

AQT0258 – CDK6/cycD2 Assay Validation

PhosphoSens[®]-Kinetic Assay Format

Outline for this Study

PhosphoSens–Kinetic Assay Validation

Enzyme Source, Construct, and Lot Information:

CDK6/D2 (Proqinase Cat#/Lot#: 005 1-0375/005) amino acids CDK6(1-326) and CyclinD2(1-289) both with N-terminal GST-tag

Reference Compound Information:

Staurosporine (MCE catalog #HY-15141, Batch # 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 MgCl₂

15 μM AQT0258

0, 0.04, 0.08, 0.16, 0.33, 0.65, 1.3, 2.5, 5, 10, 20, and 40 nM CDK6/D2

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.

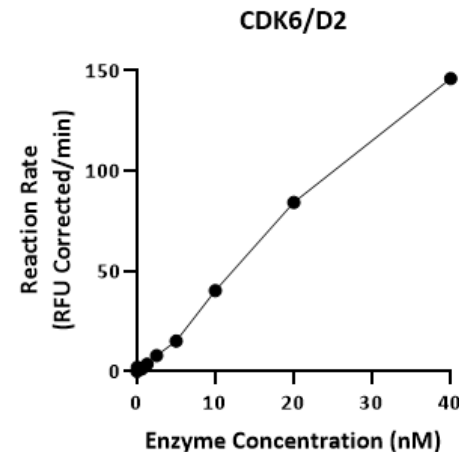
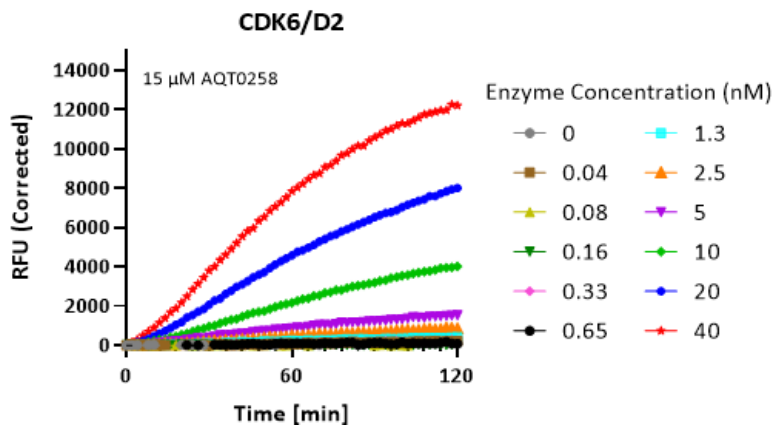
Notes:

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.

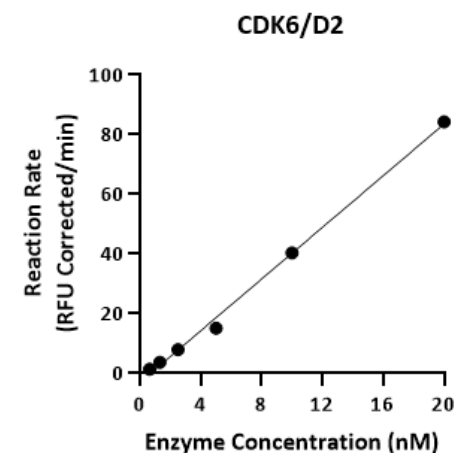
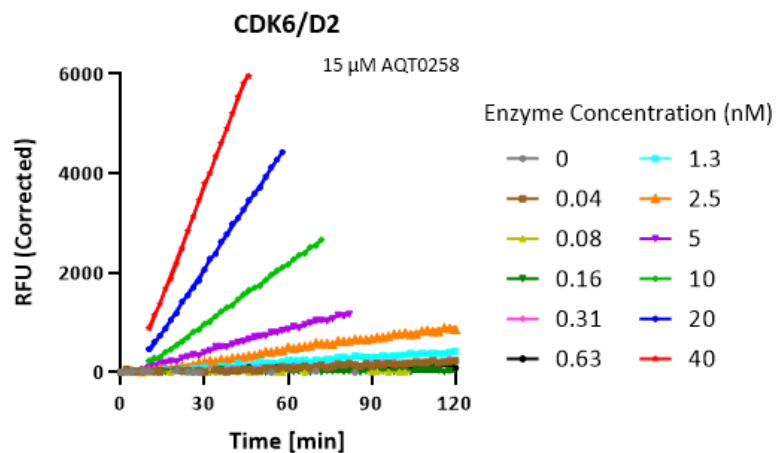
Enzyme Titration

