

# AQT0032 – ABL1 Assay Validation

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*PhosphoSens<sup>®</sup>*-Kinetic Assay Format

# Outline for this Study

## PhosphoSens–Kinetic Assay Validation

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

Carna ABL1 (Cat#/Lot#: 08-001/14CBS-0812R) amino acid full length; N-terminal His 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<sub>2</sub>

10 μM AQT0032

0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, and 5 nM ABL1

### 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 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.

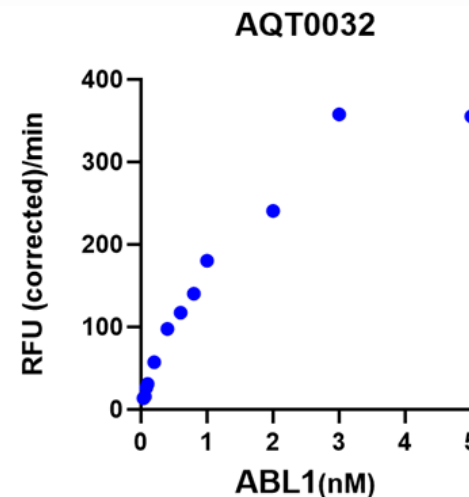
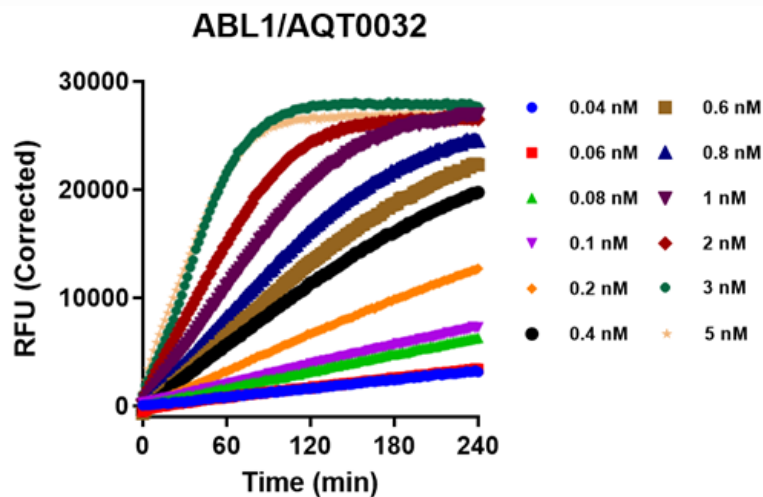
### Notes:

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.

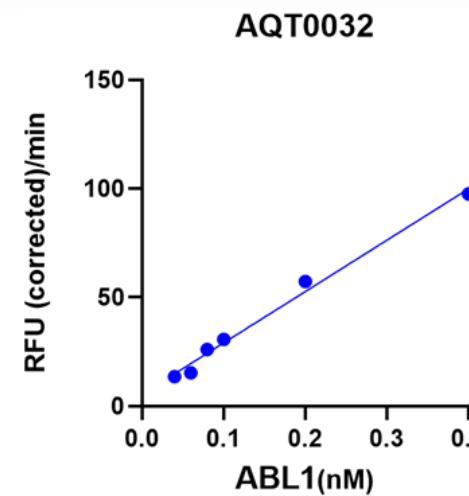
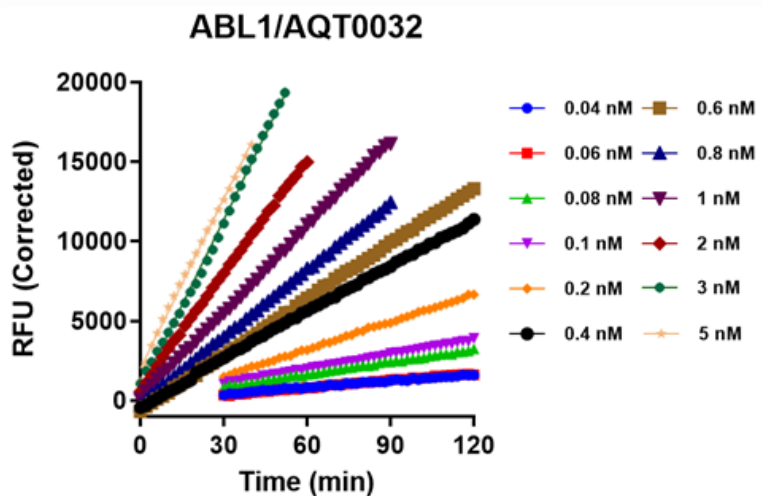
# Enzyme Titration

## 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)
0.04	16,875	284
0.06	12,800	151
0.08	16,300	136
0.1	15,345	83
0.2	14,325	56
0.4	12,199	47
0.6	9,750	16
0.8	8,750	16
1	9,000	22
2	6,015	22
3	5,958	62
5	3,551	49

The reaction is linear from 0.06 – 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  $\mu M$  AQT0032

2 nM ABL1

### Reaction Set Up:

2 or 2.5  $\mu L$

10x Sensor Peptide

14 or 17.5  $\mu L$

Reaction Mix with ATP & DTT

4 or 5  $\mu L$

1x EDB or Kinase dilutions (5x in EDB)

20 or 25  $\mu L$

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  $\mu L$  final well volume or in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20  $\mu L$  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.

