

AQT0280 – DDR1 Assay Validation

PhosphoSens[®]-Kinetic Assay Format

Outline for this Study

PhosphoSens–Kinetic Assay Validation

Enzyme Source, Construct, and Lot Information:

Carna DDR1 (Cat#/Lot#: 08-132/20CBS-0660B) amino acids 444-876(end); N-terminal GST tag

Reference Compound Information:

Staurosporine

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 AQT0280

0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 5, 10, 15 and 20 nM DDR1

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.

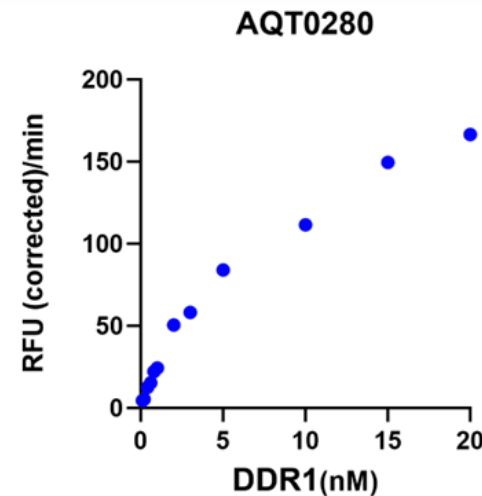
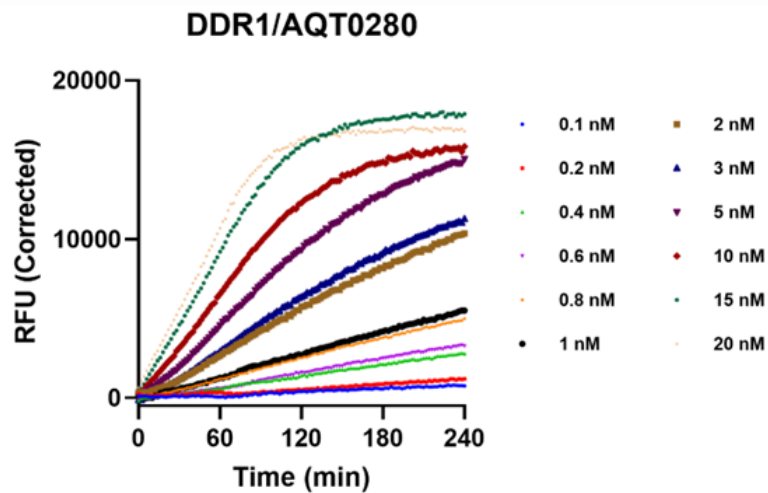
Notes:

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.

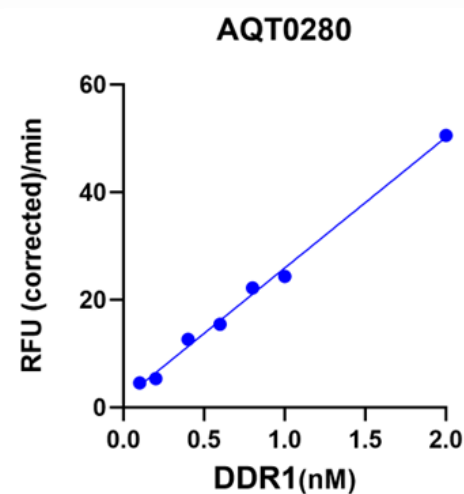
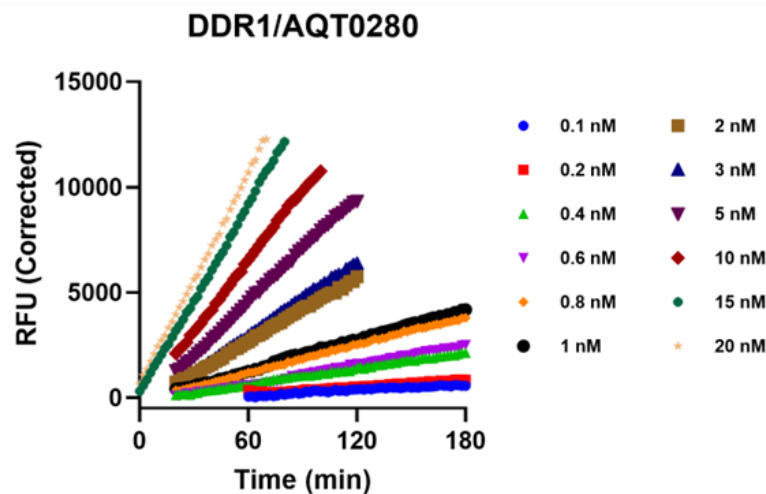
Enzyme Titration

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 Std Error (RFU/pmole/min)
0.1	2,292	70
0.2	1,340	34
0.4	1,581	10
0.6	1,290	9
0.8	1,389	5
1	1,218	6
2	1,265	6
3	971	4
5	841	3
10	558	3
15	498	2
20	417	2

The reaction is linear from 0.2 – 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, and 100 μM AQT0280

5 nM DDR1

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.