Progress Curves

Complete
Progress
Curves



Linear
Region of
Progress
Curves



Linear
Range

Enzyme Titration

Reaction Rate Table

[Enzyme], nM	Reaction Rate (RFU/min)	Normalized Rate (RFU/pmole/min)
0.04	2.0	2604
0.08	-0.42	-270
0.16	0.23	75
0.31	1.6	259
0.63	1.3	108
1.3	3.7	147
2.5	7.9	159
5	15	151
10	40	202
20	84	211
40	146	183

The reaction is linear from 0.63–20 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, or 200 μM AQT0258

15 nM CDK6/cycD2

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.

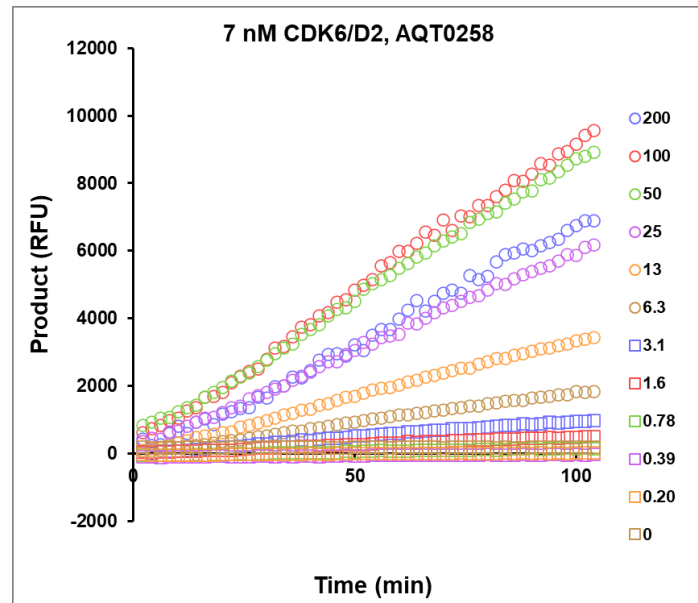
Notes:

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.

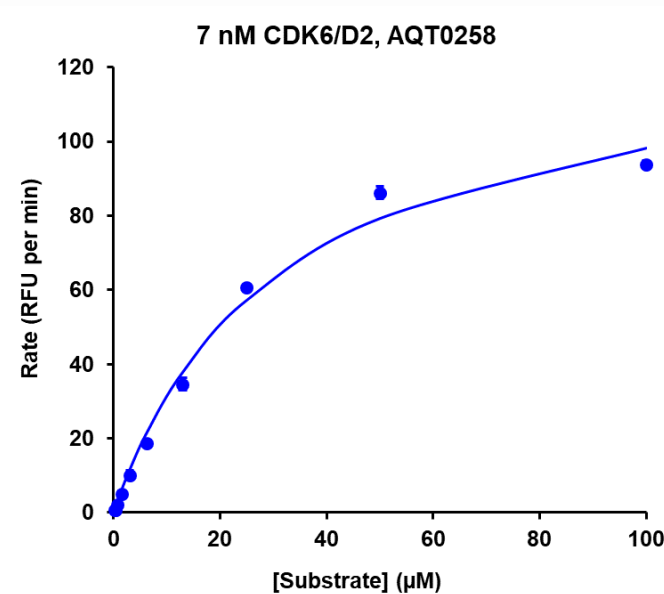
Sensor Peptide K_m Determination

Titration Curves and K_m Plot and Table

Sensor Peptide Titration Curves



Sensor Peptide K_m Plot



Sensor Peptide K_m Table

Parameter	Value	Approx SE
Vmax (RFU per min)	129.2	4.2
Vmax (RFU per pmol per min)	923	30
K_m (μM)	31.5	0.6
R squared	0.992	

Sensor Peptide K_m is 32 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 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 AQT0258
15 nM CDK6/cycD2

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.

