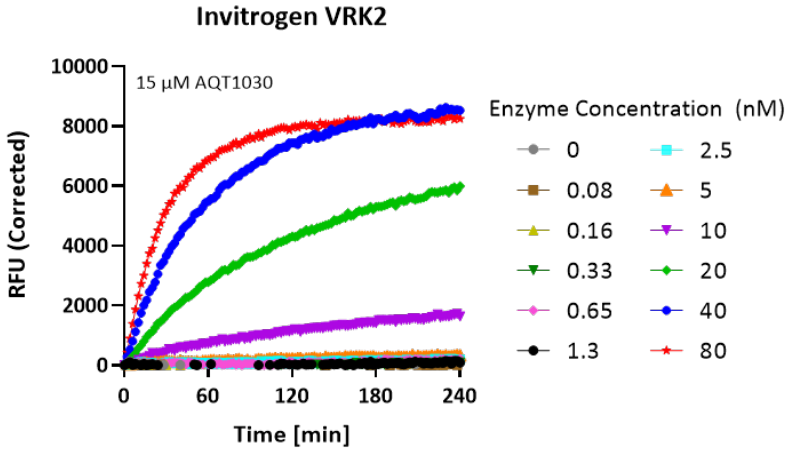
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

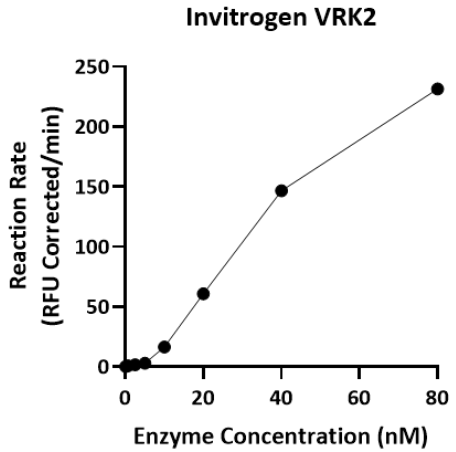
Enzyme Titration

Progress Curves

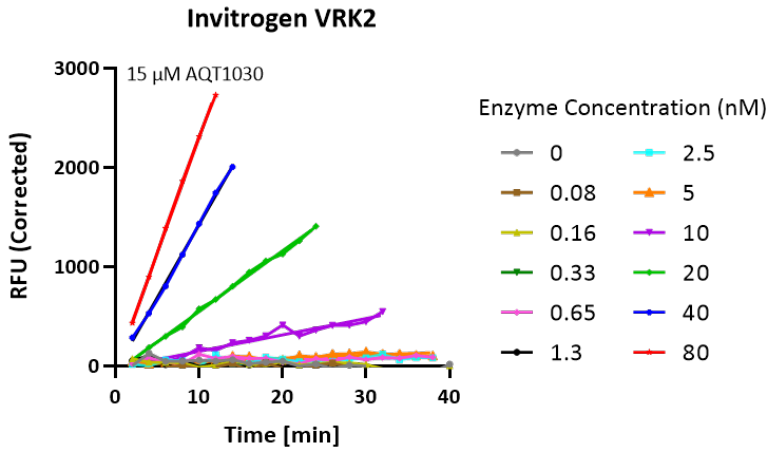
AssayQuant TECHNOLOGIES INC.

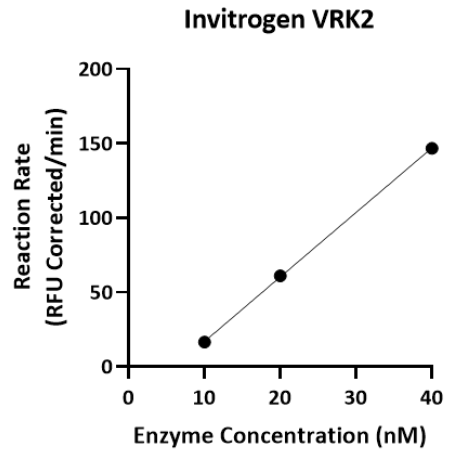
Complete Progress Curves





Linear Region of Progress Curves





Linear Range

Enzyme Titration

Reaction Rate Table

Enzyme Conc. (nM)	Reaction Rate (RFU/min)	Normalized Reaction Rate (RFU/pmole/min)
0.08	0.39	241
0.16	-0.71	-222
0.33	0.48	72
0.65	0.39	30
1.3	-2.6	-99
2.5	1.6	32
5	4.1	41
10	21	103
20	61	153
40	147	184
80	232	145

The reaction is linear from 10-40 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 $MgCl_2$

0, 0.6, 1.2, 2.3, 4.7, 9.4, 18.8, 37.5, 75, 150, 300, and 600 µM AQT1030

20 nM VRK2

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.

