

AQT0794 – FGFR4 Assay Validation

PhosphoSens[®]-Kinetic Assay Format

Outline for this Study

PhosphoSens–Kinetic Assay Validation

Enzyme Source, Construct, and Lot Information:

FGFR4 (Cat#/Lot#: 08-136/12CBS-0076M) amino acids 460-802(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 MgCl₂

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 FGFR4

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.

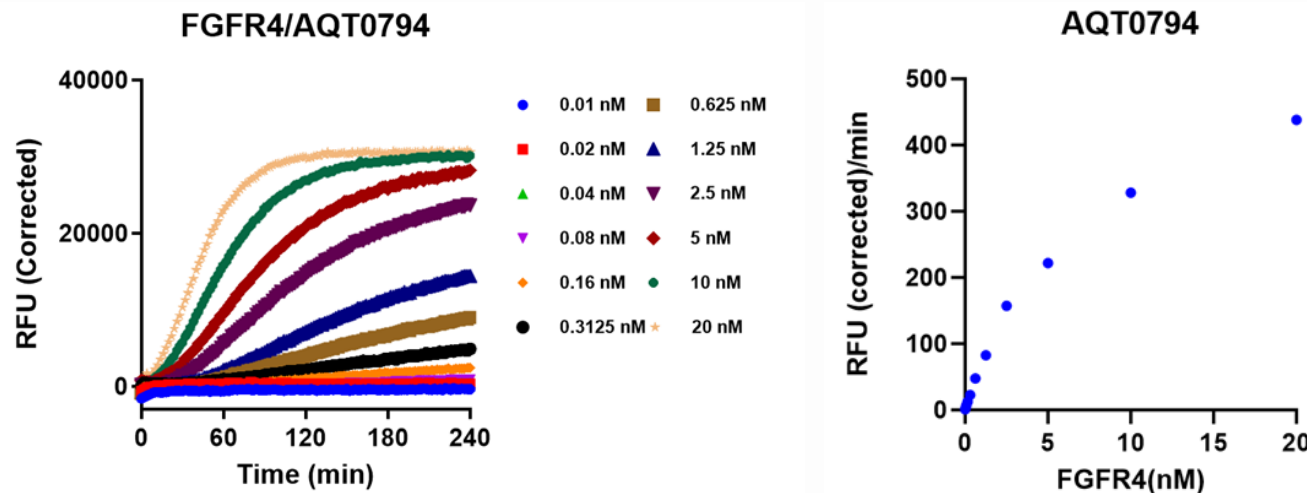
Notes:

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.

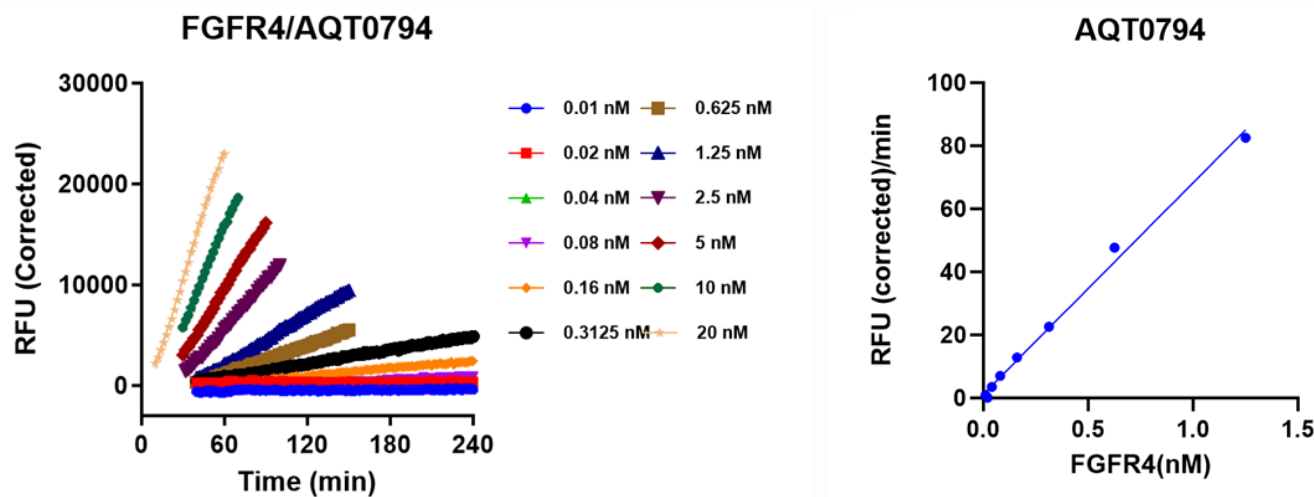
Enzyme Titration

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 Std Error (RFU/pmole/min)
0.01	4,629	554
0.02	215	258
0.04	8,785	242
0.08	8,771	107
0.16	8,044	69
0.3125	7,059	34
0.625	7,629	40
1.25	6,605	36
2.5	6,392	37
5	4,436	23
10	3,281	33
20	2,190	20

The reaction is linear from 0.04 – 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

2.5 nM Carna FGFR4

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.