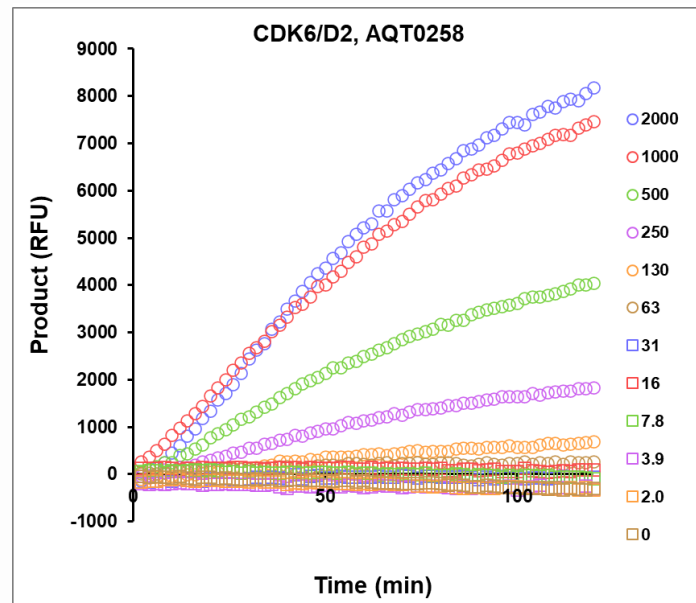
Notes:

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.

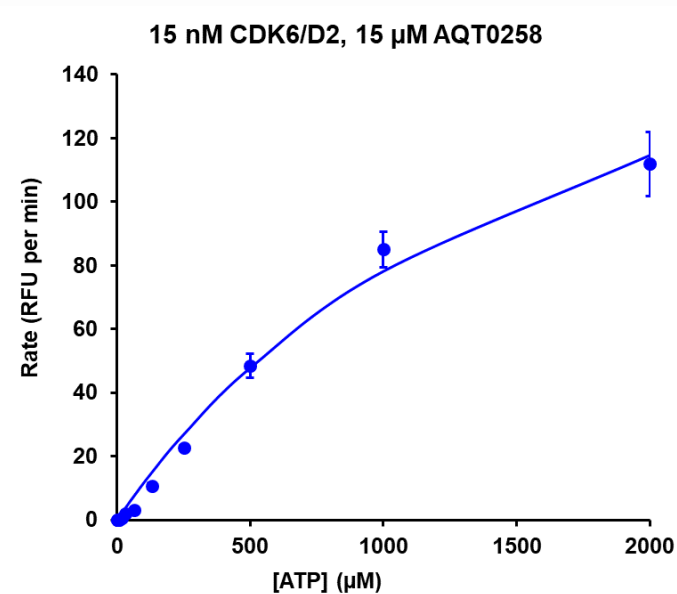
ATP K_m Determination

Titration Curves and K_m Plot and Table

ATP Titration Curves



ATP K_m Plot



ATP K_m Table

Parameter	Value	Approx SE
Vmax (RFU per min)	214.9	5.4
1/2 Vmax (RFU per pmol per min)	716	18
K_m (μM)	1746.6	420.4
R squared	0.991	

ATP K_m is 1700 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

50 μM AQT0258

7 nM CDK6/cycD2

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.

