AssayQuant TECHNOLOGIES INC.

PhosphoSens® Cell Lysate Activity Assay Format

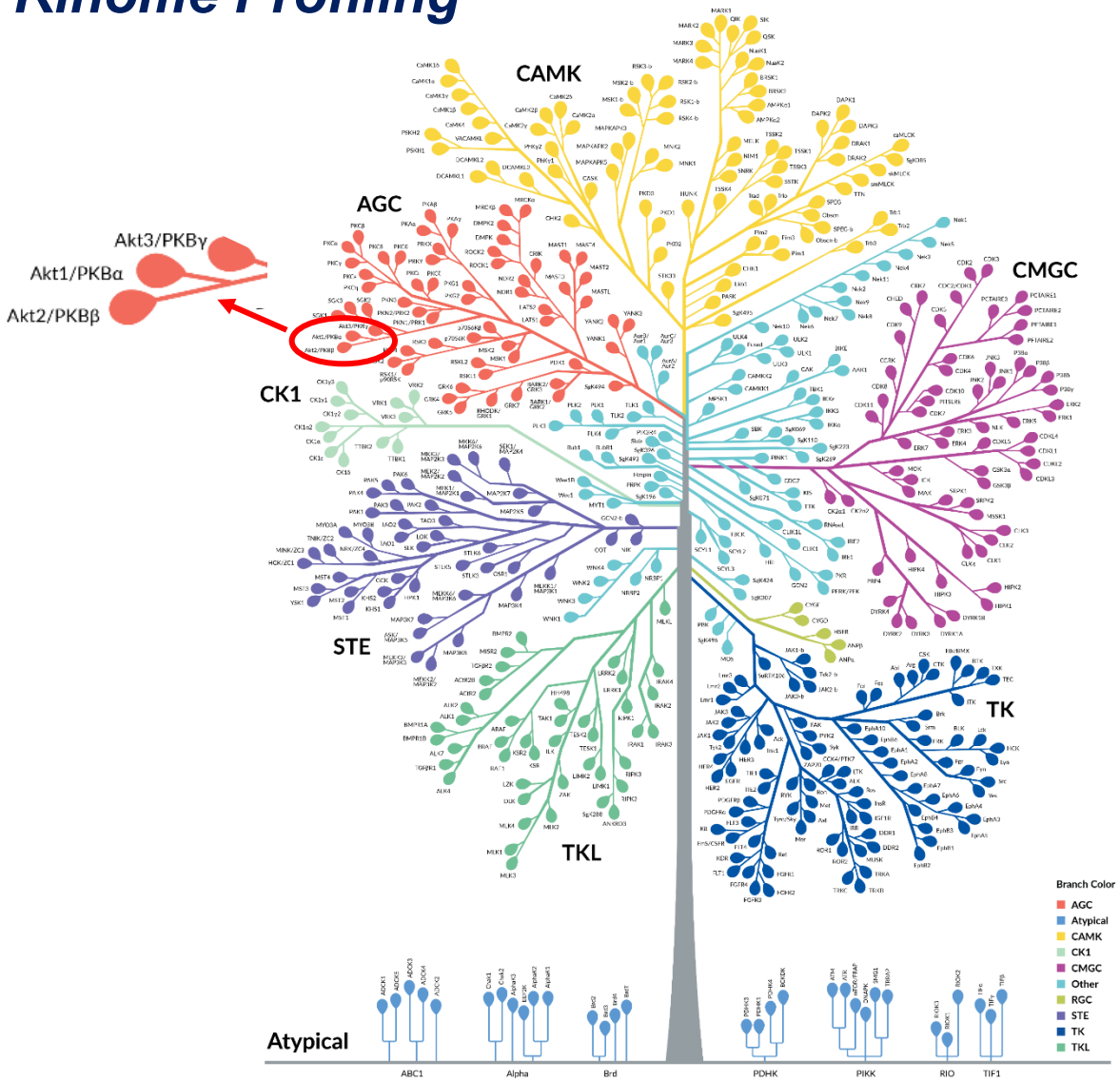
AKT1/2/3 Assay Validation Using the AQ0982 Sensor Peptide

HGNC Name: AKT1 (PKB), AKT2 (PKBbeta), and AKT3 (PKBgamma)
Long Names: RAC-alpha / RAC-beta / RAC-gamma serine/threonine-protein kinase