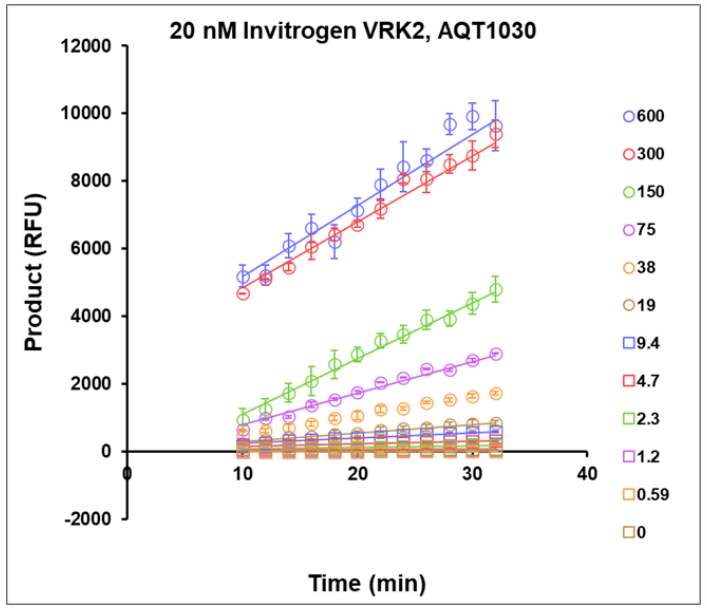
Notes:

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.

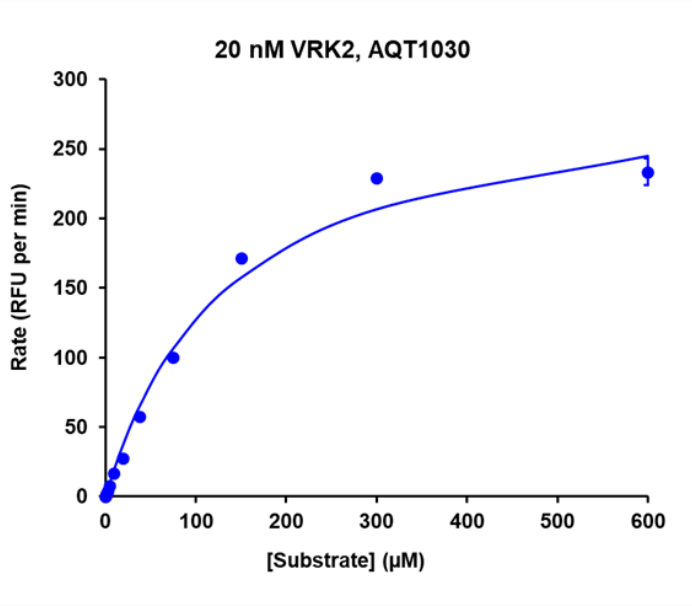
Sensor Peptide K_m Determination

Titration Curves and K_m Plot and Table

Sensor Peptide Titration Curves



Sensor Peptide K_m Plot



Sensor Peptide K_m Table

Parameter	Value	Approx SE
Vmax (RFU per min)	301.0	7.6
Vmax (RFU per pmol per min)	752	19
Km (µM)	136.6	7.4
R squared	0.983	

Sensor Peptide K_m is 137 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 AQT1030

20 nM VRK2

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.

