AQT0258 – CDK4/cycD1 Assay Validation

PhosphoSens®-Kinetic Assay Format

Outline for this Study

PhosphoSens-Kinetic Assay Validation

AssayQuant TECHNOLOGIES INC.

Enzyme Source, Construct, and Lot Information:

SignalChem CDK4/D1 (Cat#/Lot#: C31-10G/R2640-7) amino acids full length and Cyclin D1 amino acids full length; N-terminal GST tags

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 $MgCl_2$

15 µM AQT0258

0, 0.08, 0.16, 0.31, 0.63, 1.3, 2.5, 5, 10, 20, 40, and 80 nM CDK4/cycD1

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.

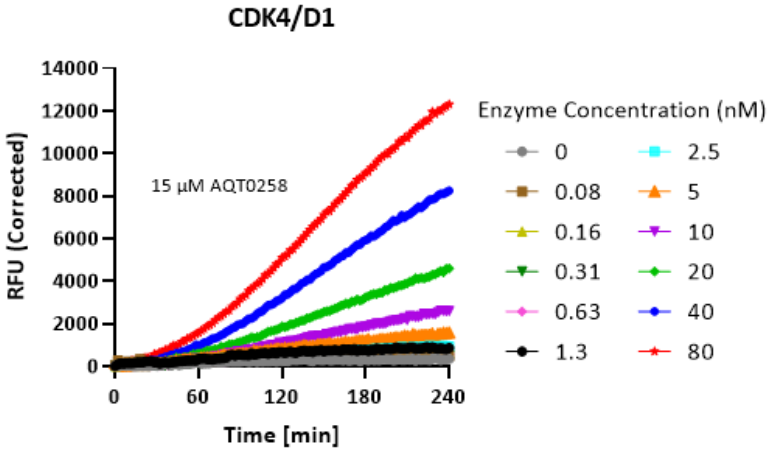
Notes:

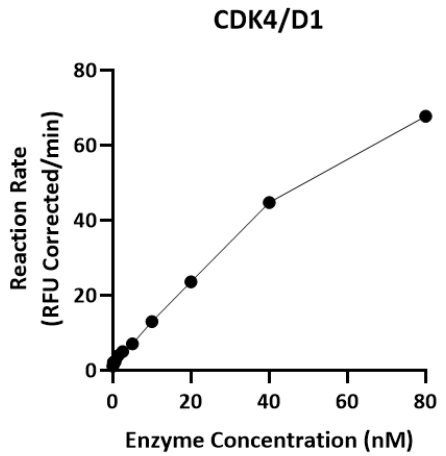
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