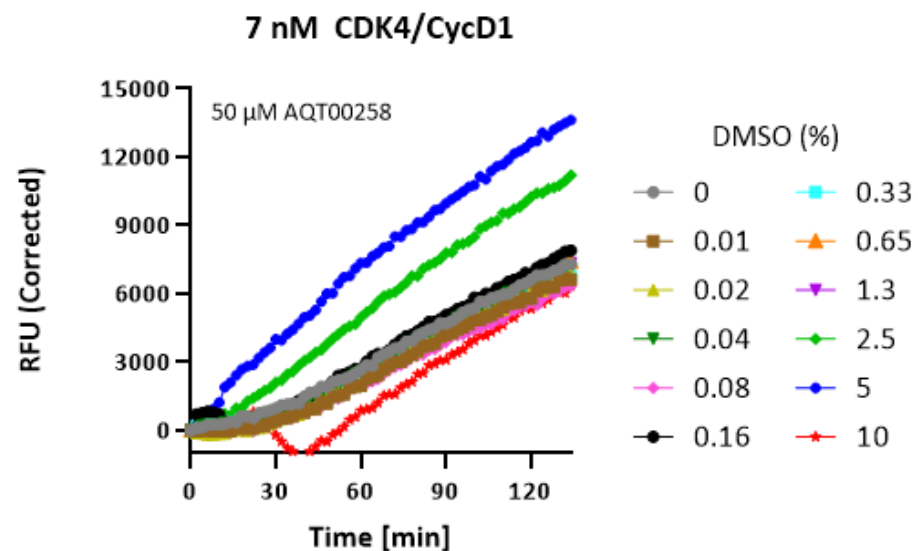
Notes:

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.

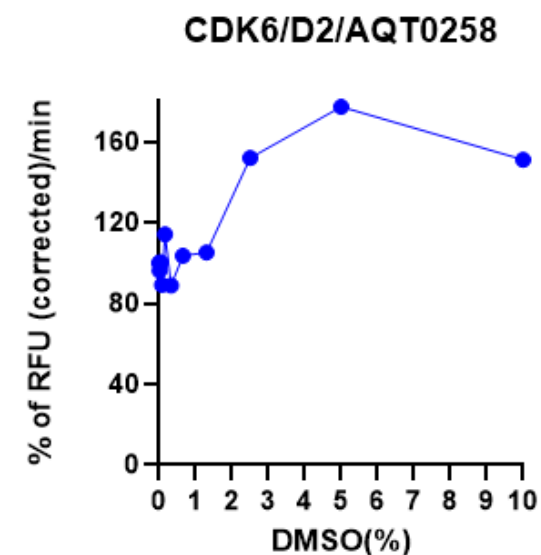
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No change in enzyme activity out to 10% 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

2% DMSO

50 μM AQT0258

7 nM CDK6/cycD2

0, 0.0169, 0.0508, 0.152, 0.457, 1.37, 4.12, 12.3, 37.0, 111, 333, 1000 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).

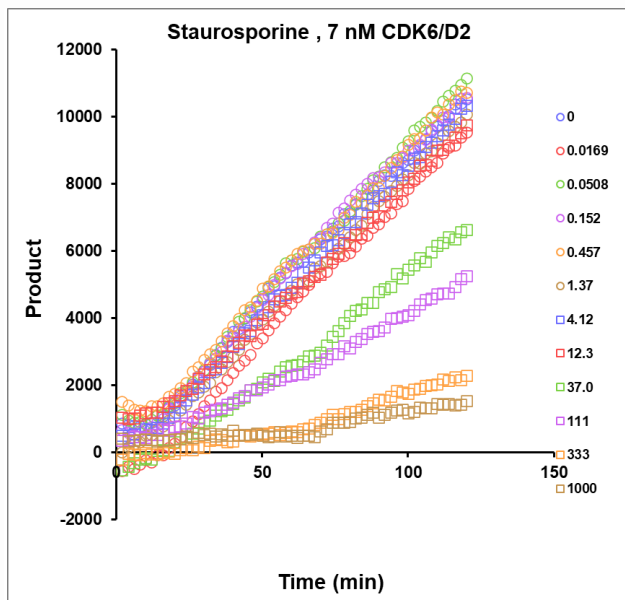
Notes:

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.