High selectivity for AKT1/2/3 with the AQT0982 Sensor Peptide

Top 25 Hits with AQT0982 in Kinome Profiling

Kinase Target	Enzyme Conc (nM)	Average (RFU/pmol/min)	Rank	Selectivity Ratio	% Activity
AKT3	1.0	2921	1	0.92	109
AKT1	1.5	2186	2	1.2	82
AKT2	1.5	1889	3	1.4	70
RSK4	1.0	165	4	16	6.2
IRAK4	2.0	113	5	24	4.2
TOPK	5.0	112	6	24	4.2
AMPK212	0.14	106	7	25	3.9
SGK3	1.0	99	8	27	3.7
RSK3	1.0	96	9	28	3.6
PAK3	1.0	88	10	31	3.3
CAMK2D	3.0	79	11	34	2.9
NEK9	0.50	68	12	39	2.5
MAP3K5	1.0	66	13	41	2.5
RSK2	1.0	64	14	42	2.4
AMPK113	0.34	60	15	45	2.2
PASK	2.5	59	16	46	2.2
CAMK1G	2.7	58	17	47	2.1
MSK1	1.0	57	18	47	2.1
NEK1	0.50	57	19	47	2.1
CAMK2A	4.9	53	20	51	2.0
AMPK111	1.0	50	21	54	1.8
AMPK121	1.0	49	22	55	1.8
MAP3K8	1.0	48	23	56	1.8
CAMK2G	4.3	48	24	56	1.8
DYRK3	5.0	45	25	60	1.7



The top off-target kinase is RSK4 (HGNC name is RPS6KA6). When tested manually at 0.3 nM AKT and 1 or 2 nM RSK1-4 and SGK1-3, RSK4 was only 2.5%, and SGK3 was 4.5%.

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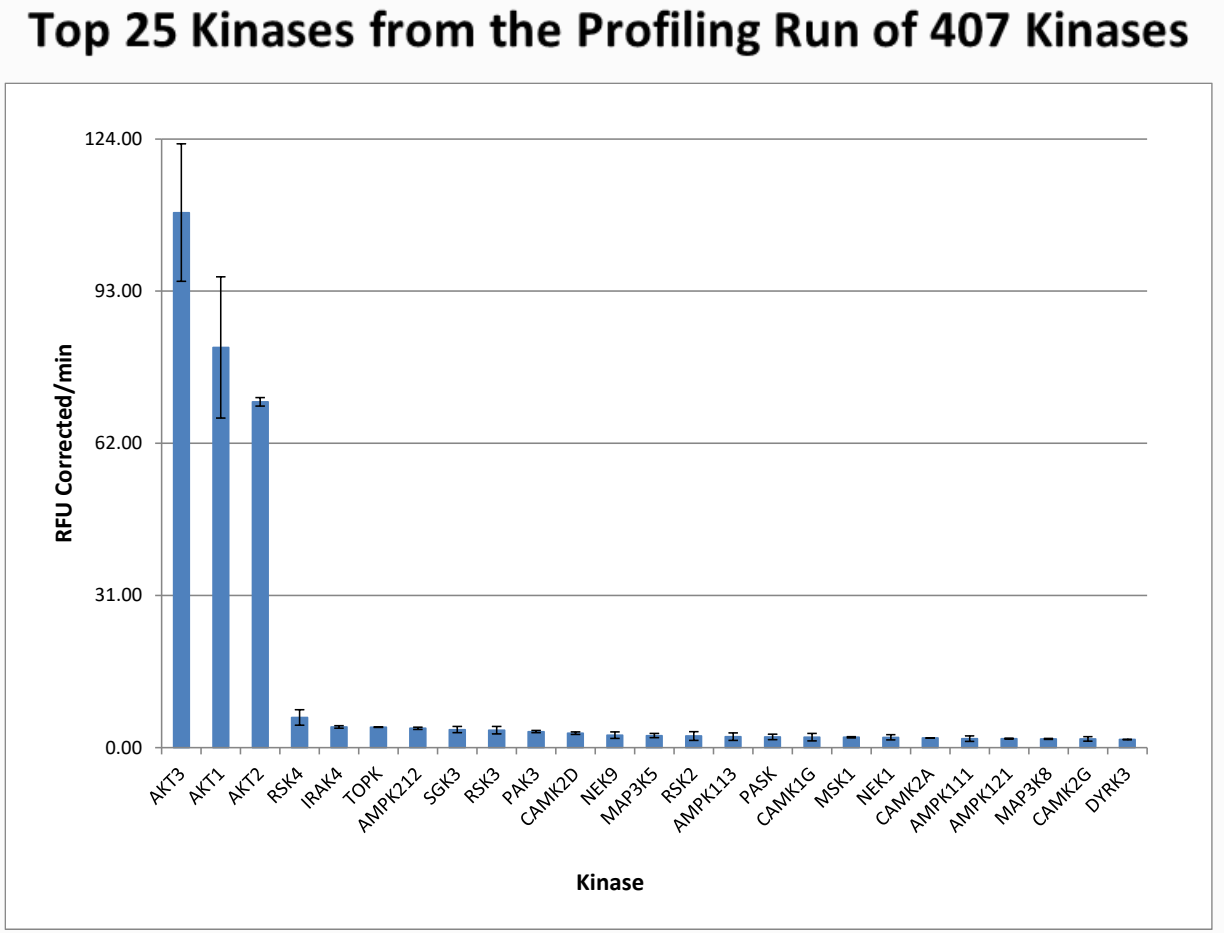
Selectivity of AQT0982 via AQT's Kinome Profiling



