# AQT0794 – CSK Assay Validation

*PhosphoSens*®-Kinetic Assay Format

# Outline for this Study

## PhosphoSens-Kinetic Assay Validation

AssayQuant TECHNOLOGIES INC.

### Enzyme Source, Construct, and Lot Information:

Carna CSK (08-111/12CBS-0040M) amino acids full length; N-terminal GST tag

### Reference Compound Information:

Staurosporine MedChem Express

### 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 AQT0794

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

### 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 volumeafter 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:

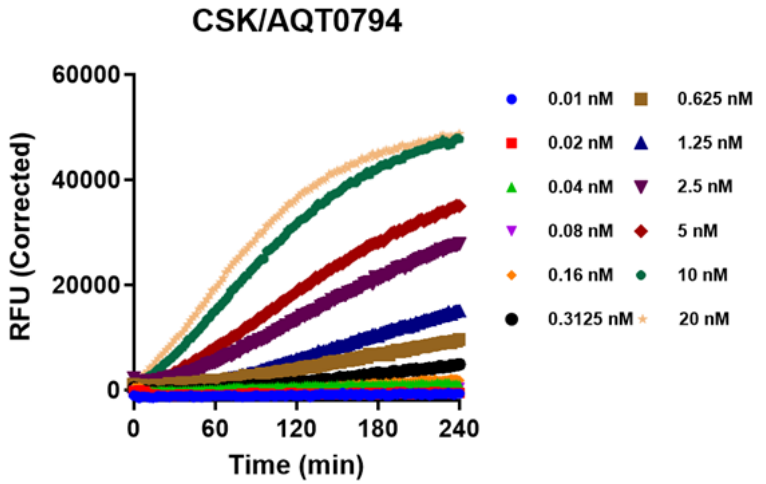
Enzyme Dilution Buffer (EDB):20 mMHEPES, pH 7.5,0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine SerumAlbumin.

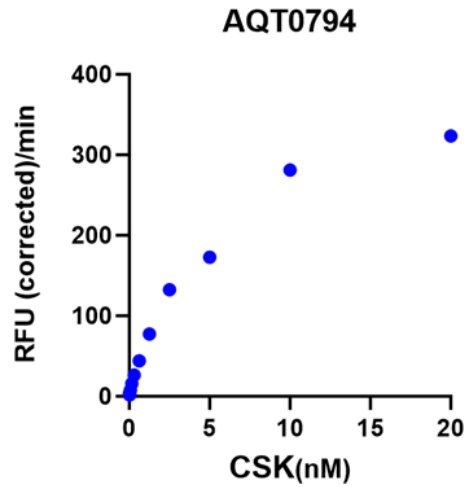
# Enzyme Titration

## Progress Curves

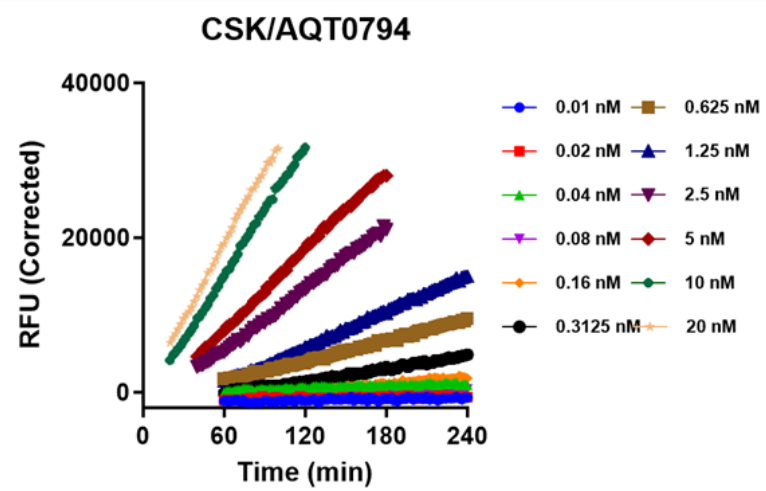
AssayQuant TECHNOLOGIES INC.

