# AQT0794 – TXK Assay Validation

*PhosphoSens*®-Kinetic Assay Format

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# Outline for this Study

## PhosphoSens-Kinetic Assay Validation

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

Carna TXK (Cat#/Lot#: 08-183/13CBS-0602E) amino acids 260-527 (end); N-terminal GST tag

**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 $K_m$

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# 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

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

### 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.
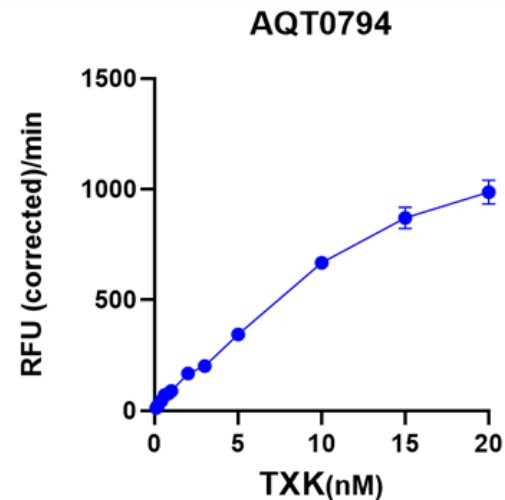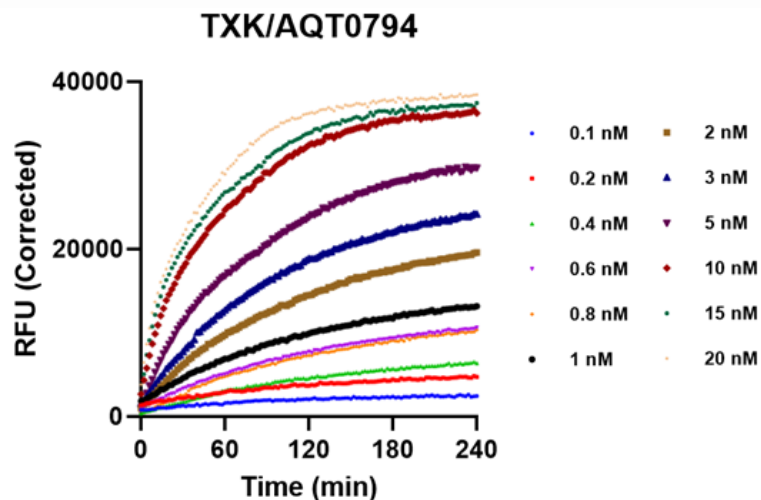
### Notes:

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.
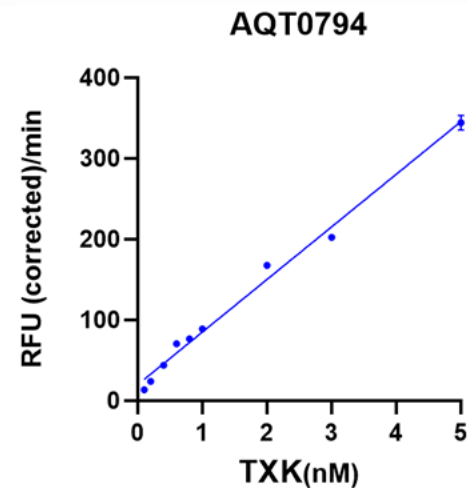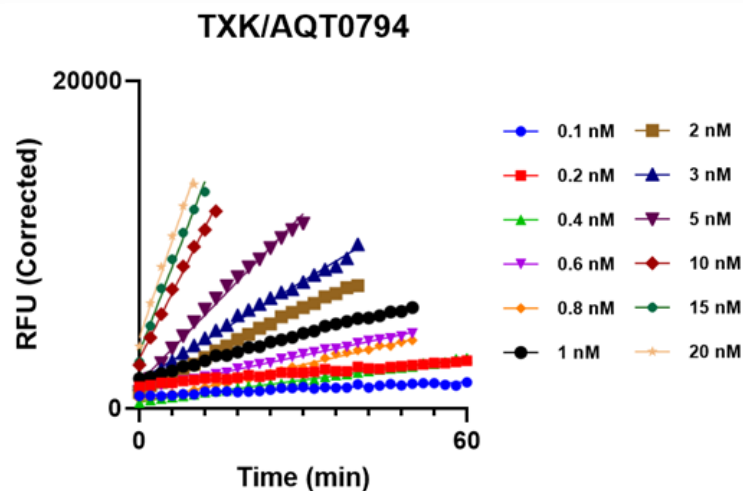
# Enzyme Titration

