# AQT0794 – LCK Assay Validation

*PhosphoSens*®-Kinetic Assay Format

# Outline for this Study

## PhosphoSens-Kinetic Assay Validation

AssayQuant TECHNOLOGIES INC.

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

Carna LCK (Cat#/Lot#: 08-170/20CBS-0111F) amino acids full length; N-terminal GST tag

### Reference Compound Information:

Staurosporine MedChemExpress (Cat. HY-15141)

### 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

0.02, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, and 5 nM LCK

### 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.

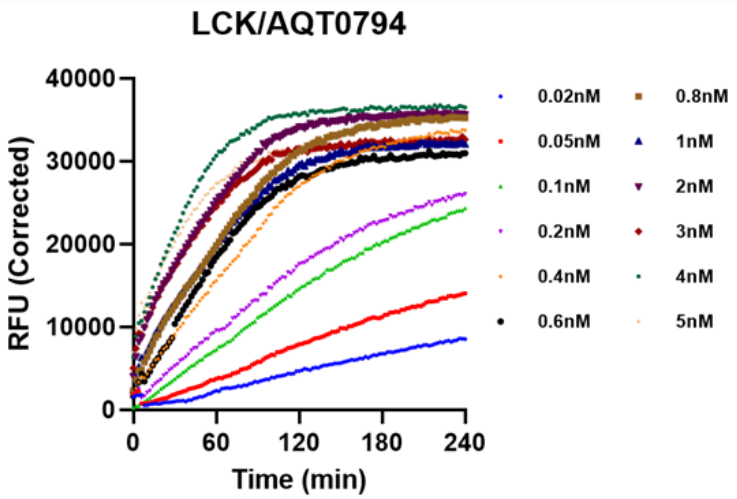
### Notes:

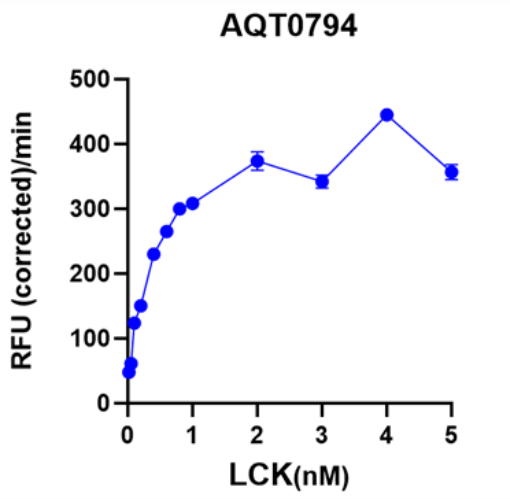
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

