AQT0794 – FGFR3 Assay Validation

PhosphoSens®-Kinetic Assay Format

Outline for this Study

PhosphoSens-Kinetic Assay Validation

AssayQuant TECHNOLOGIES INC.

Enzyme Source, Construct, and Lot Information:

FGFR3 (Cat#/Lot#: 08-135/12CBS-0744V) amino acids 436-806(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

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 Carna FGFR3

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:

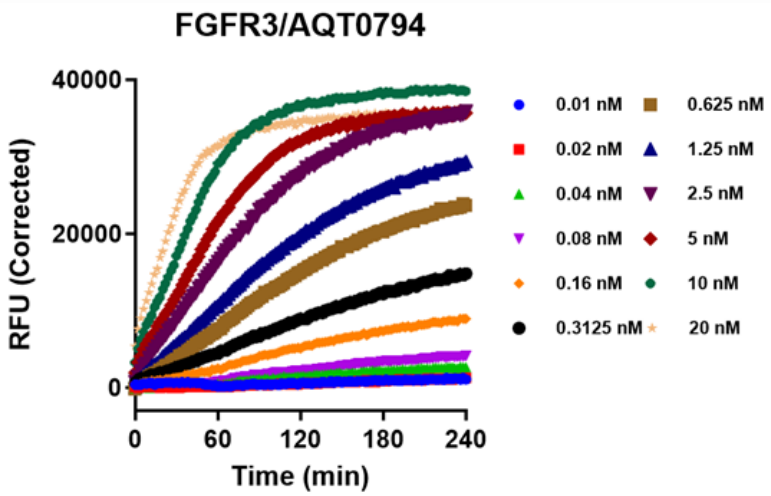
Enzyme Dilution Buffer (EDB):20 mMHEPES, pH 7.5,0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine SerumAlbumin.

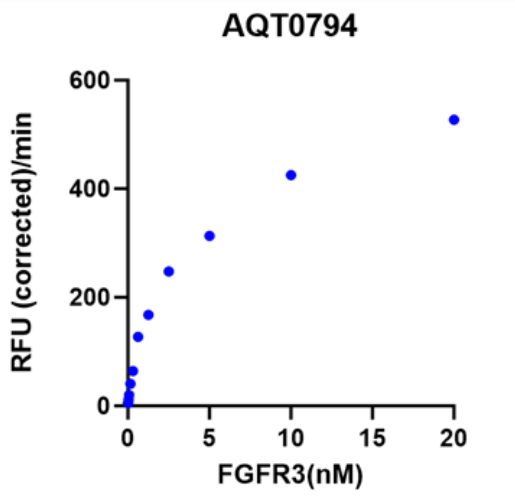
Enzyme Titration

Progress Curves

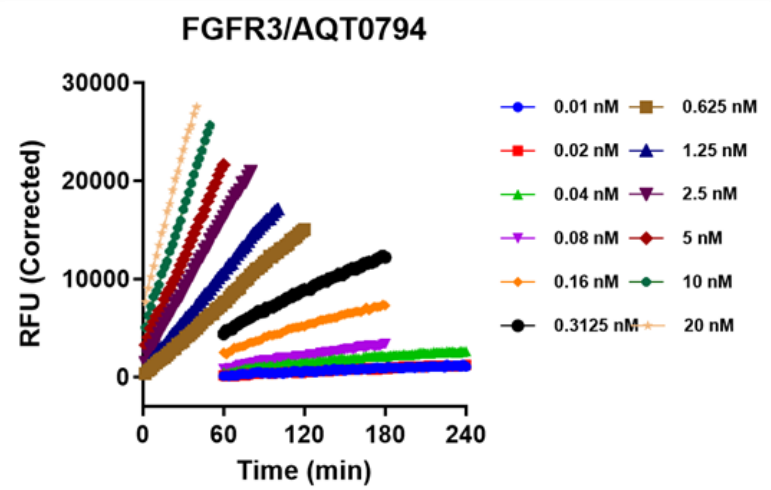
AssayQuant TECHNOLOGIES INC.

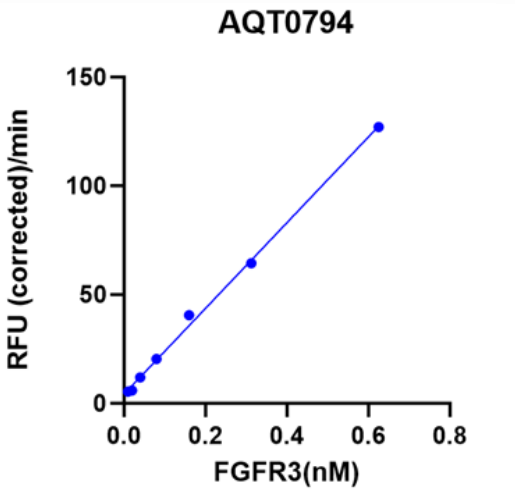
Complete Progress Curves





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	27,335	684
0.02	14,893	277
0.04	29,775	559
0.08	25,538	426
0.16	25,363	336
0.3125	20,147	181
0.625	20,320	81
1.25	13,416	44
2.5	9,912	45
5	6,266	38
10	4,253	25
20	2,636	31

The reaction is linear from 0.04 - 0.31 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

1 nM Carna FGFR3

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.

