

AQT0232 – RPS6KA2 (RSK3) Assay Validation

PhosphoSens®-Kinetic Assay Format

Outline for this Study

PhosphoSens–Kinetic Assay Validation

Enzyme Source, Construct, and Lot Information:

Carna RSK3 (Cat#/Lot#: 01-151/09CBS-0721F) amino acids full length; N-terminal GST tag

Reference Compound Information:

Staurosporine MedChemExpress (Cat#/Lot#: HY-15141/125391) CAS No.: 62996-74-1

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 MgCl₂

15 μM AQT0232

0.01, 0.02, 0.04, 0.08, 0.16, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 nM RSK3

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 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.

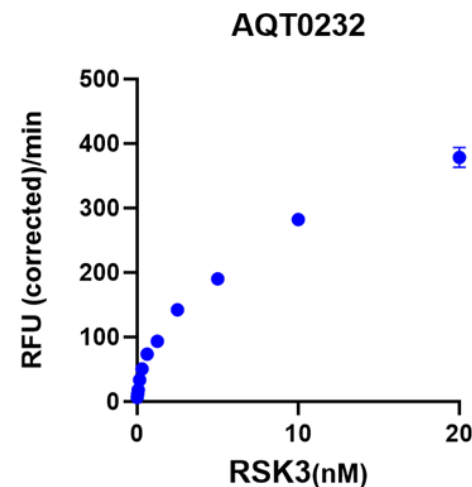
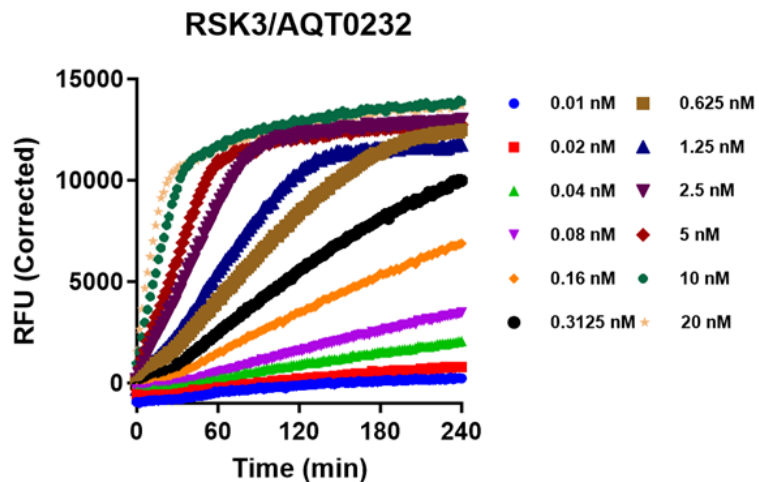
Notes:

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.

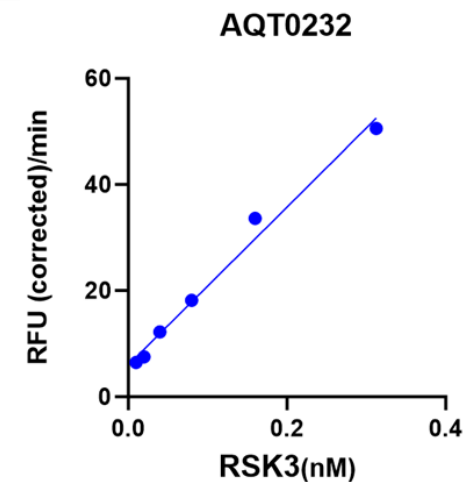
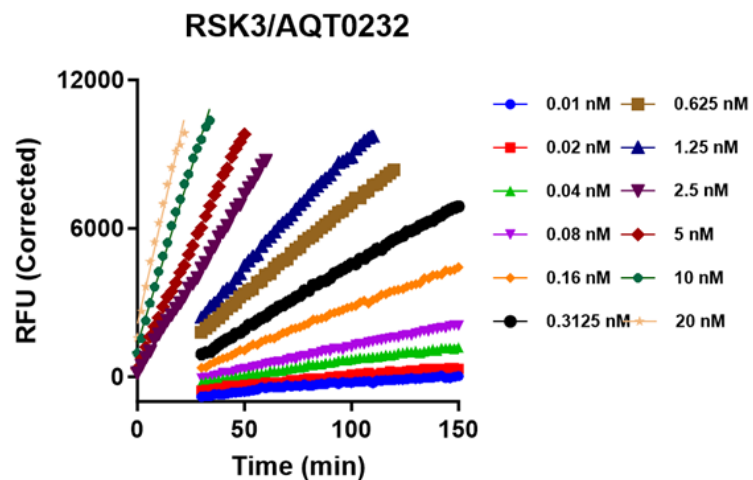
Enzyme Titration

Progress Curves

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)
0.01	32,290	929
0.02	18,830	310
0.04	15,263	197
0.08	11,356	69
0.16	10,503	60
0.3125	8,091	35
0.625	5,871	24
1.25	3,742	33
2.5	2,842	16
5	1,900	10
10	1,411	29
20	946	39

The reaction is linear from 0.04 – 0.3125 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, and 100 μM AQT0232

0.5 nM RSK3

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 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.