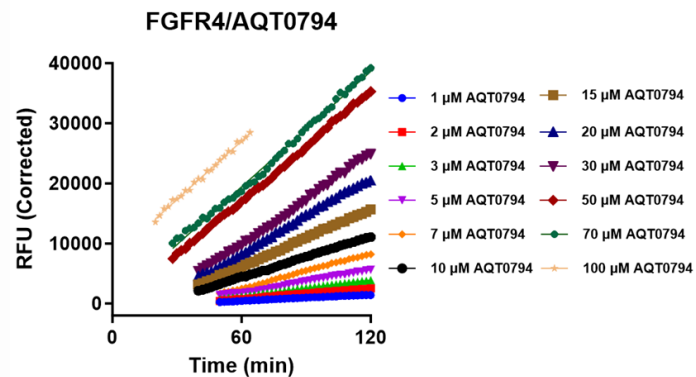
Notes:

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.

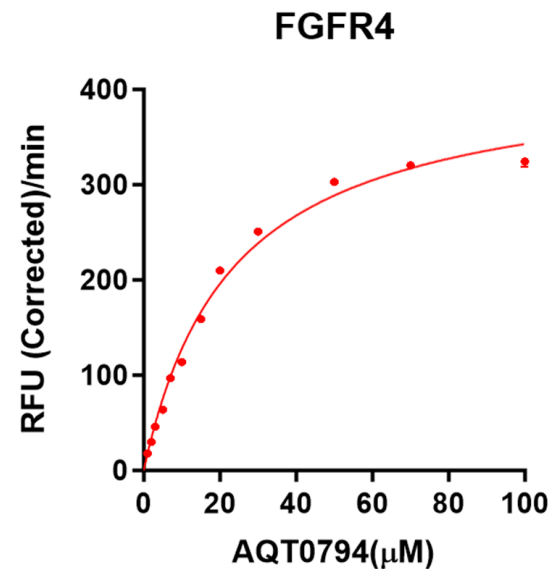
Sensor Peptide K_m Determination

Titration Curves and K_m Plot and Table

Sensor Peptide Titration Curves



Sensor Peptide K_m Plot



Sensor Peptide K_m Table

Michaelis-Menten	
Best-fit values	
Vmax	421.6
Km	23.05
Std. Error	
Vmax	16.12
Km	2.243
95% CI (profile likelihood)	
Vmax	389.0 to 458.8
Km	18.76 to 28.43
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9912

Sensor Peptide K_m is 23 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 AQT0794

5 nM Carna FGFR4

Reaction Set Up:

2 or 2.5 μ L

10x Sensor Peptide

14 or 17.5 μ L

Reaction Mix with ATP & DTT

4 or 5 μ L

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.

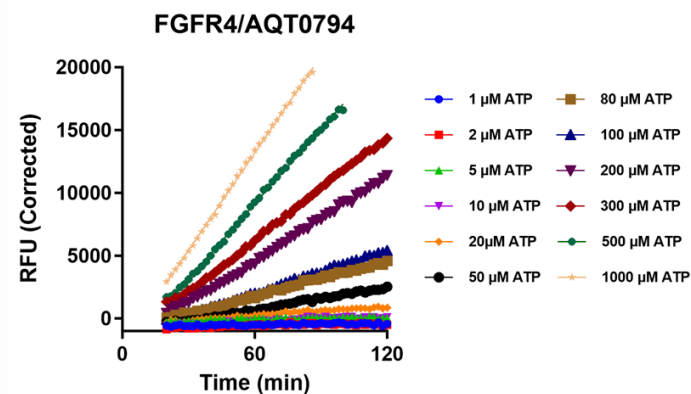
Notes:

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.