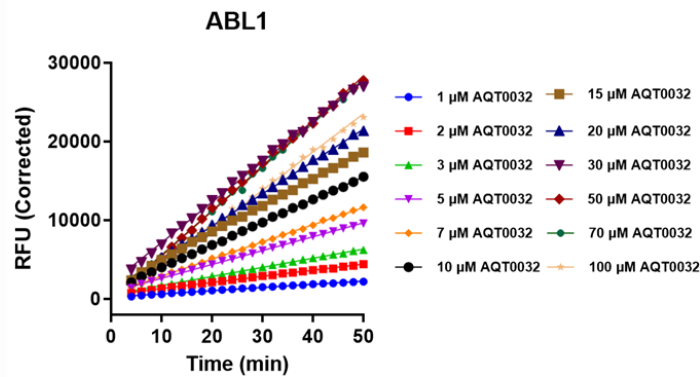
### Notes:

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.

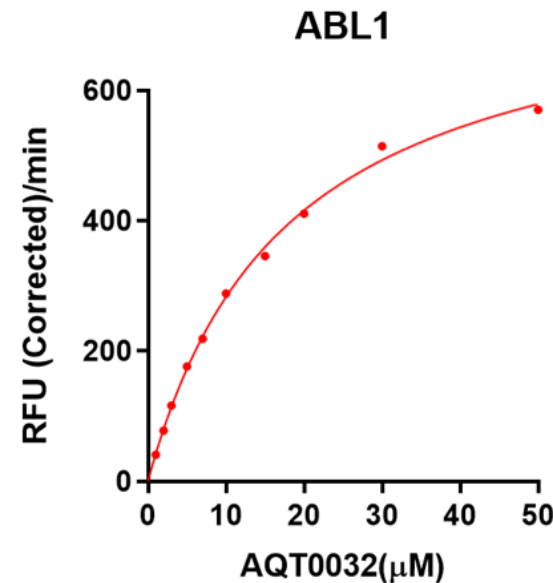
# Sensor Peptide $K_m$ Determination

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	784.4
Km	17.75
Std. Error	
Vmax	21.89
Km	1.075
95% CI (profile likelihood)	
Vmax	736.7 to 837.8
Km	15.46 to 20.43
Goodness of Fit	
Degrees of Freedom	8
R squared	0.9973

Sensor Peptide  $K_m$  is 18  $\mu\text{M}$



# 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  $\mu$ M ATP  
1.2 mM DTT  
0.012% Brij-35  
1% glycerol  
0.2 mg/ml BSA  
0.55 mM EGTA  
10 mM  $MgCl_2$   
10  $\mu$ M AQT0032  
1 nM ABL1

### Reaction Set Up:

2 or 2.5 $\mu$ L	10x Sensor Peptide
14 or 17.5 $\mu$ L	Reaction Mix with ATP & DTT
<u>4 or 5 <math>\mu</math>L</u>	1x EDB or Kinase dilutions (5x in EDB)
20 or 25 $\mu$ 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  $\mu$ L final well volume or in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20  $\mu$ 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:

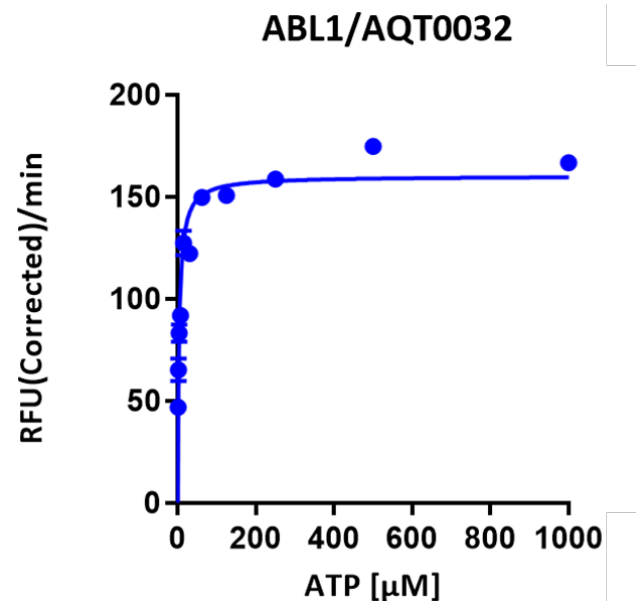
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.