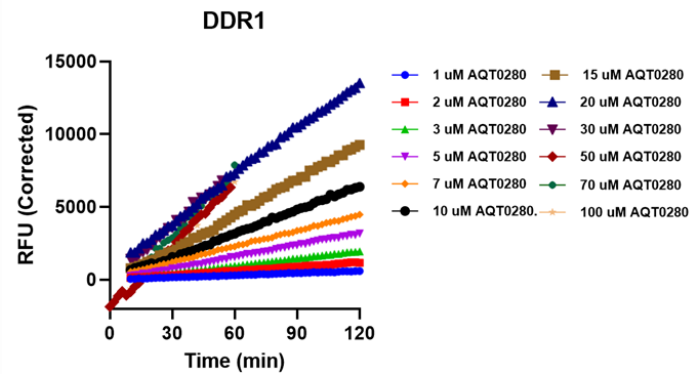
Notes:

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.

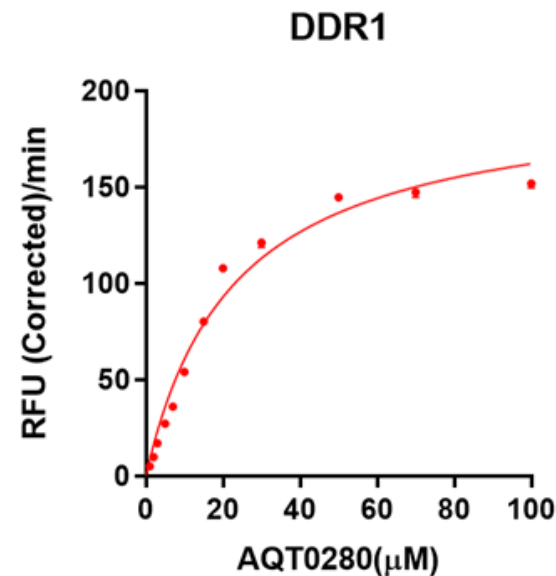
Sensor Peptide K_m Determination

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	199.2
Km	22.95
Std. Error	
Vmax	12.47
Km	3.663
95% CI (profile likelihood)	
Vmax	175.4 to 228.5
Km	16.58 to 32.08
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9781

Sensor Peptide K_m is 23 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 AQT0280

5 nM DDR1

Reaction Set Up:

2 or 2.5 μ L

10x Sensor Peptide

14 or 17.5 μ L

Reaction Mix with ATP & DTT

4 or 5 μ L

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.

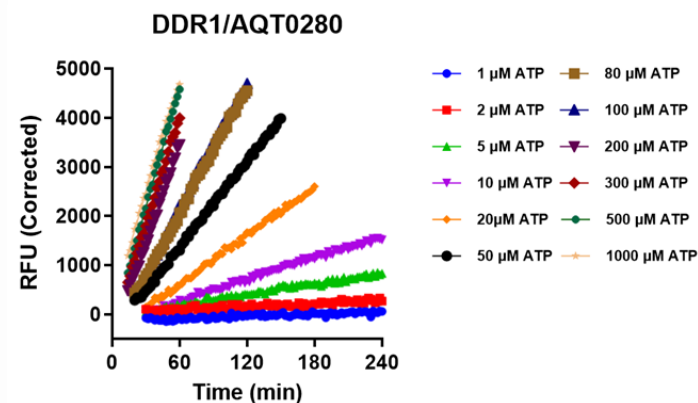
Notes:

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.