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) |
|---|---|---|
| 0.1 | 6,775 | 357 |
| 0.2 | 5,985 | 172 |
| 0.4 | 5,484 | 46 |
| 0.6 | 5,870 | 63 |
| 0.8 | 4,788 | 86 |
| 1 | 4,446 | 59 |
| 2 | 4,190 | 50 |
| 3 | 3,368 | 58 |
| 5 | 3,440 | 91 |
| 10 | 3,343 | 107 |
| 15 | 2,901 | 159 |
| 20 | 2,468 | 134 |

## The reaction is linear from 0.4 - 5 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 TXK

### 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.
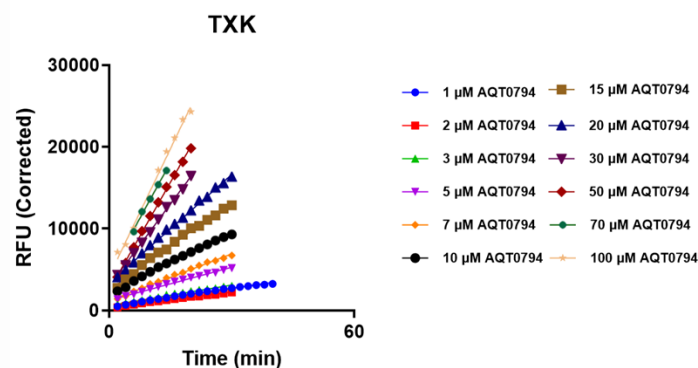
### Notes:

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.
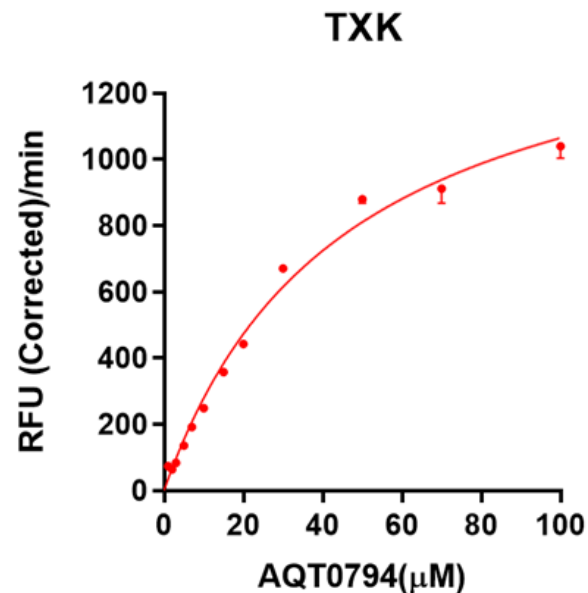
# Sensor Peptide K$_m$ Determination

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 | 1551 |
|   Km | 45.72 |
| Std. Error | |
|   Vmax | 95.98 |
|   Km | 5.824 |
| 95% CI (profile likelihood) | |
|   Vmax | 1368 to 1789 |
|   Km | 35.08 to 60.69 |
| Goodness of Fit | |
|   Degrees of Freedom | 10 |
|   R squared | 0.9902 |

## Sensor Peptide K$_m$ is 46 µM

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# 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

5 nM TXK

### 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.
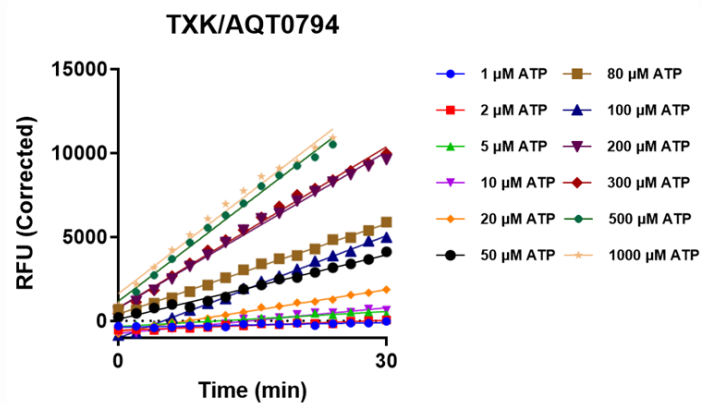
### Notes:

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.
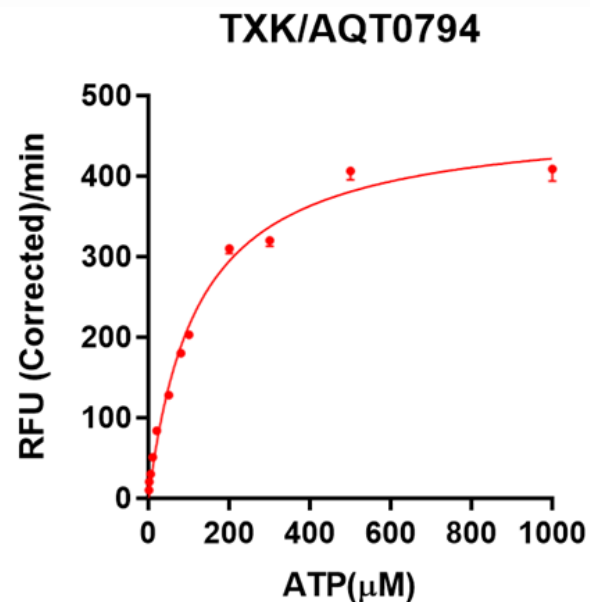
# ATP K$_m$ Determination