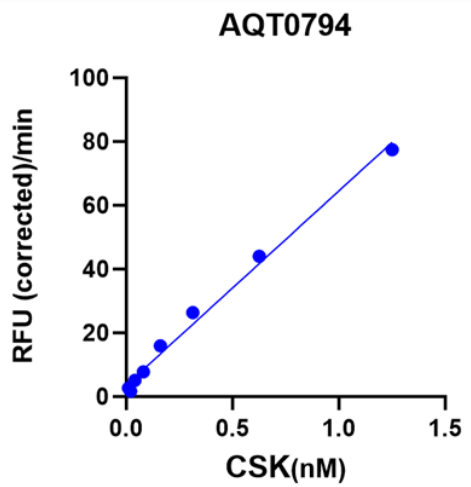
Complete Progress Curves




Linear Region of Progress Curves




Linear Range

# Enzyme Titration

## Reaction Rate Table

| Enzyme Conc. (nM) | Normalized Reaction Rate (RFU/pmole/min) | Normalized Rate Stnd Error (RFU/pmole/min) |
|---|---|---|
| 0.01 | 13,525 | 1,261 |
| 0.02 | 4,370 | 542 |
| 0.04 | 12,868 | 573 |
| 0.08 | 9,731 | 325 |
| 0.16 | 9,981 | 135 |
| 0.3125 | 8,253 | 88 |
| 0.625 | 7,051 | 49 |
| 1.25 | 6,197 | 24 |
| 2.5 | 5,300 | 27 |
| 5 | 3,454 | 14 |
| 10 | 2,813 | 12 |
| 20 | 1,617 | 11 |

The reaction is linear from 0.00-1.25 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 AQT0794

5 nM CSK

**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 volumeafter 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.

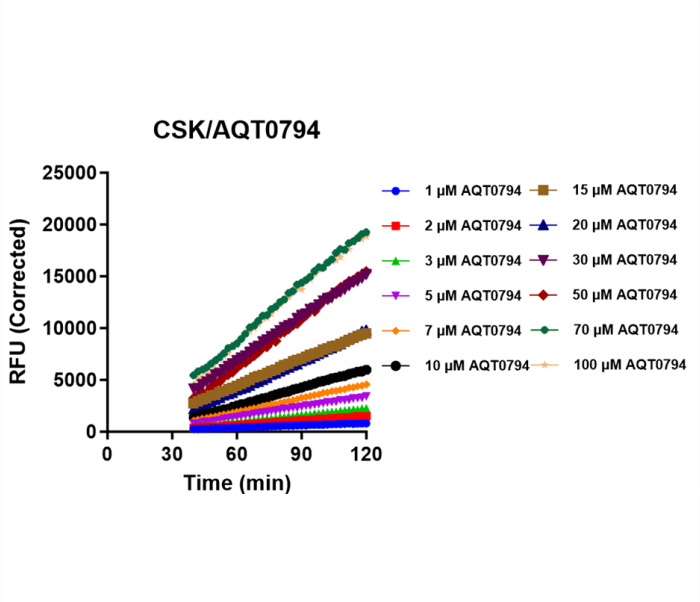
**Notes:**

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

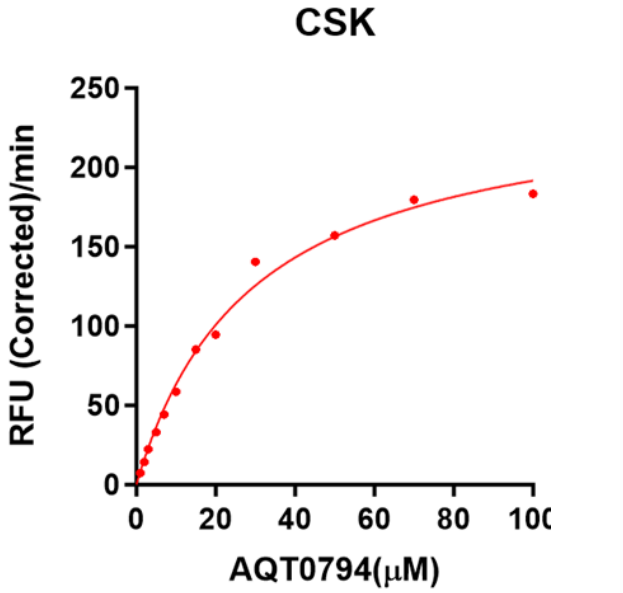
# Sensor Peptide $K_m$ Determination

## 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 | 247.5 |
| Km | 29.16 |
| Std. Error | |
| Vmax | 10.84 |
| Km | 3.028 |
| 95% CI (profile likelihood) | |
| Vmax | 225.8 to 273.1 |
| Km | 23.40 to 36.59 |
| Goodness of Fit | |
| Degrees of Freedom | 10 |
| R squared | 0.9913 |

## Sensor Peptide $K_m$ is 29 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 AQT0794

10 nM CSK

### 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 volumeafter 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.