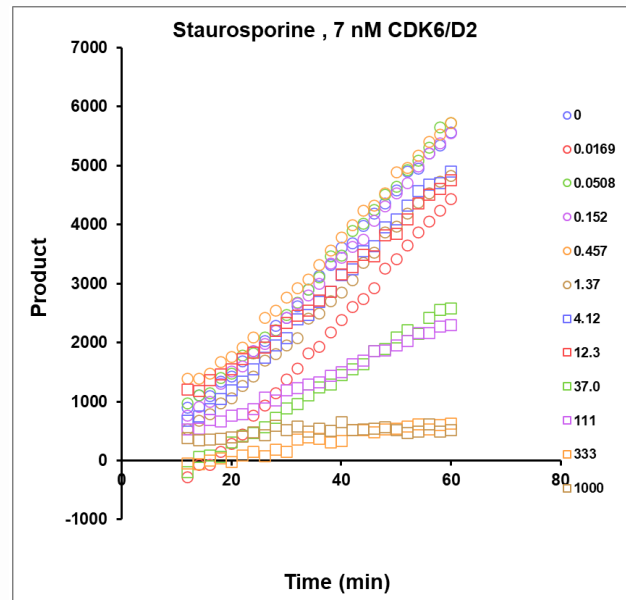
IC₅₀ Determination

Progress Curves and IC₅₀ Curves and Table

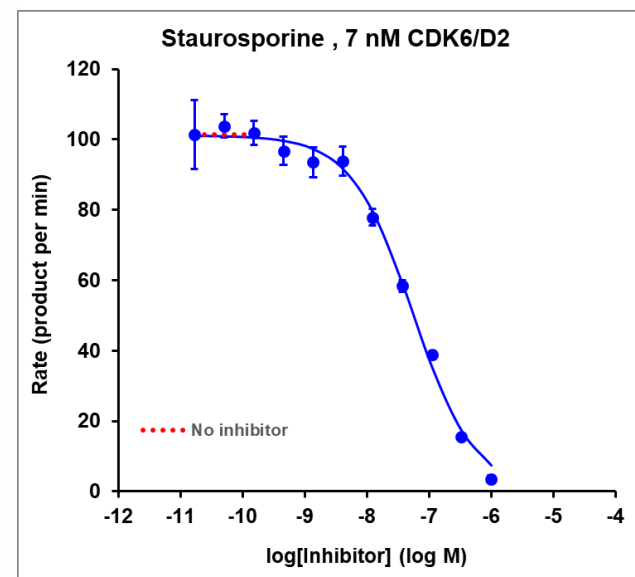
Inhibitor Titration Progress Curves



Linear Region of Progress Curves



IC₅₀ Curve



The Y-axis label is RFU/min.

IC₅₀ Table

Parameter	Value
Bottom	0.0
Top	101.0
log IC50	-7.26
IC50 (nM)	54.42
Ki (nM)	27.21
Slope	-0.877
R squared	0.994
IC50 approx SE (nM)	2.01
50% inhibition (nM)	54.42

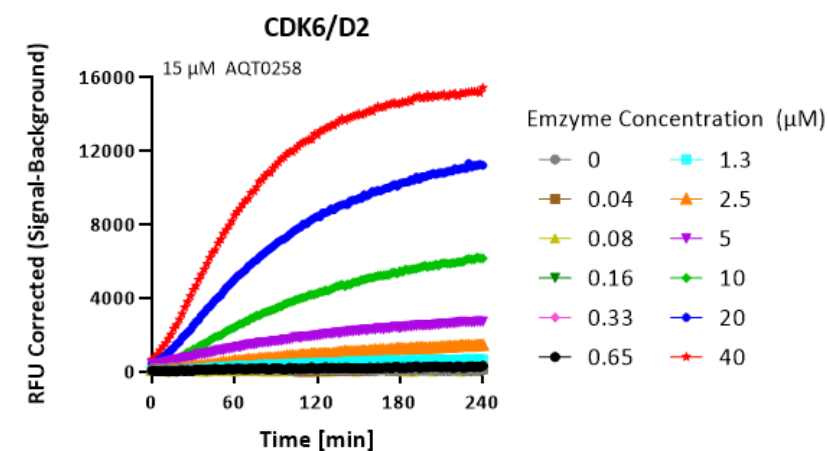
Staurosporine IC₅₀ at ATP K_m is 54 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.63-20 nM
Sensor Peptide K_m Value	32 μ M
ATP K_m Value	1700 μ M
DMSO Tolerance (highest % recommended)	10%
Staurosporine IC_{50} Determination at ATP K_m	54 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate Std Error (RFU/pmole/min)
CDK6/D2	10	AQT0258	202	1.4

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