# Enzyme Titration

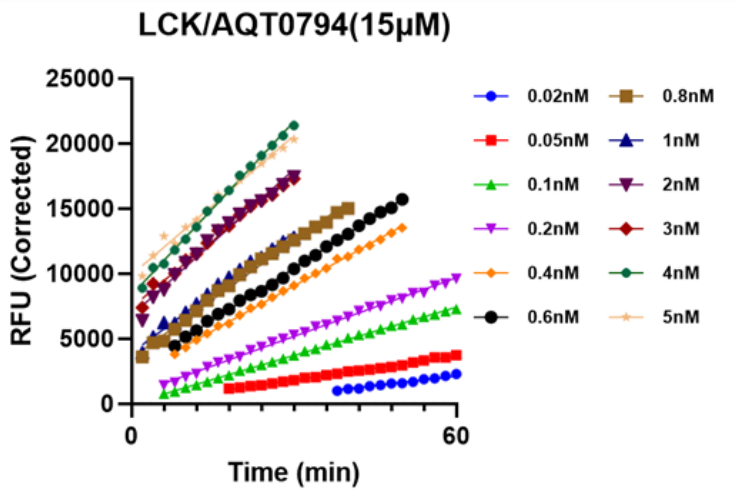
## Progress Curves

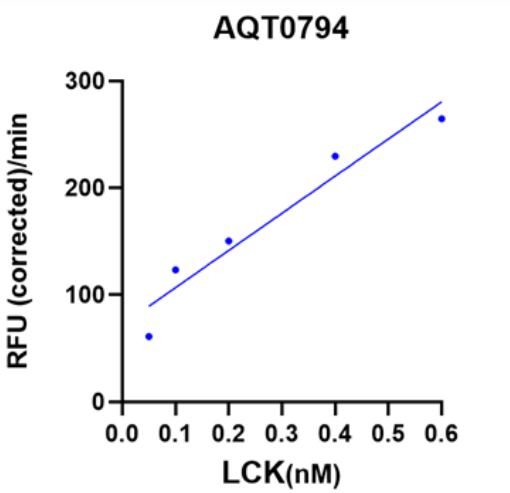
Complete Progress Curves




Linear Region of Progress Curves




Linear Range

# Enzyme Titration

## Reaction Rate Table

| Enzyme Conc. (nM) | Normalized Reaction Rate (RFU/pmole/min) | Normalized Rate Stnd Error (RFU/pmole/min) |
|---|---|---|
| 0.02 | 76,125 | 4,783 |
| 0.05 | 57,540 | 1,021 |
| 0.1 | 61,450 | 348 |
| 0.2 | 35,750 | 788 |
| 0.4 | 28,025 | 199 |
| 0.6 | 23,533 | 370 |
| 0.8 | 16,913 | 282 |
| 1 | 12,860 | 234 |
| 2 | 8,468 | 267 |
| 3 | 5,695 | 169 |
| 4 | 5,559 | 89 |
| 5 | 3,566 | 118 |

The reaction is linear from 0.1 - 0.4 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

2 nM LCK

**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.

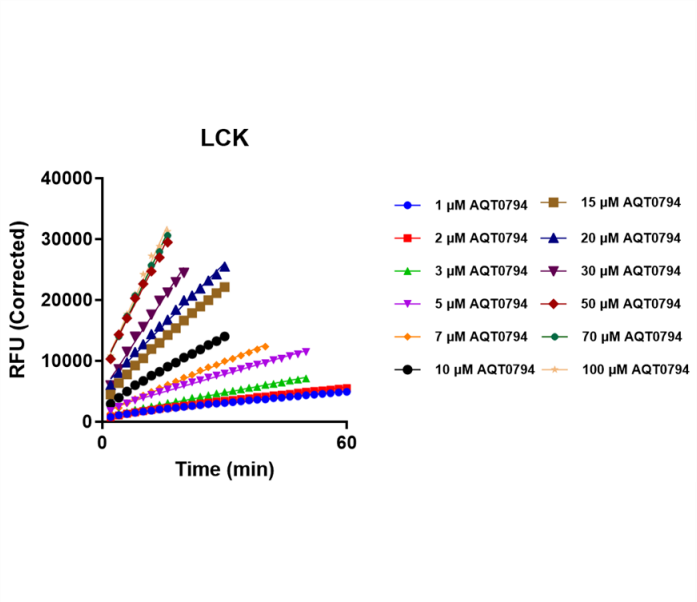
**Notes:**

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.

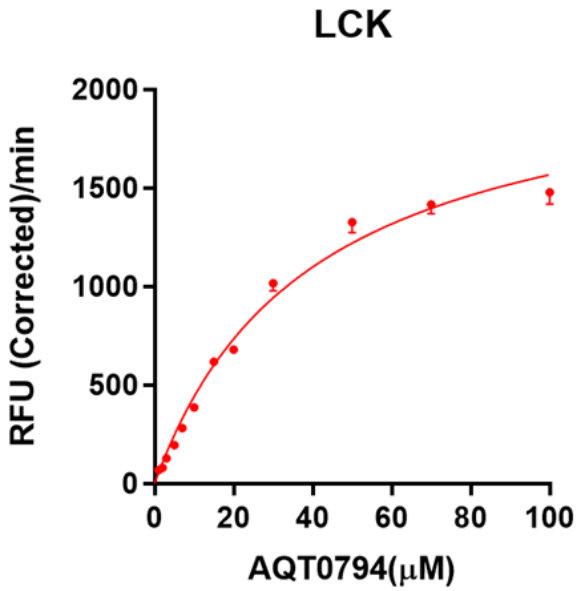
# Sensor Peptide $K_m$ Determination

## Titration Curves and $K_m$ Plot and Table

### Sensor Peptide Titration Curves




### Sensor Peptide $K_m$ Plot




### Sensor Peptide $K_m$ Table

| | |
|---|---|
| Michaelis-Menten | |
| Best-fit values | |
| Vmax | 2190 |
| Km | 39.78 |
| Std. Error | |
| Vmax | 137.0 |
| Km | 5.355 |
| 95% CI (profile likelihood) | |
| Vmax | 1931 to 2523 |
| Km | 30.19 to 53.32 |
| Goodness of Fit | |
| Degrees of Freedom | 10 |
| R squared | 0.9883 |