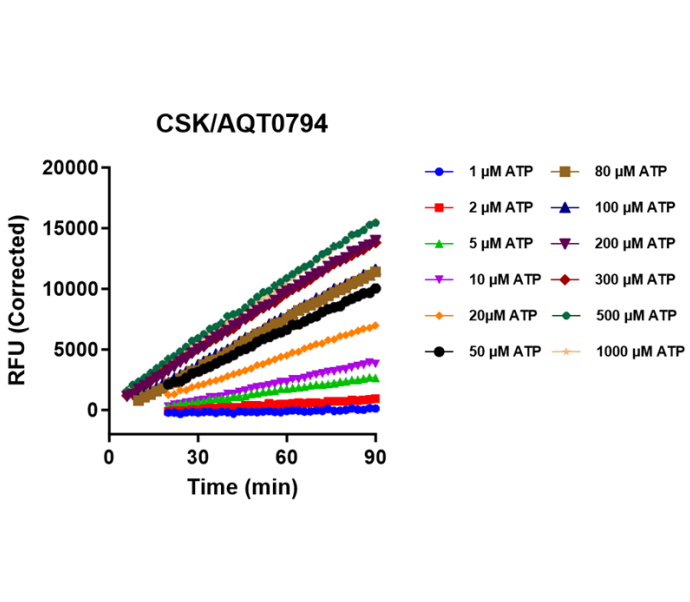
### Notes:

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

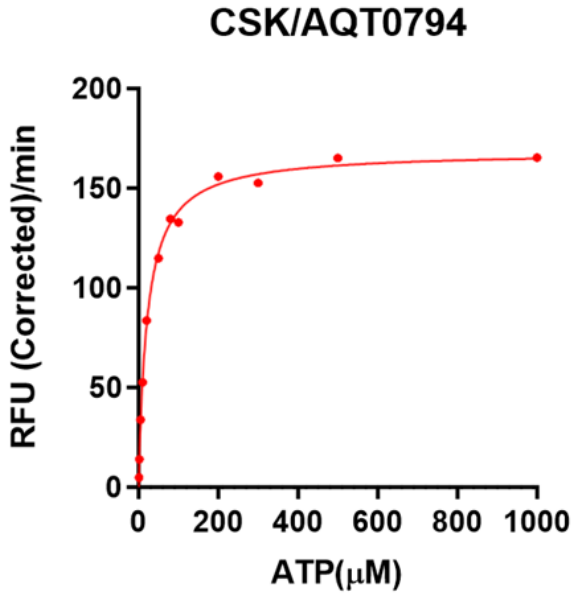
# ATP $K_m$ Determination

## 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 | 168.3 |
| Km | 21.71 |
| Std. Error | |
| Vmax | 1.945 |
| Km | 1.204 |
| 95% CI (profile likelihood) | |
| Vmax | 164.0 to 172.8 |
| Km | 19.15 to 24.58 |
| Goodness of Fit | |
| Degrees of Freedom | 10 |
| R squared | 0.9972 |

ATP $K_m$ is 22 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 AQT0794

5 nM CSK

**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 volumeafter 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.