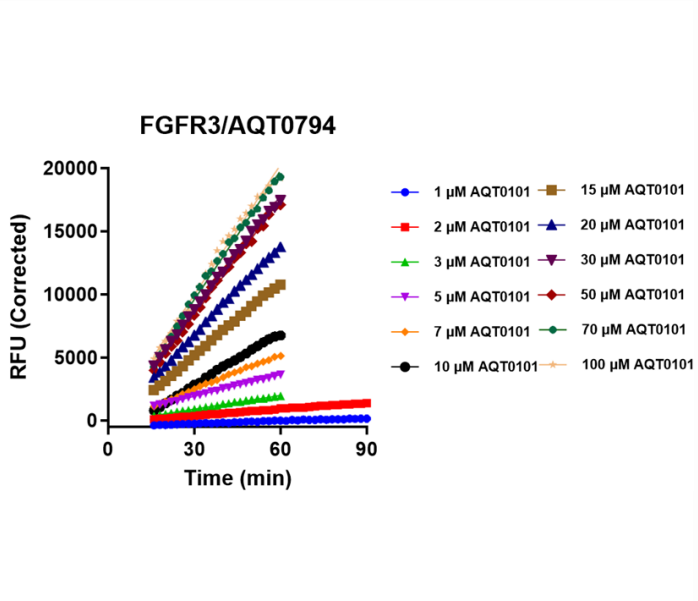
Notes:

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.

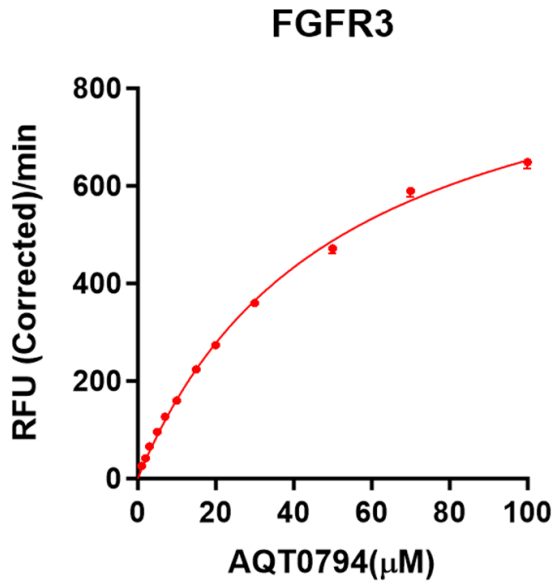
Sensor Peptide K_m Determination

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	986.0
Km	51.18
Std. Error	
Vmax	28.82
Km	2.971
95% CI (profile likelihood)	
Vmax	925.2 to 1056
Km	45.00 to 58.47
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9981

Sensor Peptide K_m is 51 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

1.5 nM Carna FGFR3

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.

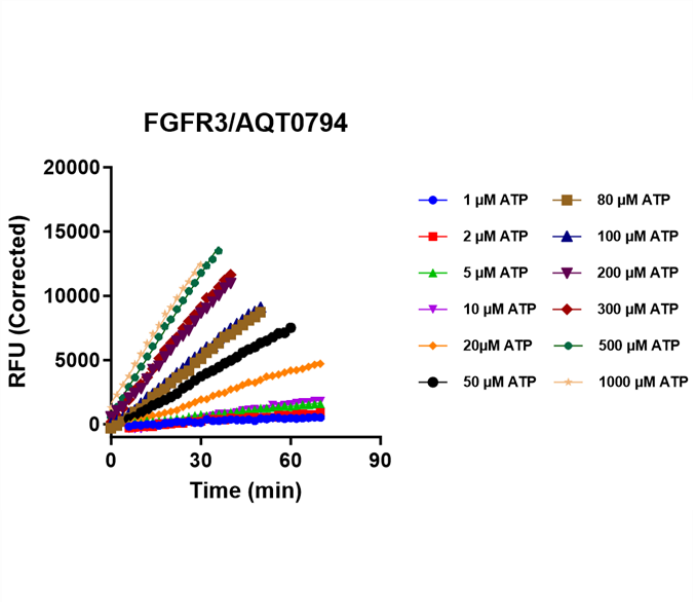
Notes:

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.