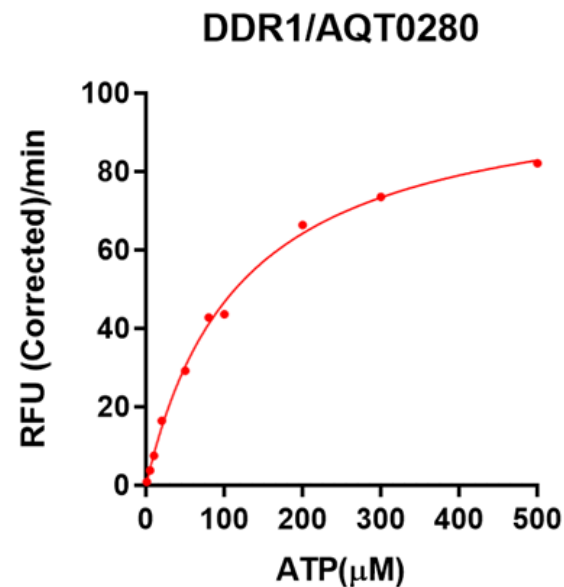
ATP K_m Determination

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	102.7
Km	120.2
Std. Error	
Vmax	2.610
Km	7.884
95% CI (profile likelihood)	
Vmax	97.05 to 108.9
Km	103.7 to 139.4
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9976

ATP K_m is 120 μ M

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 AQT0280

5 nM DDR1

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.

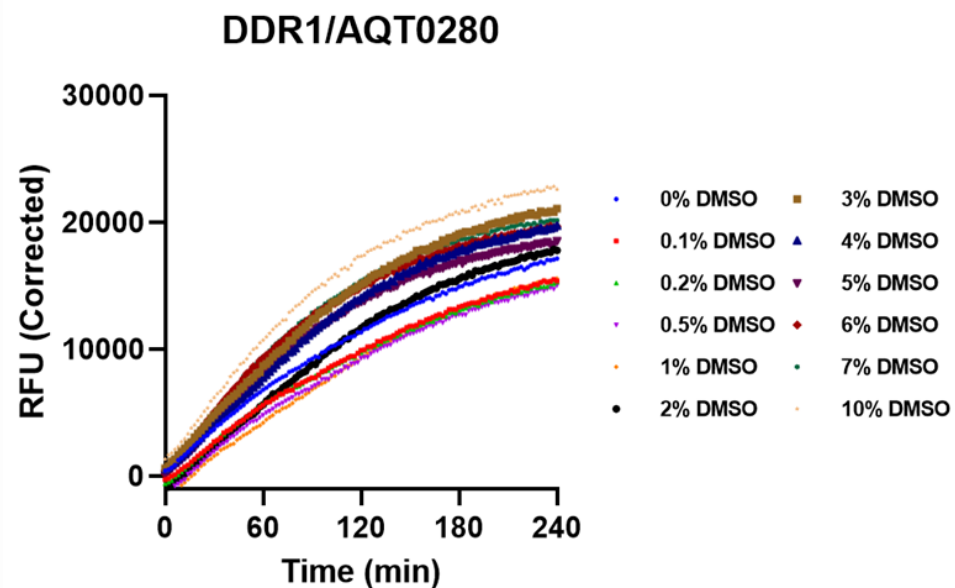
Notes:

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.

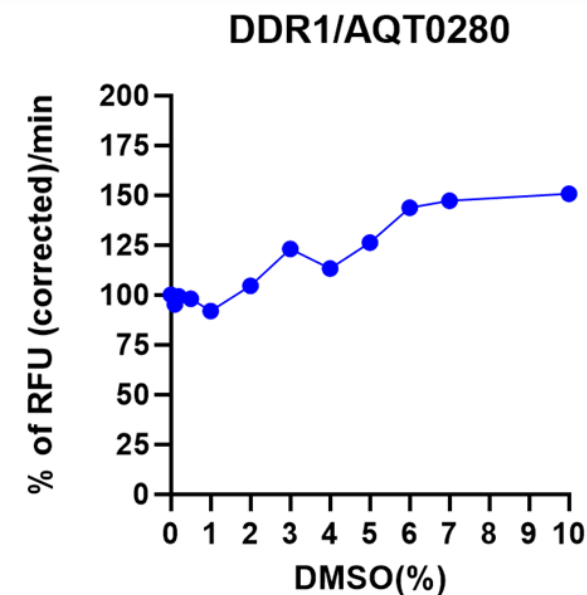
DMSO Tolerance Test

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

10 μM AQT0280

5 nM DDR1

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 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).