# ATP $K_m$ Determination

Titration Curves and  $K_m$  Plot and Table

## ATP $K_m$ Plot



## ATP $K_m$ Table

Michaelis-Menten	
Best-fit values	
Vmax	160.0
Km	3.876
Std. Error	
Vmax	4.761
Km	0.6881
95% CI (asymptotic)	
Vmax	149.4 to 170.6
Km	2.343 to 5.410
Goodness of Fit	
Degrees of Freedom	10
R squared	0.9354

ATP  $K_m$  is 4 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<sub>2</sub>

0, .01, .02, .04, .08, .16, .31, .63, 1.3, 2.5, 5.0, and 10% DMSO

10 μM AQT0032

2 nM ABL1

### 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 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.

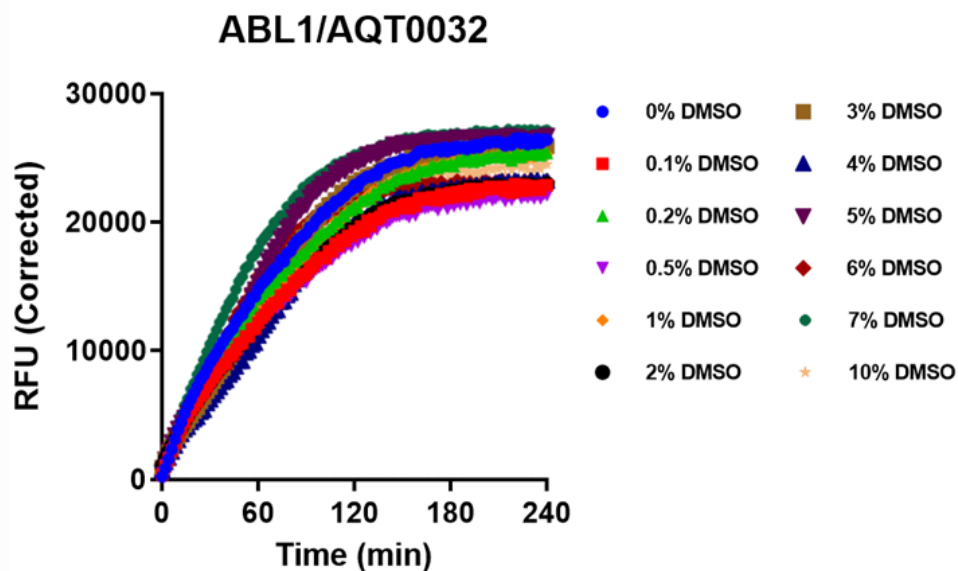
### Notes:

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.

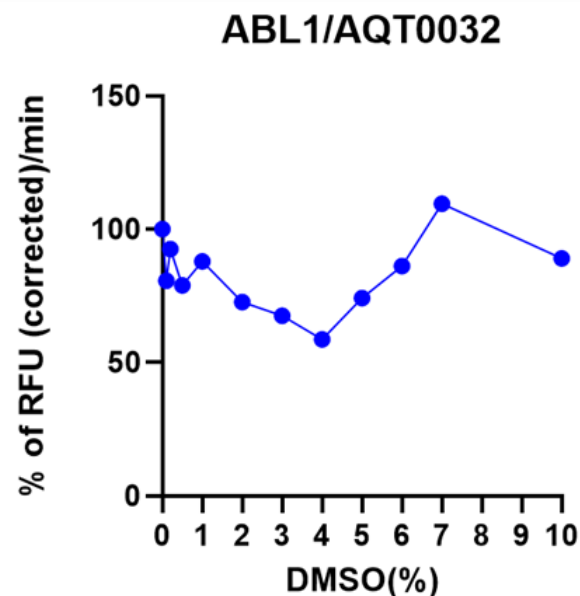
# DMSO Tolerance Test

Titration Curves and Inhibition Plot

## Complete Progress Curves



## Reaction Rate vs [DMSO] Plot



No change in enzyme activity out to 1% DMSO

# IC<sub>50</sub> Determination

## Reaction Conditions and Set Up

### Reaction Conditions:

54 mM HEPES, pH 7.5

ATP at K<sub>m</sub>

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.55 mM EGTA

10 mM MgCl<sub>2</sub>

1% DMSO

10 μM AQT0032

2 nM Carna ABL1

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).