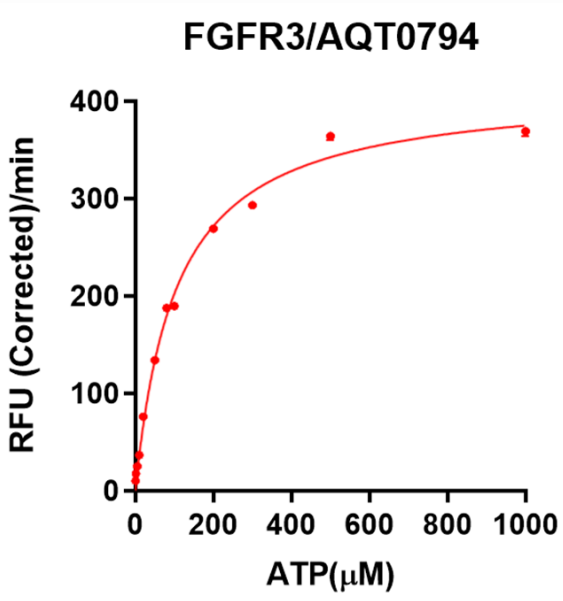
ATP K_m Determination

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	414.1
Km	104.8
Std. Error	
Vmax	11.41
Km	9.009
95% CI (profile likelihood)	
Vmax	389.3 to 441.0
Km	86.05 to 127.2
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9940

ATP K_m is 105 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

1 nM Carna FGFR3

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.

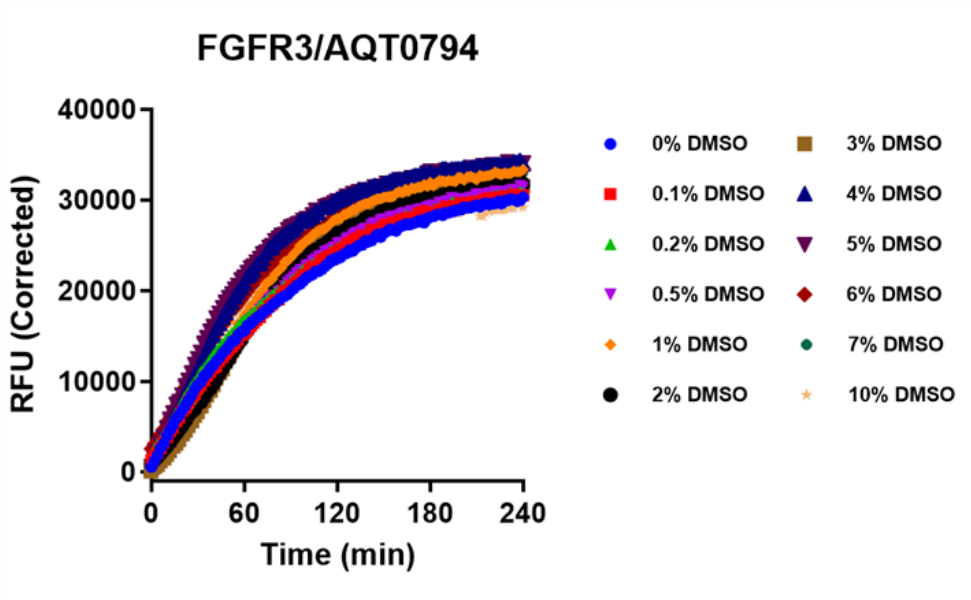
Notes:

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.

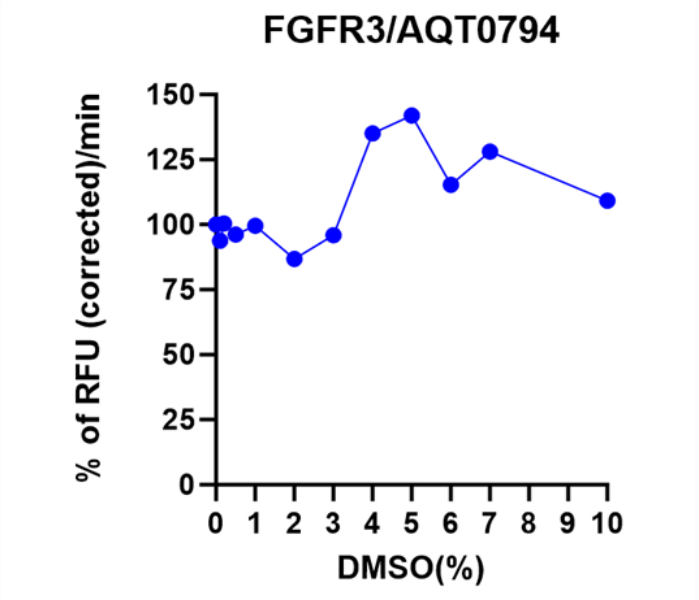
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No change in enzyme activity out to 1% 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 Carna FGFR3

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 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).