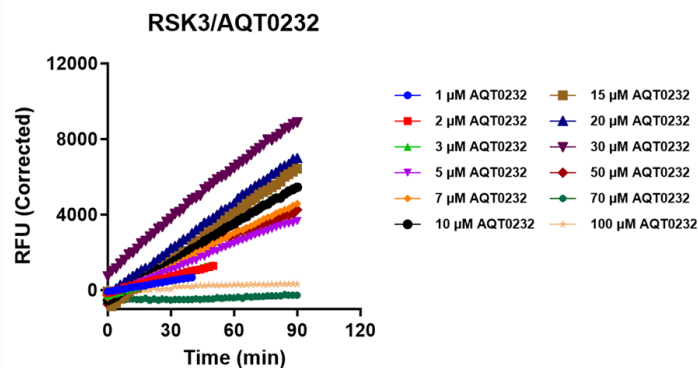
Notes:

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.

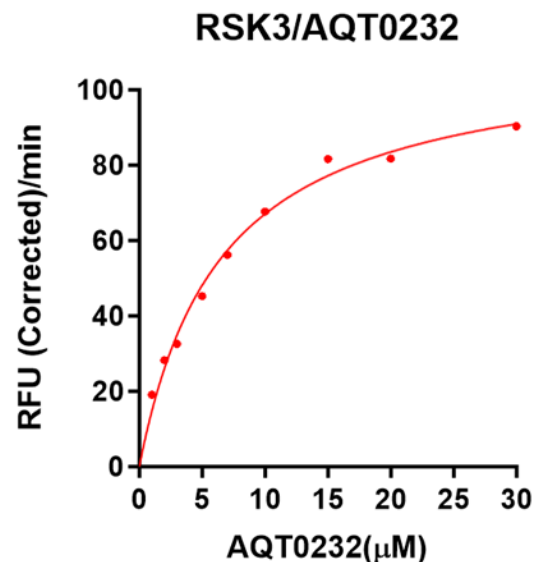
Sensor Peptide K_m Determination

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	110.6
Km	6.503
Std. Error	
Vmax	4.288
Km	0.6823
95% CI (profile likelihood)	
Vmax	101.2 to 121.8
Km	5.060 to 8.359
Goodness of Fit	
Degrees of Freedom	7
R squared	0.9885

Sensor Peptide K_m is 6.5 μ M

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 μ M 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 μ M AQT0232
0.5 nM RSK3

Reaction Set Up:

2 or 2.5 μ L	10x Sensor Peptide
14 or 17.5 μ L	Reaction Mix with ATP & DTT
<u>4 or 5 μL</u>	1x EDB or Kinase dilutions (5x in EDB)
20 or 25 μ L	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 μ L final well volume or in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 μ L 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.

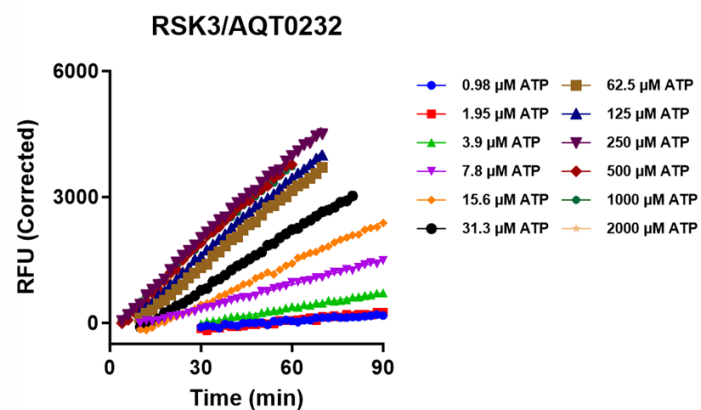
Notes:

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.