Enzyme Titration

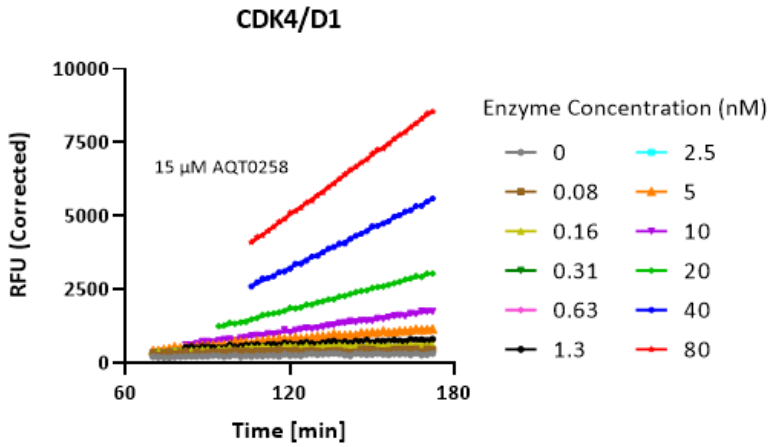
Progress Curves

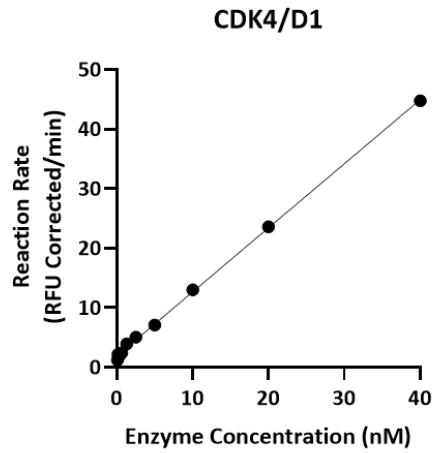
Complete Progress Curves





Linear Region of Progress Curves





Linear Range

Enzyme Titration

Reaction Rate Table

[Enzyme], nM	Reaction Rate (RFU/min)	Normalized Reaction Rate (RFU/pmole/min)
0.08	1.2	769
0.16	2.2	685
0.31	1.9	305
0.63	2.4	194
1.3	3.9	151
2.5	5.1	101
5	7.1	71
10	13	65
20	24	59
40	45	56
80	68	42

The reaction is linear from 0.08-40 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, 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 100, and 200 µM AQT0258

20 nM CDK4/cycD1

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.

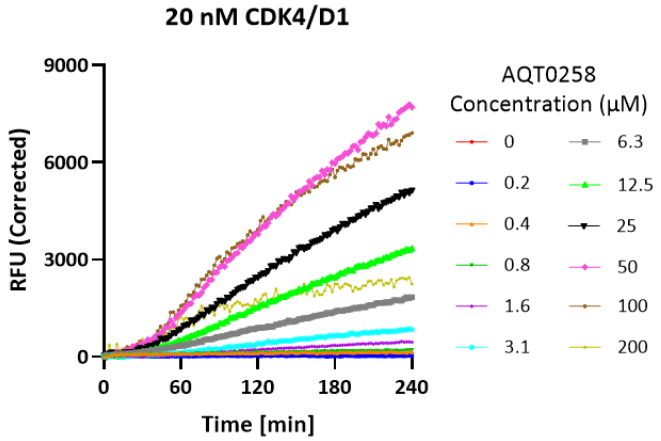
Notes:

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.

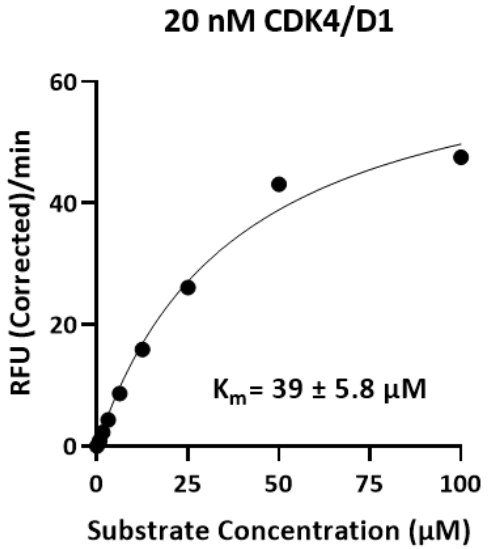
Sensor Peptide K_m Determination

Titration Curves and K_m Plot and Table

Sensor Peptide Titration Curves



Sensor Peptide K_m Plot



Sensor Peptide K_m Table

Sensor Peptide K_m is 39 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 AQT0258

40 nM CDK4/cycD1

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.