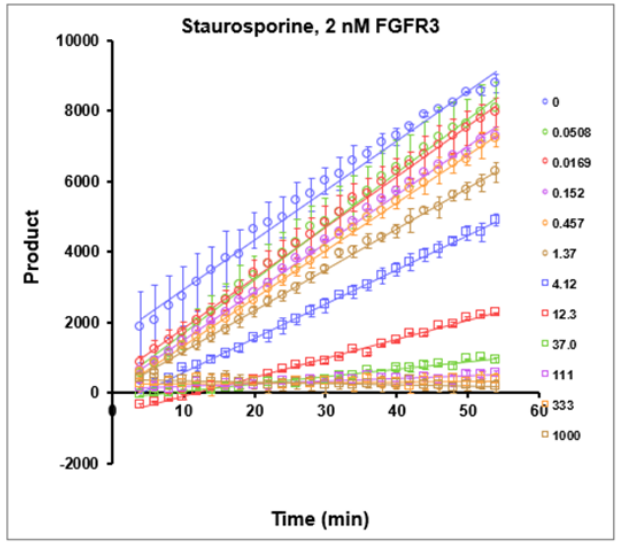
Notes:

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.

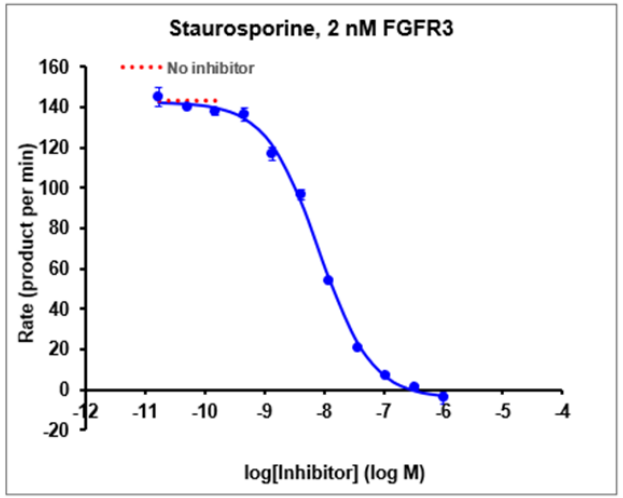
IC_{50} Determination

Progress Curves and IC_{50} Curves and Table

Linear Region of Progress Curves



IC_{50} Curve



IC_{50} Table

Parameter	Value
Bottom	-4.2
Top	142.6
log IC50	-8.09
IC50 (nM)	8.12
Ki (nM)	4.06
Slope	-0.985
R squared	0.998
IC50 approx SE (nM)	0.00
50% inhibition (nM)	7.66

The Y-axis label is RFU/min.

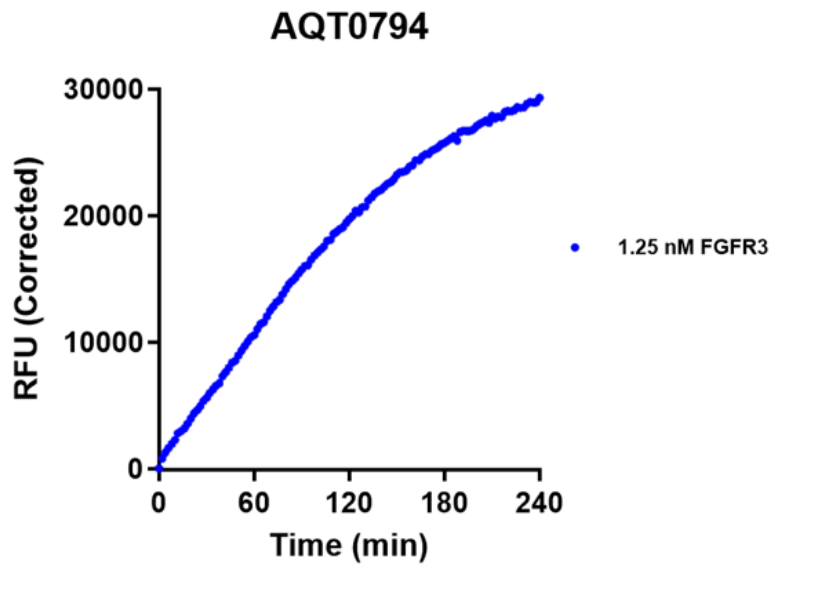
Staurosporine IC_{50} Determination at ATP K_m is 8.1 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.04 - 0.31 nM
Sensor Peptide K_m Value	51 µM
ATP K_m Value	105 µM
DMSO Tolerance (highest % recommended)	1
Staurosporine IC50 Determination at ATP Km is	8.1 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate StndError (RFU/pmole/min)	Maximum Signal:Bkgd (S/B) Kinetic
FGFR3	1.25	AQT0794	13,416	44	2.8

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