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 | 472.6 |
| Km | 121.2 |
| Std. Error | |
| Vmax | 17.59 |
| Km | 13.50 |
| 95% CI (profile likelihood) | |
| Vmax | 435.2 to 514.2 |
| Km | 94.17 to 155.3 |
| Goodness of Fit | |
| Degrees of Freedom | 10 |
| R squared | 0.9900 |

## ATP K$_m$ is 121 µM

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# 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 TXK

### 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.
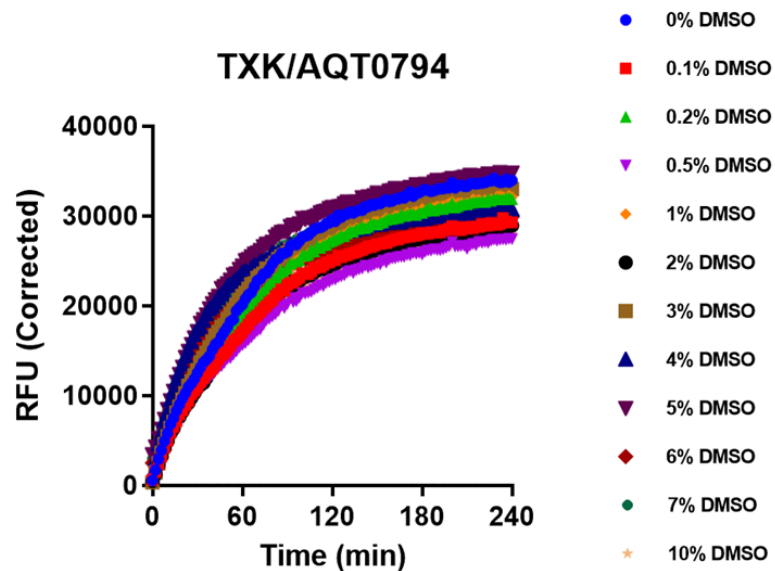
### Notes:

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.
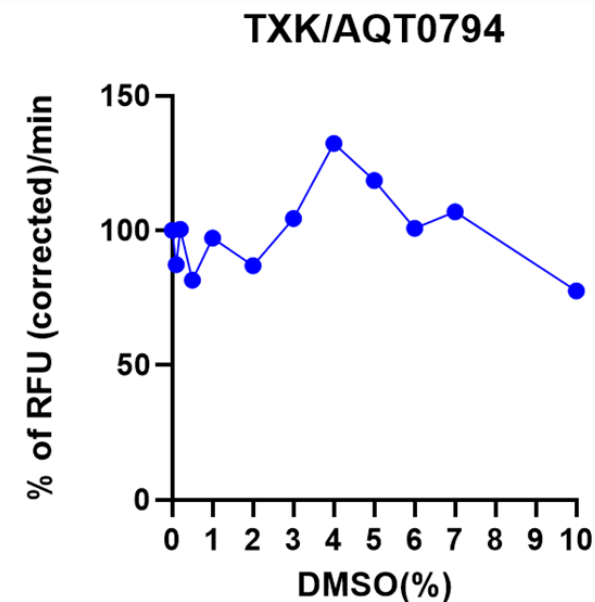
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# DMSO Tolerance Test

## Titration Curves and Inhibition Plot



No change in enzyme activity out to 2% 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

5 nM TXK

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).
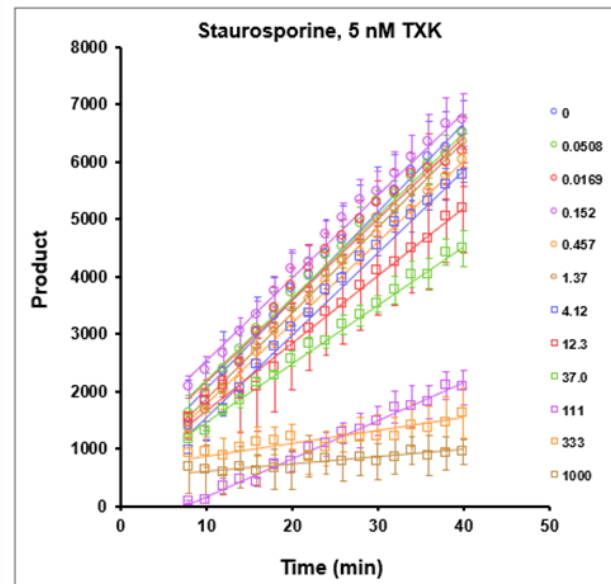
### Notes:

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.
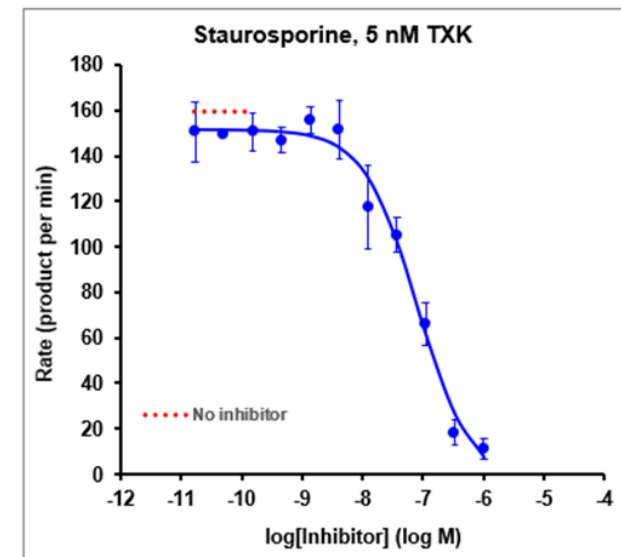
# IC$_{50}$ Determination

Progress Curves and IC$_{50}$ Curves and Table

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## Linear Region of Progress Curves



## IC$_{50}$ Curve



The Y-axis label is RFU/min.

## IC$_{50}$ Table

| Parameter | Value |
|---|---|
| Bottom | -4.5 |
| Top | 151.4 |
| log IC50 | -7.10 |
| IC50 (nM) | 79.83 |
| Ki (nM) | 39.91 |
| Slope | -0.986 |
| R squared | 0.986 |
| IC50 approx SE (nM) | 0.63 |
| 50% inhibition (nM) | 75.33 |

## Staurosporine IC$_{50}$ at ATP K$_m$ is 80 nM

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# Summary

| Experiment | Result |
|---|---|
| Enzyme Titration Linear Range | 0.4 - 5 nM |
| Sensor Peptide $K_m$ Value | 46 µM |
| ATP $K_m$ Value | 121 µM |
| DMSO Tolerance (highest % recommended) | 2 |
| Staurosporine $IC_{50}$ Determination at ATP $K_m$ | 80 nM |

## Progress Curve



AQT0794 — 3 nM TXK
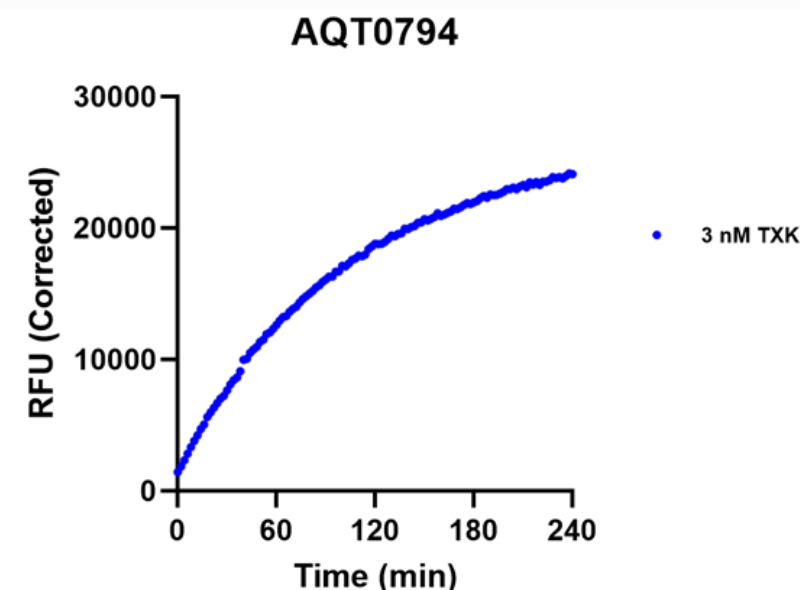
| Sox-based Substrate Name | Conc. (nM) | Sox-based Substrate Name | Normalized Reaction Rate (RFU/pmole/min) | Normalized Rate StndError (RFU/pmole/min) |
|---|---|---|---|---|
| TXK | 3 | AQT0794 | 3,368 | 58 |

**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