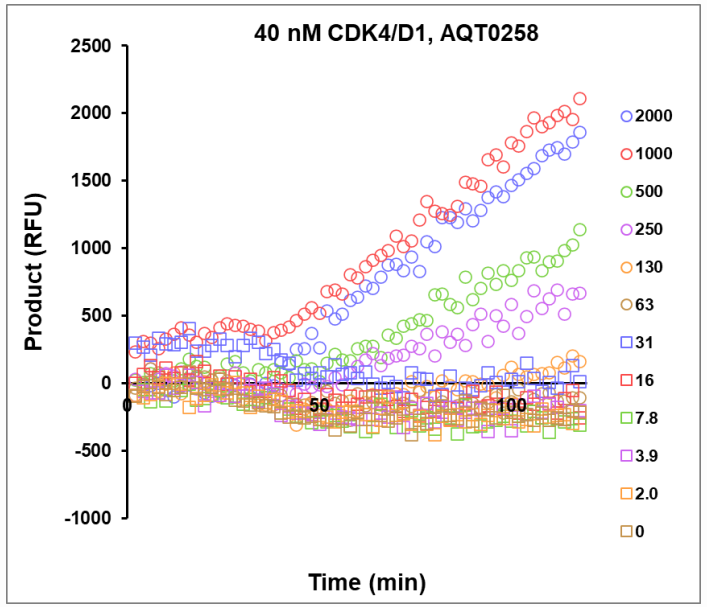
Notes:

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.

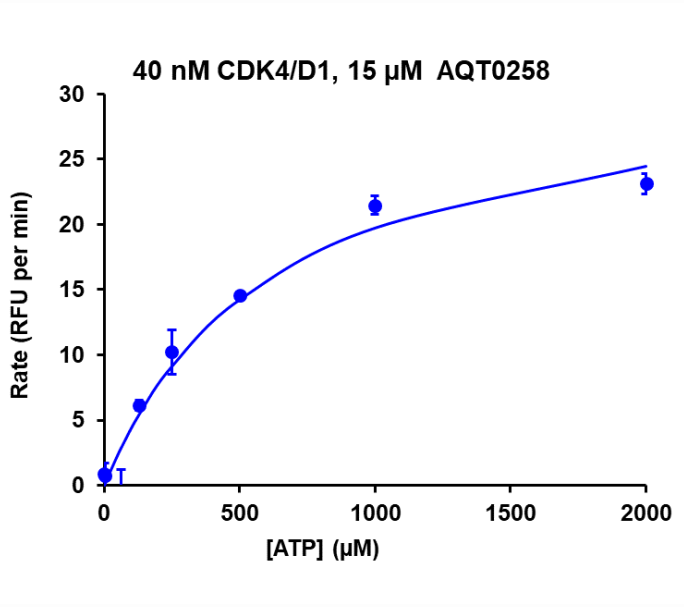
ATP K_m Determination

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)	32.1	0.5
Vmax (RFU per pmol per min)	40	1
Km (µM)	629.6	812.4
R squared	0.951	

ATP K_m is 630 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 AQT0258

40 nM CDK4/cycD1

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.

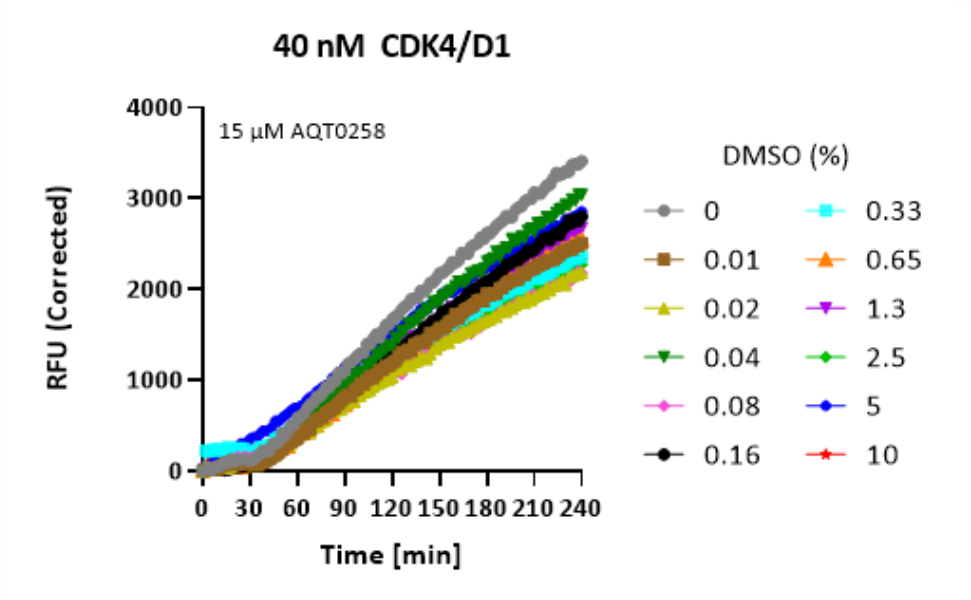
Notes:

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.

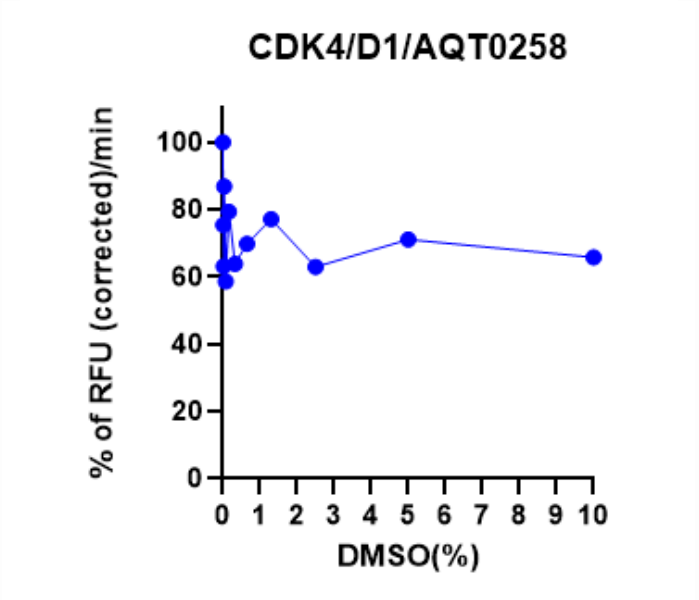
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



Some loss of kinase activity from 0.01-10% 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 $MgCl_2$

2% DMSO

40 µM AQT0258

30 nM CDK4/cycD1

0, 0.5, 1.5, 4.6, 14, 41, 123, 370, 1111, 3333, 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).

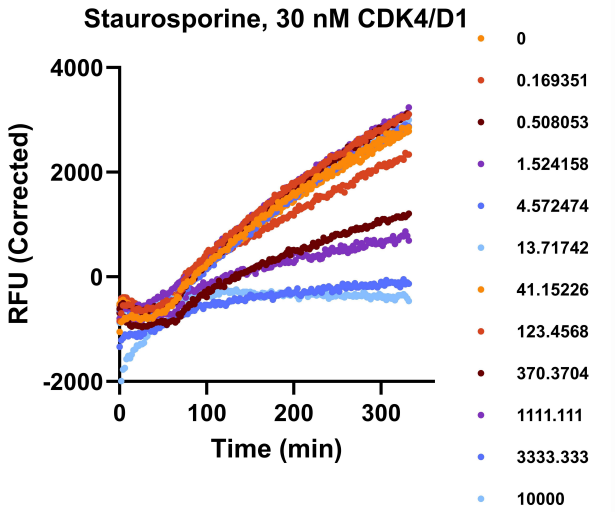
Notes:

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.