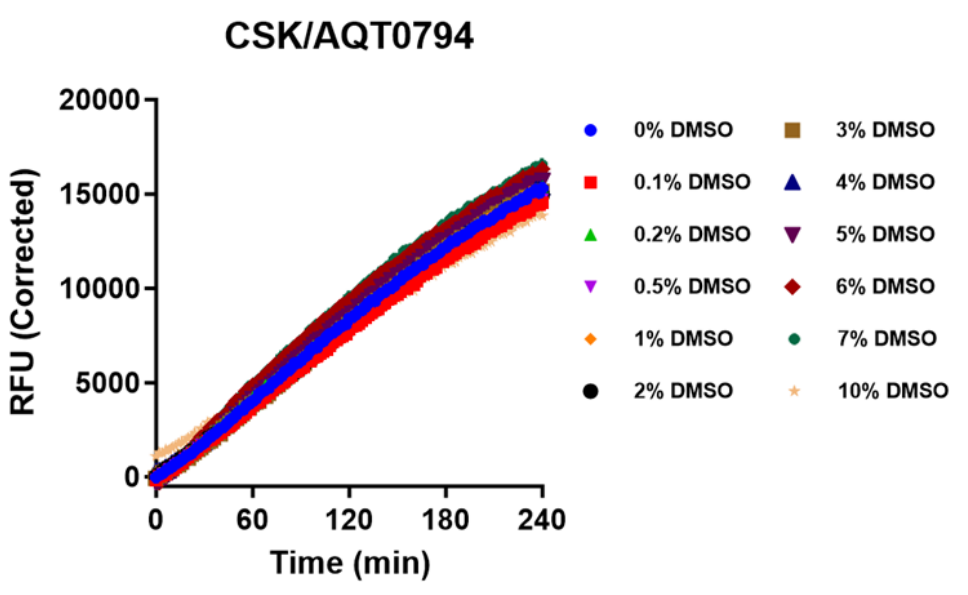
**Notes:**

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

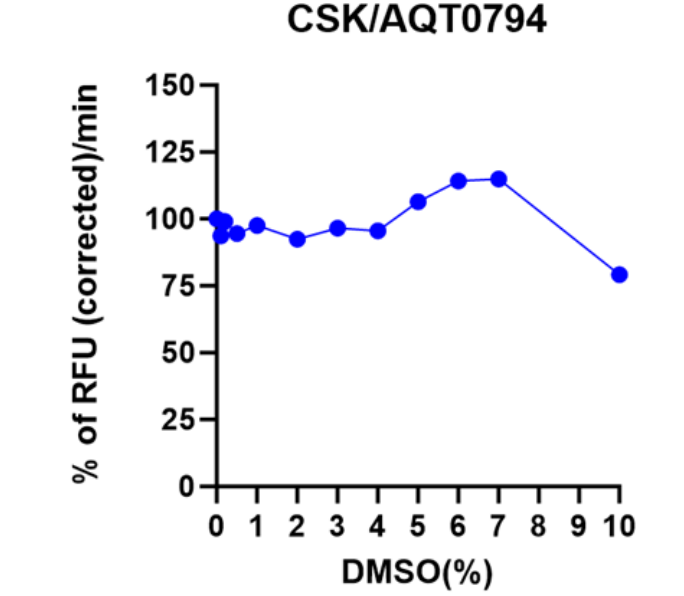
# DMSO Tolerance Test

## Titration Curves and Inhibition Plot

### Complete Progress Curves




### Reaction Rate vs [DMSO] Plot




No change in enzyme activity out to 4% 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 $MgCl_2$

1% DMSO

15 µM AQT0794

10 nM CSK

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 volumeafter 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).