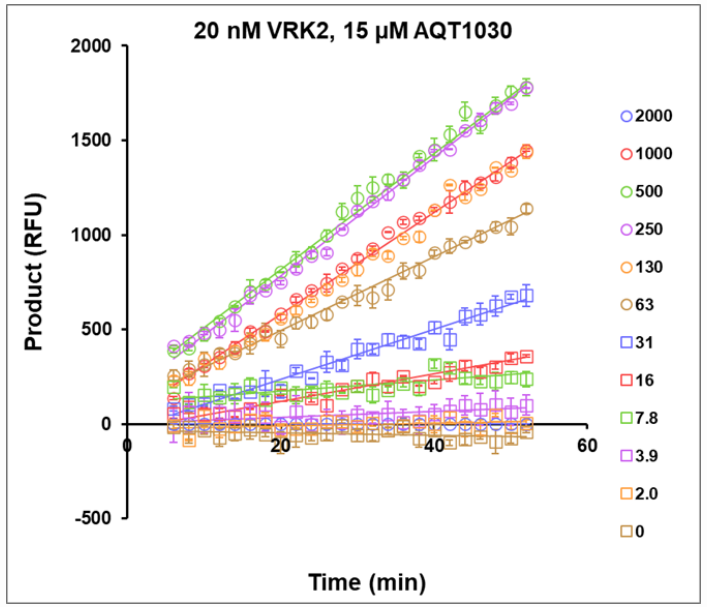
Notes:

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.

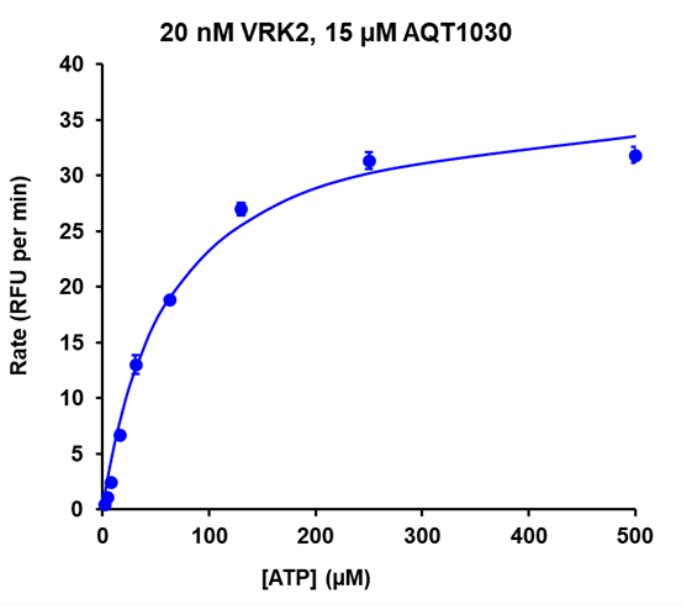
ATP K_m Determination

Titration Curves and K_m Plot and Table

ATP Titration Curves



ATP K_m Plot



ATP K_m Table

Parameter	Value	Approx SE
Vmax (RFU per min)	37.7	1.5
max (RFU per pmol per min)	94	4
Km (µM)	62.1	9.7
R squared	0.992	

ATP K_m is 62 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 AQT1030

20 nM VRK2

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.

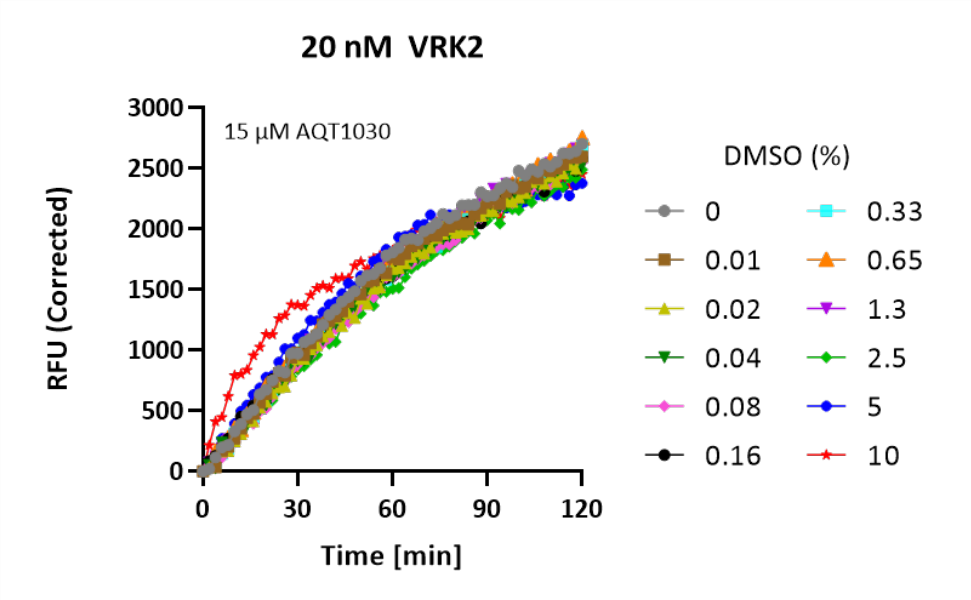
Notes:

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.

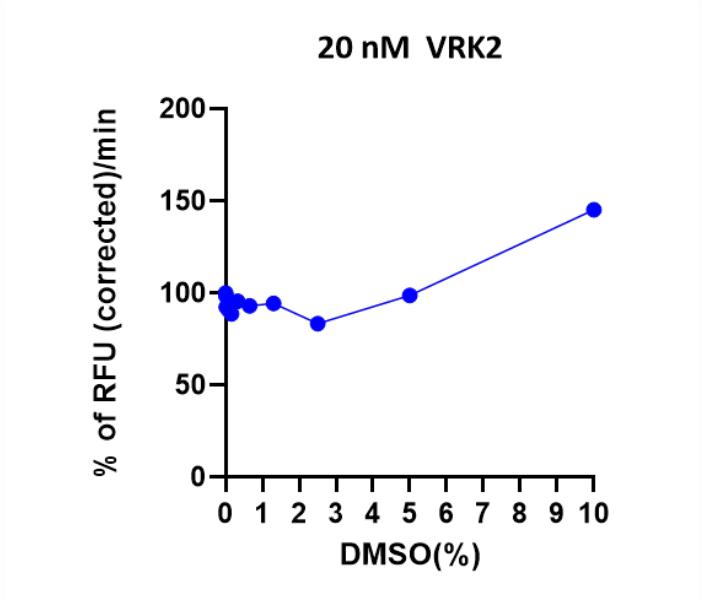
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No change in enzyme activity out to 2% DMSO, but significant activation (150%) at 5% and 10% DMSO. This has been seen with other kinases.

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 $MgCl_2$

2% DMSO

15 µM AQT1030

20 nM VRK2

0, 0.169, 0.508, 1.52, 4.57, 13.7, 41.2, 123, 370, 1110, 3330, 10000 nM Staurosporine

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).

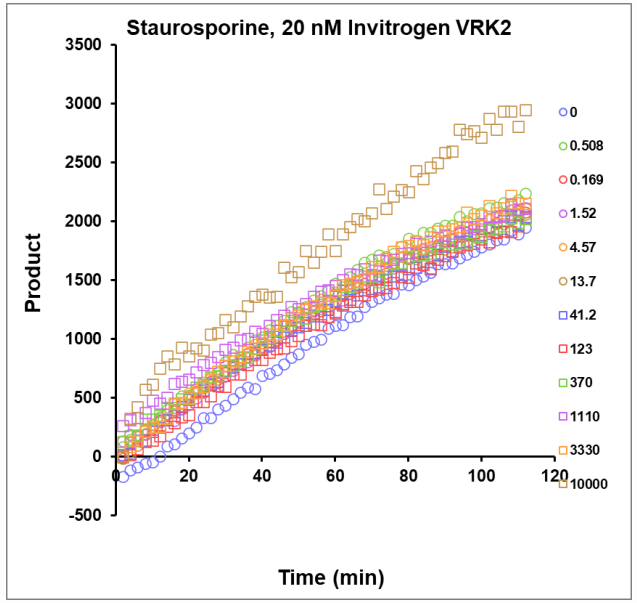
Notes:

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.