High-throughput Milestone (M1) Optimization led to the identification of the AQT0982 sensor peptide substrate.

The top 25 off-target kinases were reassessed and showed *only 6.2% or less of the AKT signal.*

AQT0982 shows exquisite selectivity for the target kinases, a critical requirement for accurately measuring the target kinase activity in crude lysates

Outline for this Study

PhosphoSens-Lysate Assay Validation

Lysate Source:

- NIH-3T3 Cells +/- 50 ng/mL PDGF-bb

Reference Compound Information:

- Rizavasertib (A-443654)
- Vevorisertib trihydrochloride (ARQ 751 trihydrochloride)

Experimental Validation at AssayQuant:

- NIH-3T3 cell lysate (activated with 50 ng/mL PDGF) titration.
- AQT0982 substrate K_m determination
- DMSO Tolerance Test
- Reference Compound IC_{50} Determinations
- Full-length AKT1, which has been activated by AQT with the addition of PDK1, MK5, and lipids/detergent to serve as a positive control
- Phosphopeptide control (AQT1146)

Preparation of Crude Cell Lysates from NIH3T3 Cells Treated +/- PDGF to Activate AKT1/2/3

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1) NIH-3T3 Cells were plated in T-225 flasks and grown to 80-90% confluency over 48 hours at 37°C in DMEM Medium with 10% FBS and PenStrep in an atmosphere of 5% CO_2. Cells were then serum-starved in culture medium with 0.1% FBS for 24 hours and then incubated for 15 minutes with or without 50 ng/mL PDGF-bb. Cells were then washed with PBS and lysed with 500 µL lysis buffer containing:

- 50 mM HEPES, pH 7.4
- 150 mM NaCl
- 2 mM EGTA
- 1 mM DTT
- 1% Triton X-100
- 30 mM NaF
- 10 mM $Na_4P_2O_7$
- 100 µM Na3VO4
- 50 mM β-glycerophosphate
- PhosphoPreserve Protease Inhibitor Cocktail (AQT60XPTIC)
- PhosphoPreserve Phosphatase Inhibitor Cocktail (AQT60XPPIC)

2) The DNA strands were broken by briefly sonicating on ice for 2 seconds on low power or using an 18-gauge needle and syringe. Lysates were aliquoted and frozen at –80 °C, and each aliquot was **used only once (one freeze/thaw cycle)**. Except for the lysate titration, analysis of kinase activity using the PhosphoSens Lysate Assay for AKT used 1.3-2.0 µg/well Western Blotting detection was performed using phospho-specific or pan-AKT antibodies, with 4 µg of each lysate per lane.

Lysate 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

0.54 mM EGTA

10 mM $MgCl_2$

15 µM AQT0982

0, 0.020, 0.039, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, 5.0, 10, and 20 µg/well **crude cell lysate** from NIH-3T3 cells activated with 50 ng/mL PDGF

Reaction Set Up:

20 µL Reaction Mix with AQT0982, ATP, & DTT
5 µL Enzyme dilution buffer (EDB) with AKT Lysate Buffer (1x) or AKT Lysate in Lysate Buffer (5x in EDB)
25 µL Final reaction volume

Reaction was 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.