## Sensor Peptide $K_m$ is 40 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

2 nM LCK

### 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.

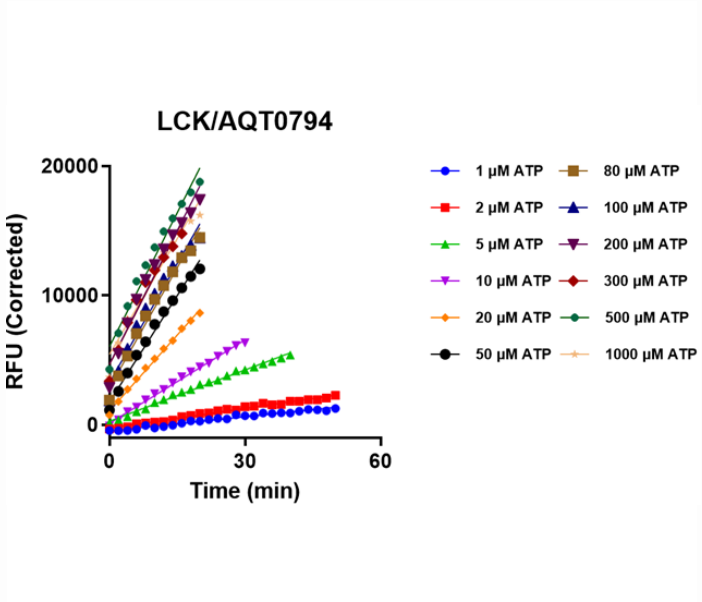
### Notes:

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.

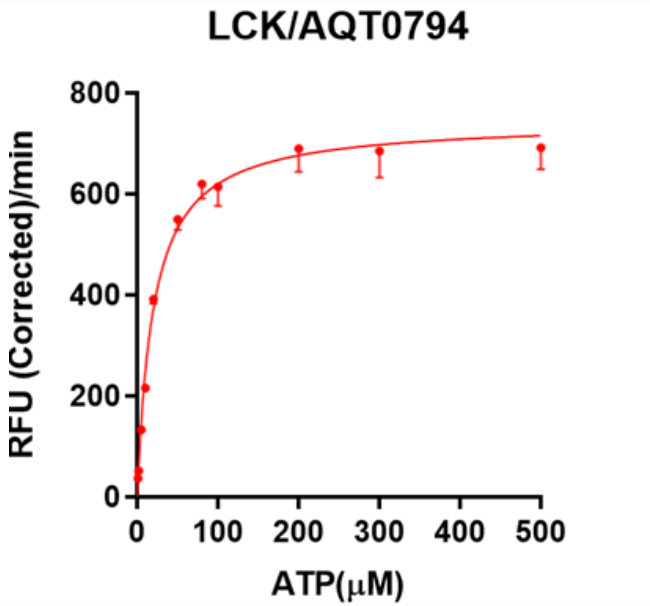
# ATP $K_m$ Determination

## Titration Curves and $K_m$ Plot and Table

### ATP Titration Curves




### ATP $K_m$ Plot




### ATP $K_m$ Table

| | |
|---|---|
| Michaelis-Menten | |
| Best-fit values | |
| Vmax | 743.2 |
| Km | 20.01 |
| Std. Error | |
| Vmax | 13.61 |
| Km | 1.638 |
| 95% CI (profile likelihood) | |
| Vmax | 713.7 to 773.9 |
| Km | 16.75 to 23.88 |
| Goodness of Fit | |
| Degrees of Freedom | 9 |
| R squared | 0.9947 |