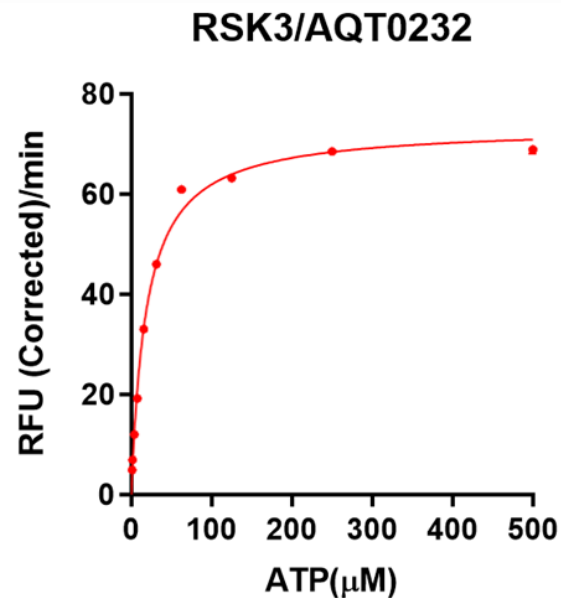
ATP K_m Determination

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	73.52
Km	18.76
Std. Error	
Vmax	1.431
Km	1.468
95% CI (profile likelihood)	
Vmax	70.33 to 76.83
Km	15.73 to 22.33
Goodness of Fit	
Degrees of Freedom	8
R squared	0.9950

ATP K_m is 19 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 MgCl₂

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

15 μM AQT0232

0.5 nM RSK3

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 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.

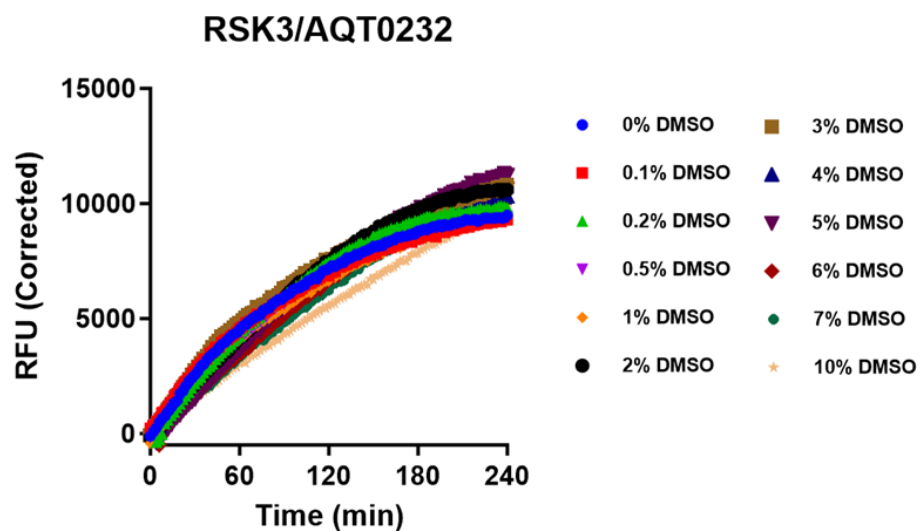
Notes:

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.

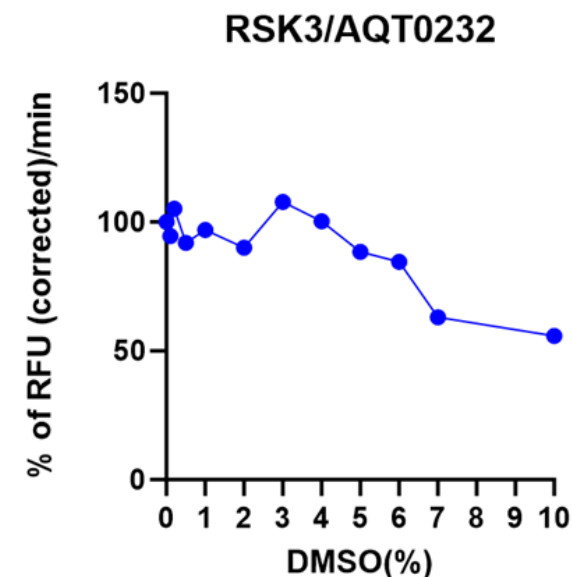
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No change in enzyme activity out to 2% DMSO

IC₅₀ 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 MgCl₂

1% DMSO

15 μM AQT0232

0.5 nM RSK3

0.1 mM Staurosporine was serially diluted (3-fold, 11-point) in 100%DMSO. The series was then diluted 10-fold into BSA (with a final concentration of 0.2 mg/mL BSA in 10% DMSO) to prepare the 10x compound stocks.

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).