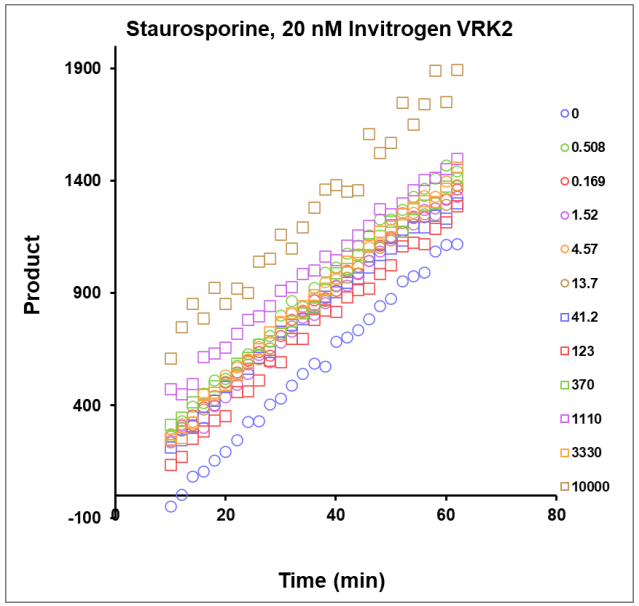
IC_{50} Determination

Progress Curves and IC_{50} Curves and Table

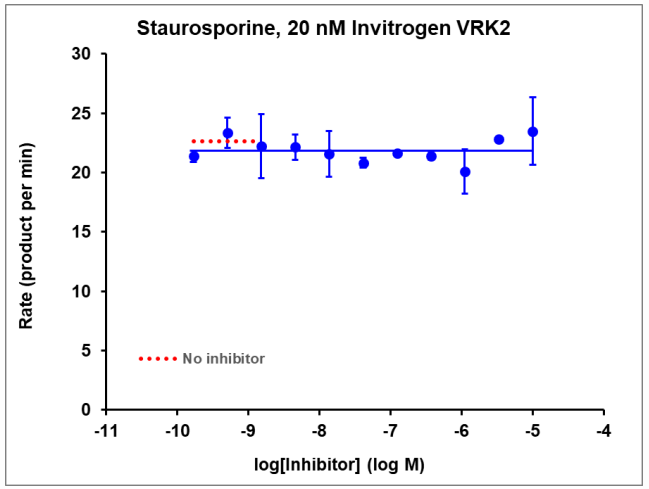
Inhibitor Titration Progress Curves



Linear Region of Progress Curves



IC_{50} Curve



IC_{50} Table

Parameter	Value
Bottom	-0.1
Top	21.9
log IC50	0.35
IC50 (nM)	> 10 µM
Ki (nM)	> 10 µM
Slope	-1.810
R squared	0.643
IC50 approx SE (nM)	ND
50% inhibition (nM)	ND

The Y-axis label is RFU/min.

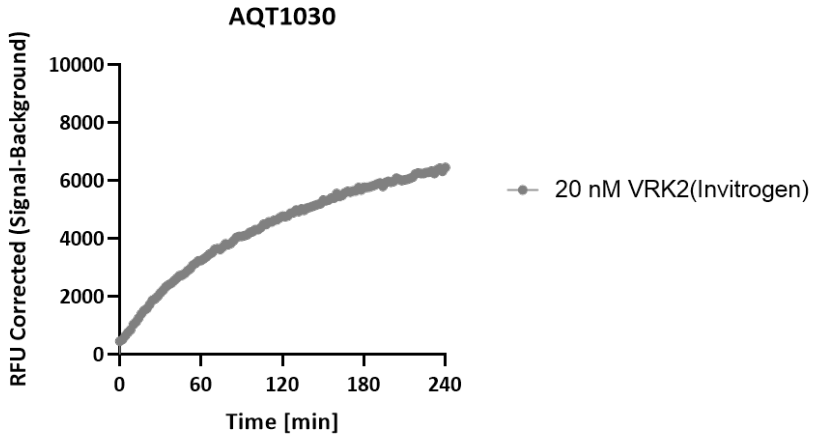
Staurosporine IC_{50} at ATP K_m is > 10000 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	10-40 nM
Sensor Peptide K_m Value	137 µM
ATP K_m Value	62 µM
DMSO Tolerance (highest % recommended)	2%
Staurosporine IC_{50} Determination at ATP K_m	> 10000 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate Stnd Error (RFU/pmole/min)
VRK2	20	AQT1030	153	2.3

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 Moderate