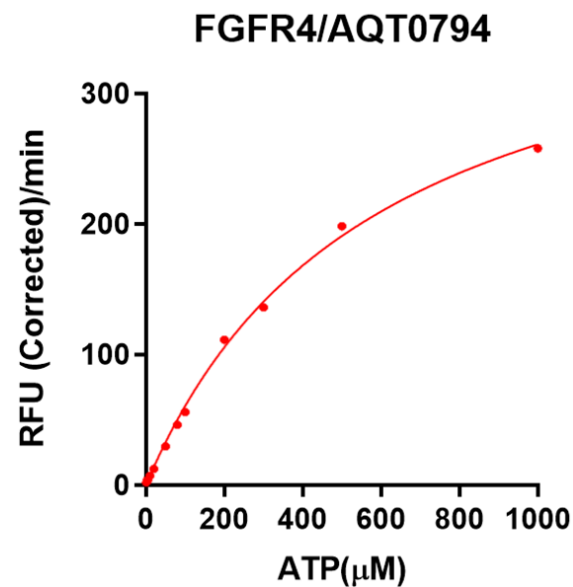
ATP K_m Determination

Titration Curves and K_m Plot and Table

ATP Titration Curves



ATP K_m Plot



ATP K_m Table

Michaelis-Menten	
Best-fit values	
Vmax	413.2
Km	582.9
Std. Error	
Vmax	15.19
Km	40.36
95% CI (profile likelihood)	
Vmax	382.3 to 449.2
Km	502.5 to 680.1
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9980

ATP K_m is 538 μ M

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

15 μM AQT0794

2.5 nM Carna FGFR4

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.

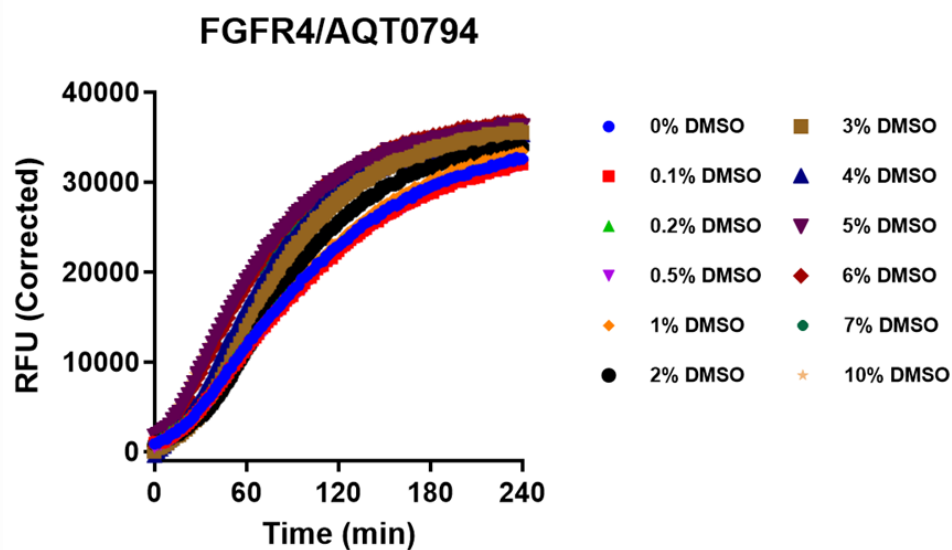
Notes:

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.

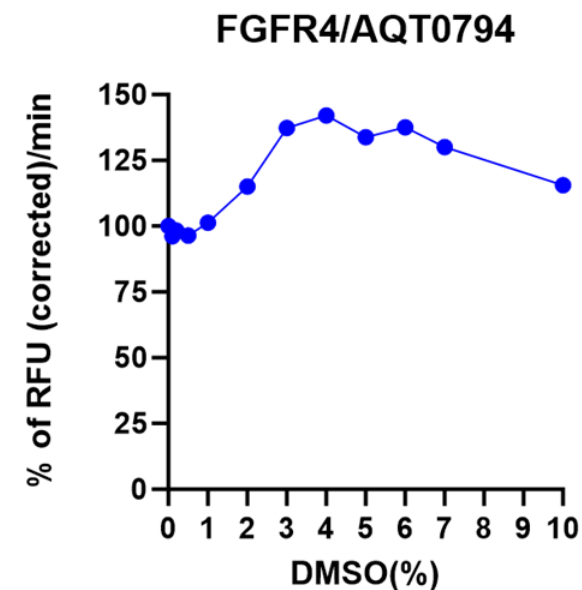
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No change in enzyme activity out to 2% 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

1% DMSO

15 μM AQT0794

5 nM Carna FGFR4

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 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).