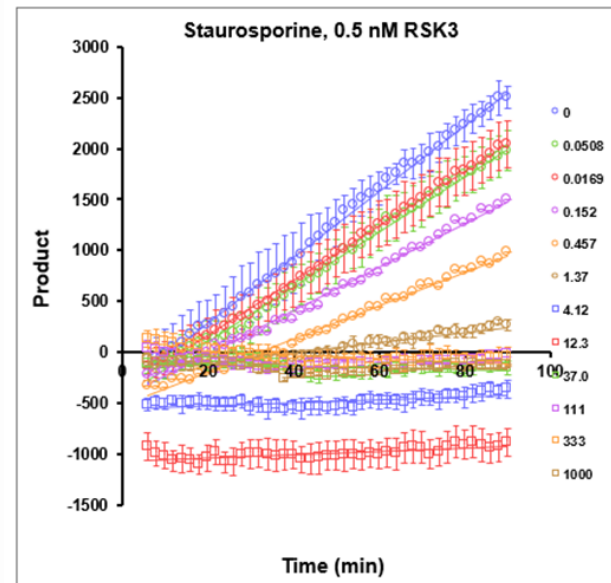
Notes:

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.

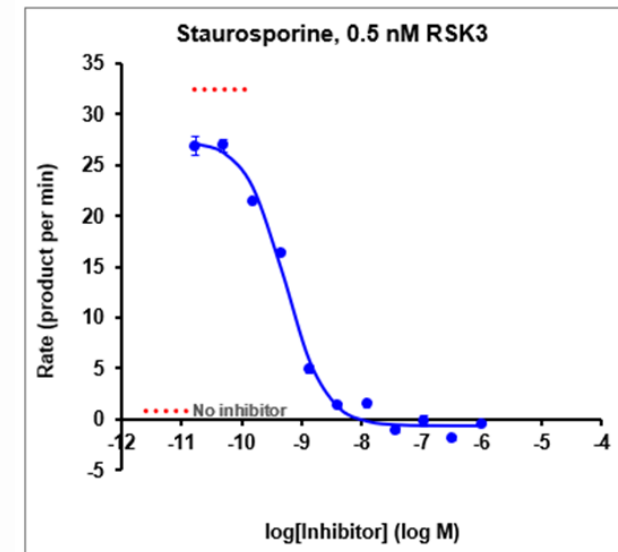
IC₅₀ Determination

Progress Curves and IC₅₀ Curves and Table

Linear Region of Progress Curves



IC₅₀ Curve



IC₅₀ Table

Parameter	Value
Bottom	-0.6
Top	27.5
log IC50	-9.28
IC50 (nM)	0.53
Ki (nM)	0.27
Slope	-1.311
R squared	0.991
IC50 approx SE (nM)	0.02
50% inhibition (nM)	0.51

The Y-axis label is RFU/min.

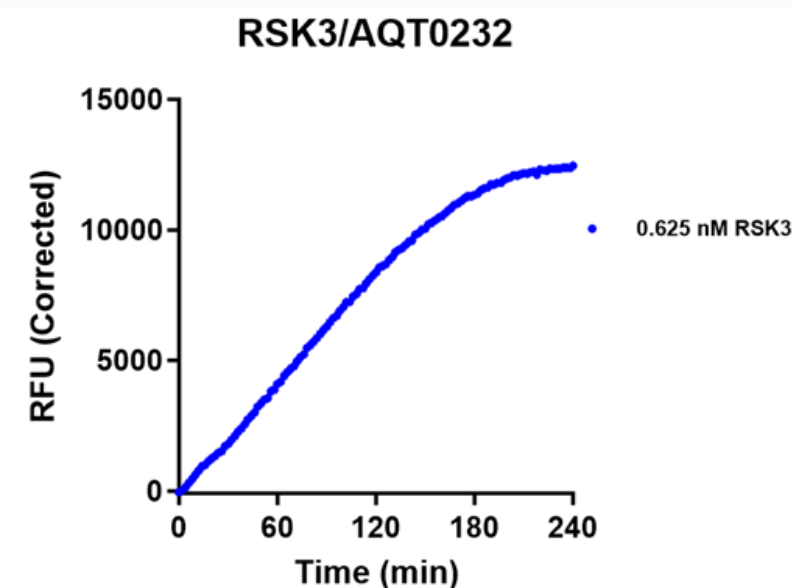
Staurosporine IC₅₀ at ATP K_m is 0.53 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.04 - 0.3125 nM
Sensor Peptide K_m Value	6.5 μ M
ATP K_m Value	19 μ M
DMSO Tolerance (highest % recommended)	2
Staurosporine IC_{50} Determination at ATP K_m	0.53 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate StdError (RFU/pmole/min)
RSK3	0.625	AQT0232	5,871	24

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