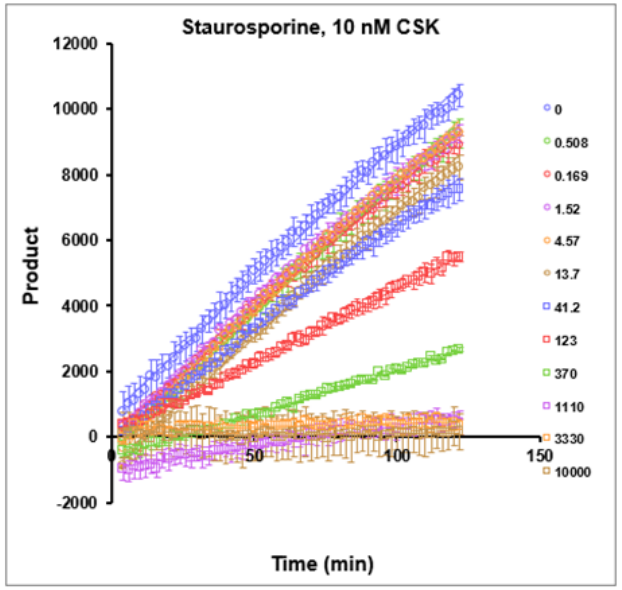
**Notes:**

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

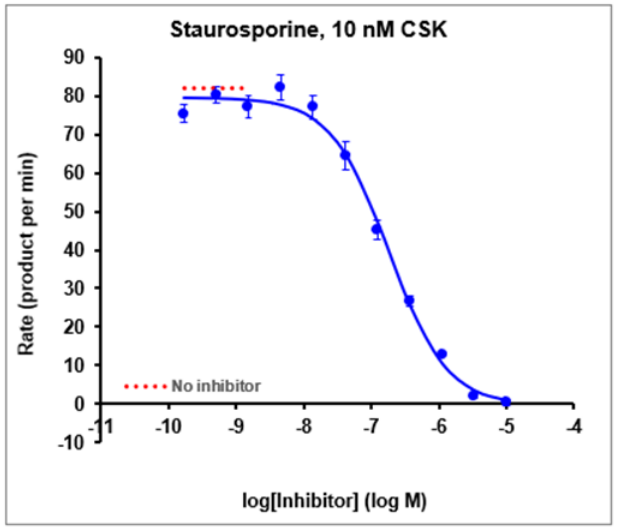
# $IC_{50}$ Determination

## Progress Curves and $IC_{50}$ Curves and Table

### Linear Region of Progress Curves




### $IC_{50}$ Curve




### $IC_{50}$ Table

| Parameter | Value |
|---|---|
| Bottom | -0.6 |
| Top | 79.8 |
| log IC50 | -6.74 |
| IC50 (nM) | 182.50 |
| Ki (nM) | 91.25 |
| Slope | -1.010 |
| R squared | 0.995 |
| IC50 approx SE (nM) | 8.62 |
| 50% inhibition (nM) | 179.91 |

The Y-axis label is RFU/min.

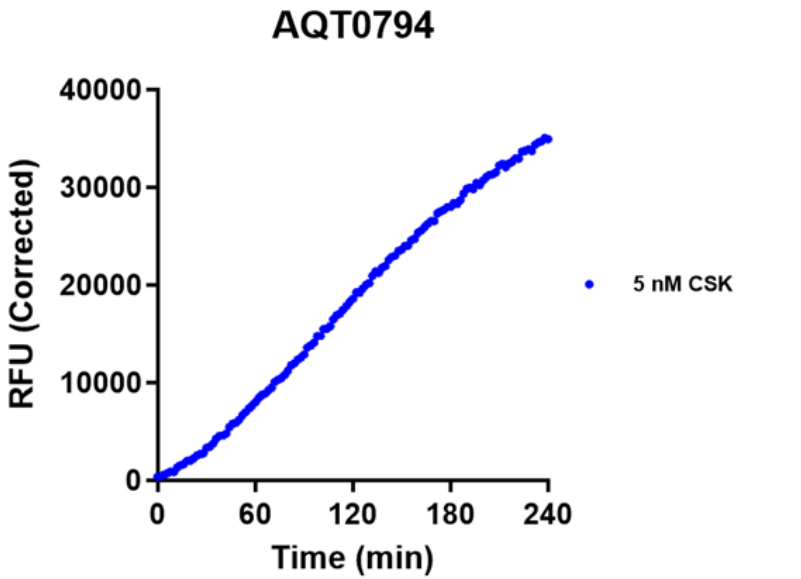
## Staurosporine $IC_{50}$ Determination at ATP $K_m$ is 183 nM

# Summary

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

| Experiment | Result |
|---|---|
| Enzyme Titration Linear Range | 0.00-1.25 nM |
| Sensor Peptide $K_m$ Value | 29 µM |
| ATP $K_m$ Value | 22 µM |
| DMSO Tolerance (highest % recommended) | 4% |
| Staurosporine $IC_{50}$ Determination at ATP $K_m$ | 183 nM |

## Progress Curve




| Kinase Name | Conc. (nM) | Sox-based Substrate Name | Normalized Reaction Rate (RFU/pmole/min) | Normalized Rate StndError (RFU/pmole/min) |
|---|---|---|---|---|
| CSK | 5 | AQT0794 | 3,454 | 14 |

| 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