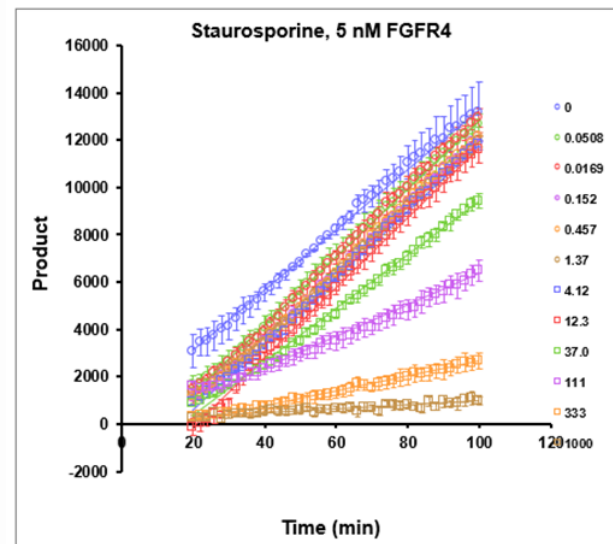
Notes:

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.

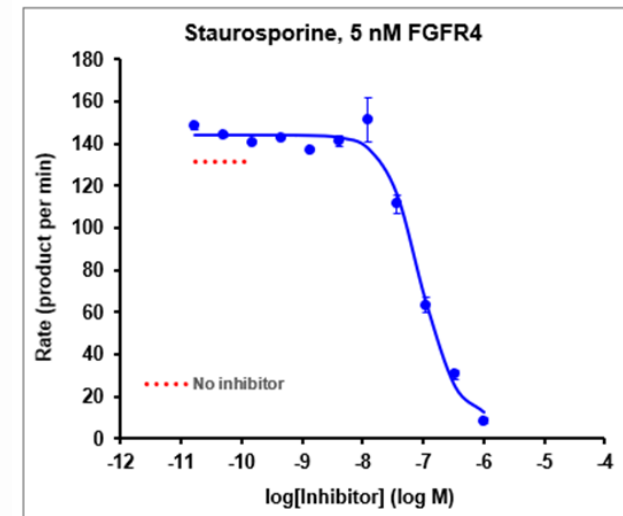
IC₅₀ Determination

Progress Curves and IC₅₀ Curves and Table

Linear Region of Progress Curves



IC₅₀ Curve



IC₅₀ Table

Parameter	Value
Bottom	9.2
Top	144.1
log IC50	-7.06
IC50 (nM)	87.72
Ki (nM)	43.86
Slope	-1.532
R squared	0.988
IC50 approx SE (nM)	4.39
50% inhibition (nM)	95.86

The Y-axis label is RFU/min.

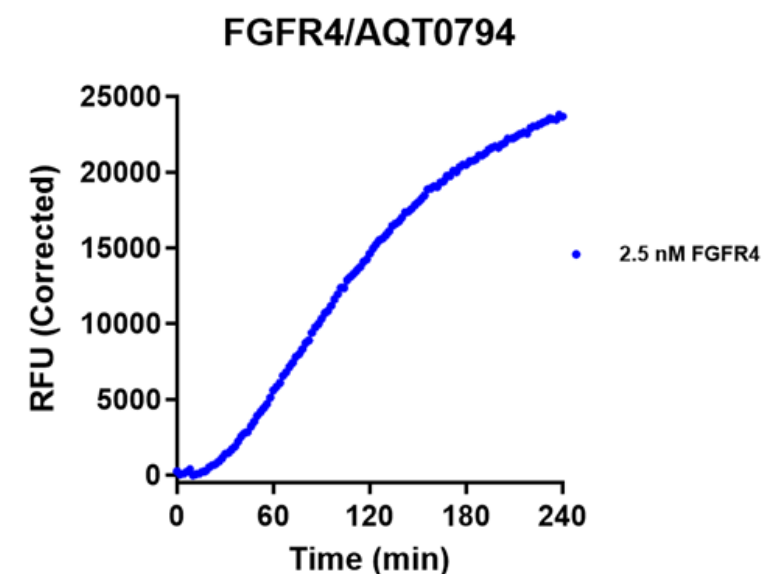
Staurosporine IC₅₀ Determination at ATP K_m is 88 nM

Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.04 - 1.25 nM
Sensor Peptide K_m Value	23 μ M
ATP K_m Value	538 μ M
DMSO Tolerance (highest % recommended)	2
Staurosporine IC50 Determination at ATP K_m is	88 nM

Progress Curve



Kinase Name	Conc. (nM)	Sox-based Substrate Name	Normalized Reaction Rate (RFU/pmole/min)	Normalized Rate StdError (RFU/pmole/min)	Maximum Signal:Bkgd (S/B) Kinetic
FGFR4	2.5	AQT0794	6,392	37.3	2.4

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 Very Strong