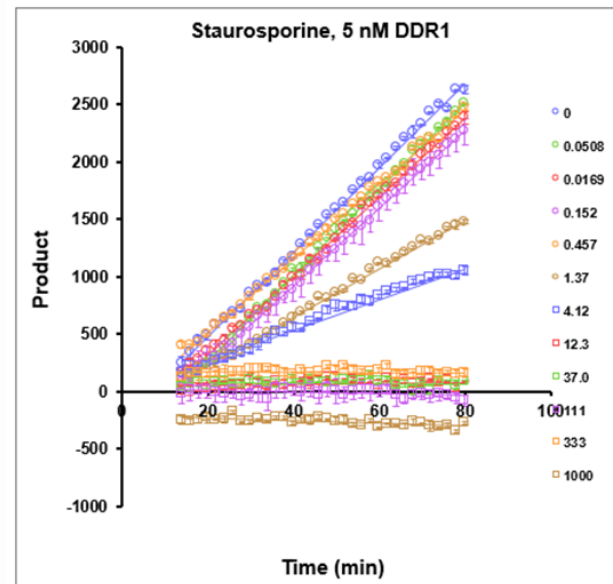
Notes:

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.

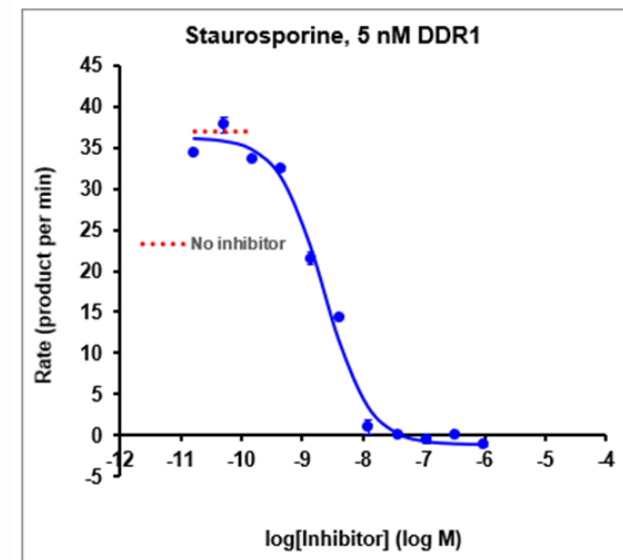
IC₅₀ Determination

Progress Curves and IC₅₀ Curves and Table

Linear Region of Progress Curves



IC₅₀ Curve



The Y-axis label is RFU/min.

IC₅₀ Table

Parameter	Value
Bottom	-1.1
Top	36.3
log IC50	-8.63
IC50 (nM)	2.33
Ki (nM)	1.17
Slope	-1.174
R squared	0.990
IC50 approx SE (nM)	0.11
50% inhibition (nM)	2.22

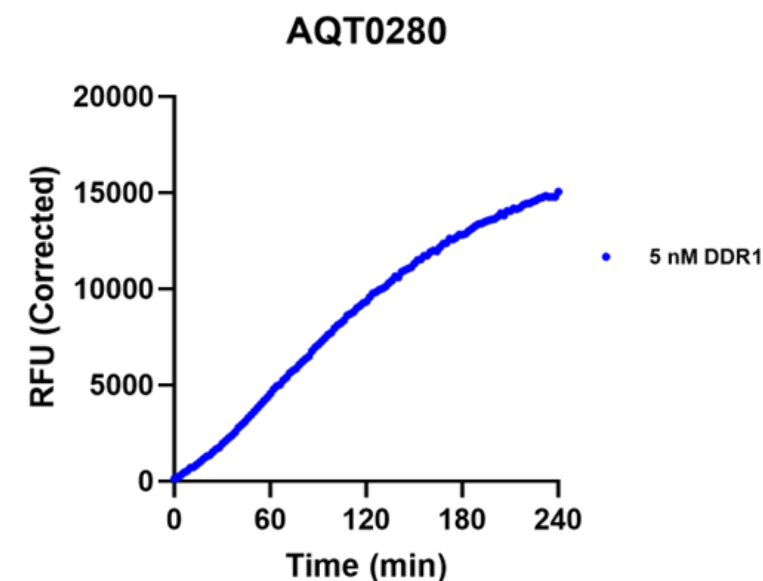
Staurosporine IC₅₀ Determination at ATP K_m is 2.3 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.2 - 2.0 nM
Sensor Peptide K_m Value	23 μ M
ATP K_m Value	120 μ M
DMSO Tolerance (highest % recommended)	2
Staurosporine IC_{50} Determination at ATP K_m	2.3 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate StndError (RFU/pmole/min)
DDR1	5	AQT0280	841	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 Strong