ATP $K_m$ is 20 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

2 nM LCK

### 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.

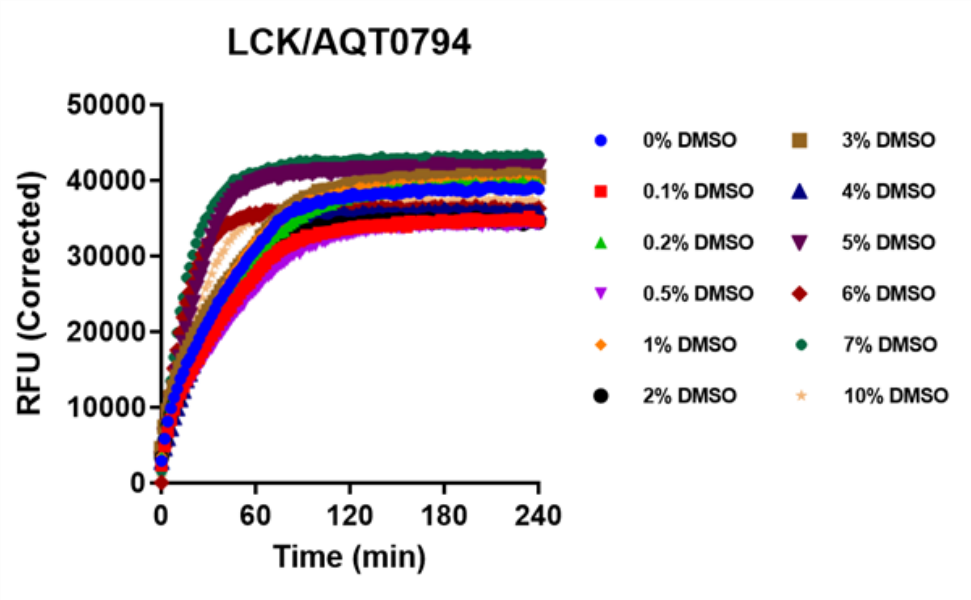
### Notes:

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.

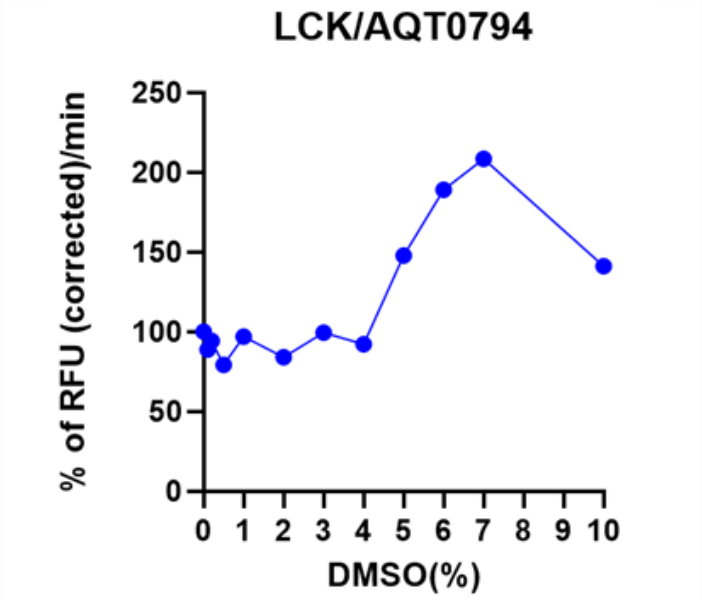
# DMSO Tolerance Test

## Titration Curves and Inhibition Plot

### Complete Progress Curves




### Reaction Rate vs [DMSO] Plot




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

2 nM LCK

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).

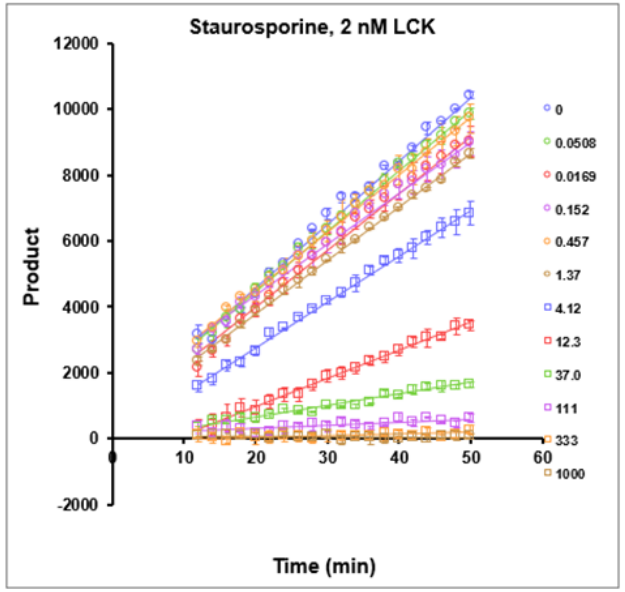
**Notes:**

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.