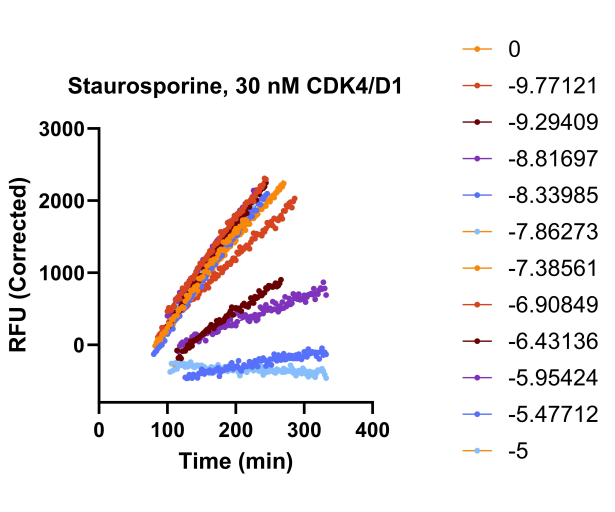
IC_{50} Determination

Progress Curves and IC_{50} Curves and Table

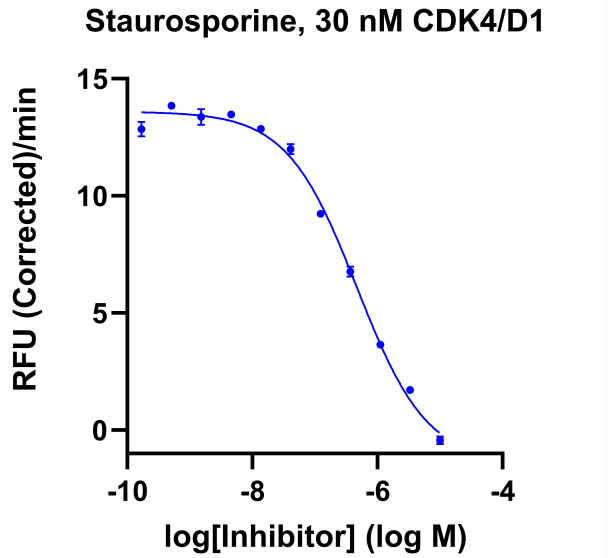
Inhibitor Titration Progress Curves



Linear Region of Progress Curves



IC_{50} Curve



The Y-axis label is RFU/min.

IC_{50} Table

log(inhibitor) vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	-1.410
Top	13.59
LogIC50	2.672
HillSlope	-0.7770
IC50	470.4
Span	15.00
Std. Error	
Bottom	0.8664
Top	0.2548
LogIC50	0.08973
HillSlope	0.09799
Span	0.9934

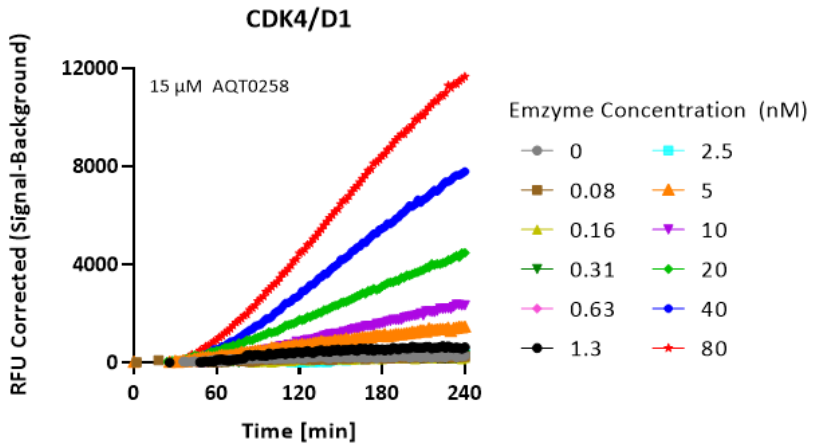
Staurosporine IC_{50} at ATP K_m is 470 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.08-40 nM
Sensor Peptide K_m Value	39 µM
ATP K_m Value	630 µM
DMSO Tolerance (highest % recommended)	Loss of kinase activity observed from 0.01-10% DMSO
Staurosporine IC_{50} Determination at ATP K_m	470 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate Stnd Error (RFU/pmole/min)
CDK4/D1	40	AQT0258	56	0.38

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 Weak