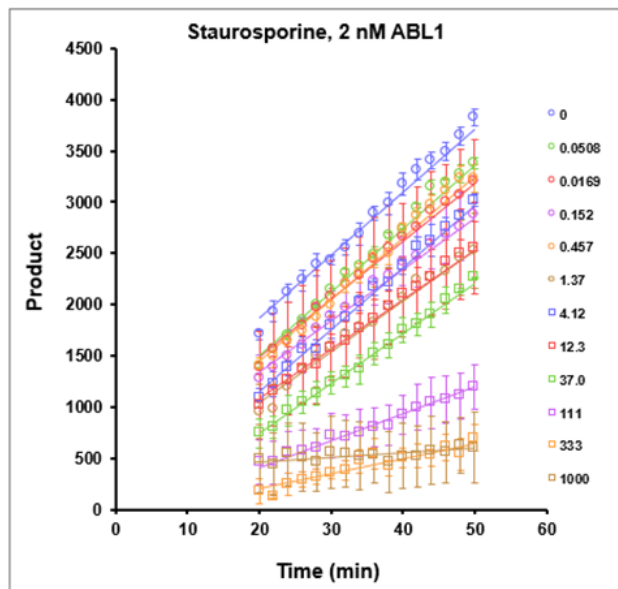
### Notes:

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.

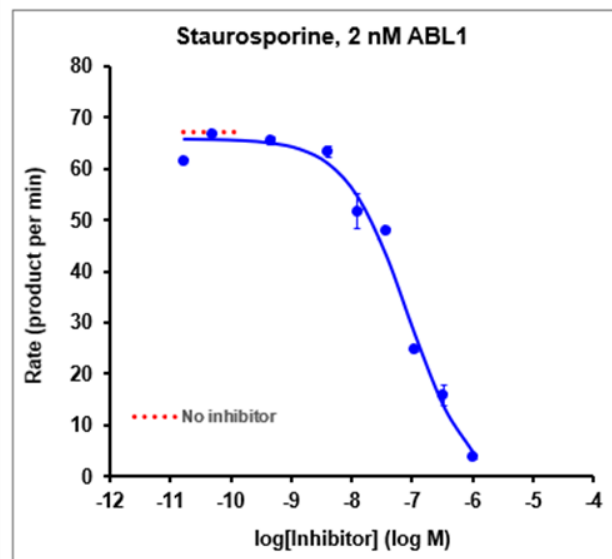
# IC<sub>50</sub> Determination

Progress Curves and IC<sub>50</sub> Curves and Table

## Linear Region of Progress Curves



## IC<sub>50</sub> Curve



The Y-axis label is RFU/min.

## IC<sub>50</sub> Table

Parameter	Value
Bottom	-2.9
Top	65.8
log IC50	-7.08
IC50 (nM)	83.93
Ki (nM)	41.97
Slope	-0.850
R squared	0.982
IC50 approx SE (nM)	4.81
50% inhibition (nM)	76.07

Staurosporine IC<sub>50</sub> Determination at ATP K<sub>m</sub> is 84 nM

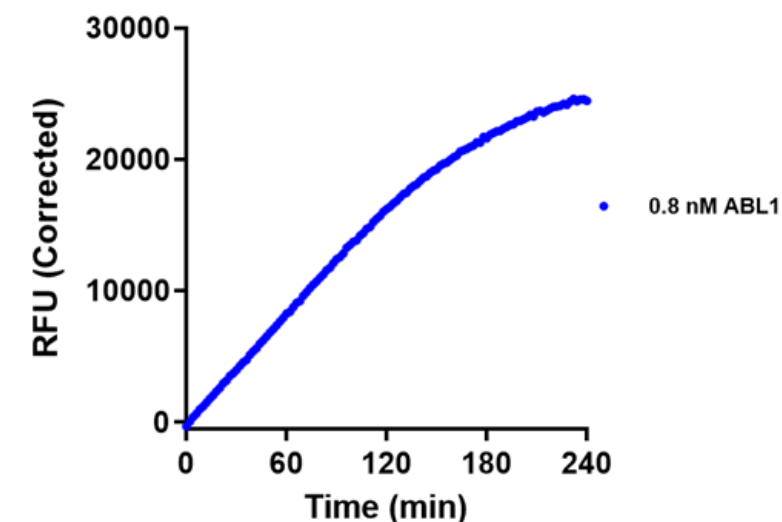
# Summary

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

Experiment	Result
Enzyme Titration Linear Range	0.06 - 0.4 nM
Sensor Peptide $K_m$ Value	18 $\mu$ M
ATP $K_m$ Value	4 $\mu$ M
DMSO Tolerance (highest % recommended)	1
Staurosporine $IC_{50}$ Determination at ATP $K_m$	84 nM

## Progress Curve

AQT0032



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
ABL1	0.8	AQT0032	8,750	16	3.5

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