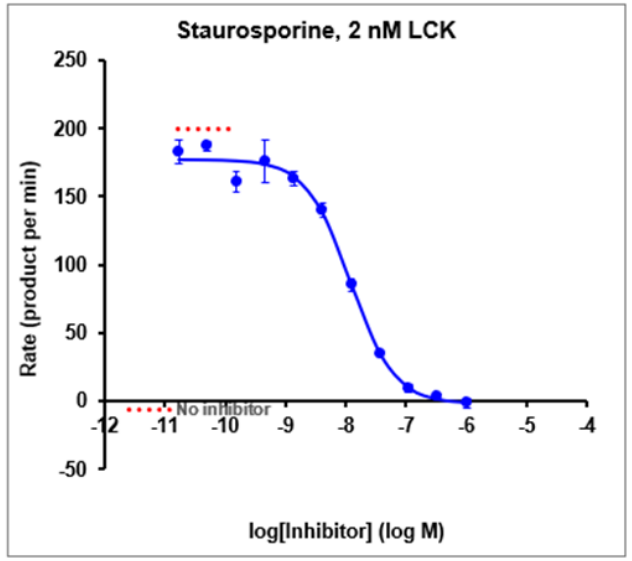
# $IC_{50}$ Determination

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

### Linear Region of Progress Curves




### $IC_{50}$ Curve




### $IC_{50}$ Table

| Parameter | Value |
|---|---|
| Bottom | -1.7 |
| Top | 177.6 |
| log IC50 | -7.93 |
| IC50 (nM) | 11.76 |
| Ki (nM) | 5.88 |
| Slope | -1.197 |
| R squared | 0.994 |
| IC50 approx SE (nM) | 0.52 |
| 50% inhibition (nM) | 11.57 |

The Y-axis label is RFU/min.

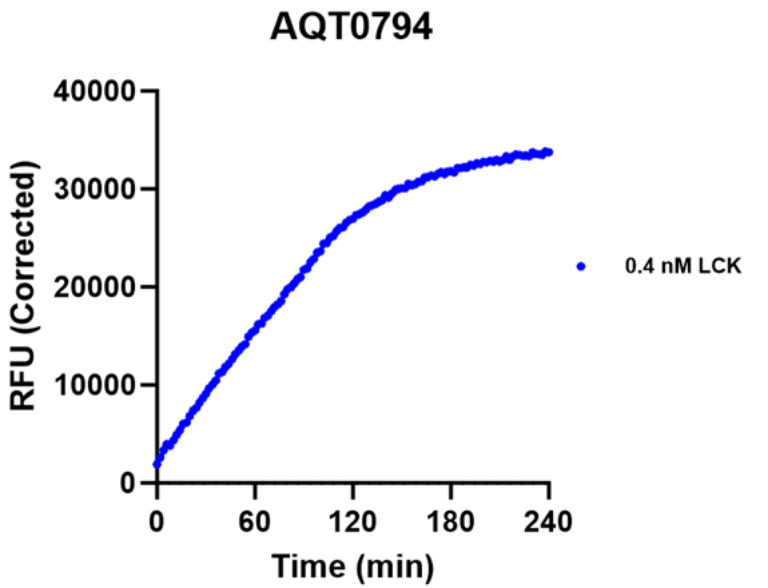
## Staurosporine $IC_{50}$ at ATP $K_m$ is 11.8 nM

# Summary

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

| Experiment | Result |
|---|---|
| Enzyme Titration Linear Range | 0.1 - 0.4 nM |
| Sensor Peptide $K_m$ Value | 40 µM |
| ATP $K_m$ Value | 20 µM |
| DMSO Tolerance (highest % recommended) | 4 |
| Staurosporine $IC_{50}$ Determination at ATP $K_m$ | 11.8 nM |

## Progress Curve




| Kinase Name | Conc. (nM) | Sox-based Substrate Name | Normalized Reaction Rate (RFU/pmole/mi | Normalized Rate StndError (RFU/pmole/mi |
|---|---|---|---|---|
| LCK | 0.4 | AQT0794 | 28,025 | 199 |

| 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