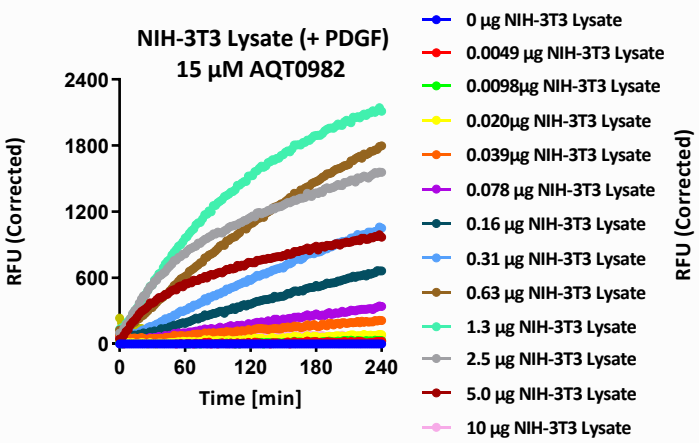
Notes:

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.

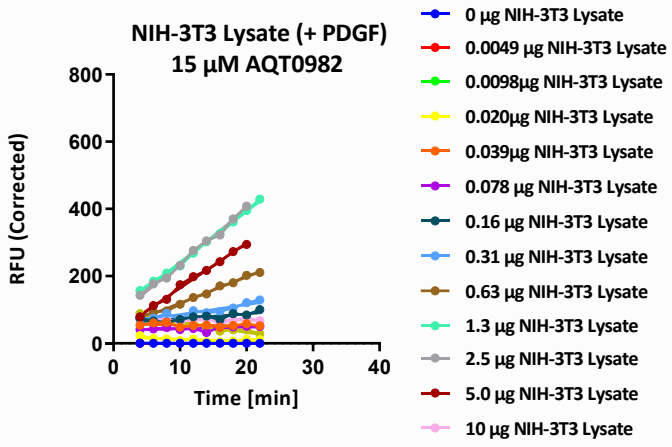
Lysate Titration

Progress Curves

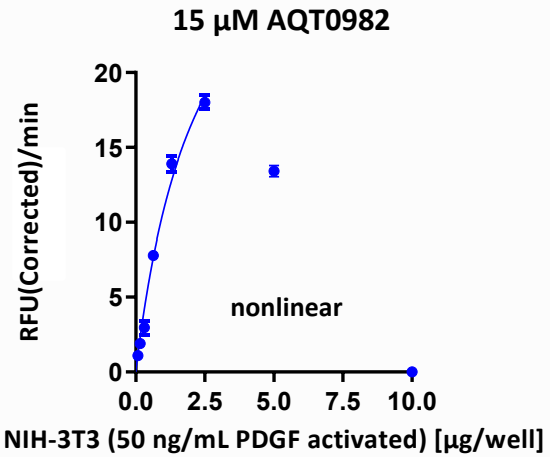
Complete Progress Curves



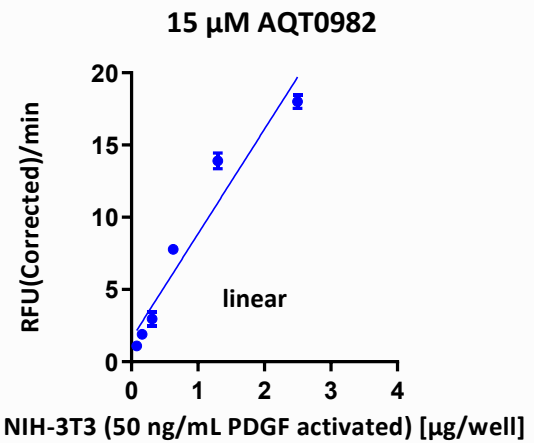
Linear Region of Progress Curves



Nonlinear Reaction



Linear Reaction



The AKT1/2/3 Lysate Assay is linear from 0.078 – 2.5 µg/well lysate

AKT1/2/3 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

0.54 mM EGTA

10 mM $MgCl_2$

0, 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 µM **AQT0982 sensor peptide**

1.3 µg/well NIH-3T3 crude cell lysate (from cells activated with 50 ng/mL PDGF)

Reaction Set Up:

2.5 µl 10X AQT0982 Substrate dilutions
17.5 µL Reaction Mix with ATP & DTT
5 µL Enzyme dilution buffer (EDB) with lysate buffer(1x) or Lysate in lysis buffer (5x in EDB)
25 µL Final reaction volume

Reaction was 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.

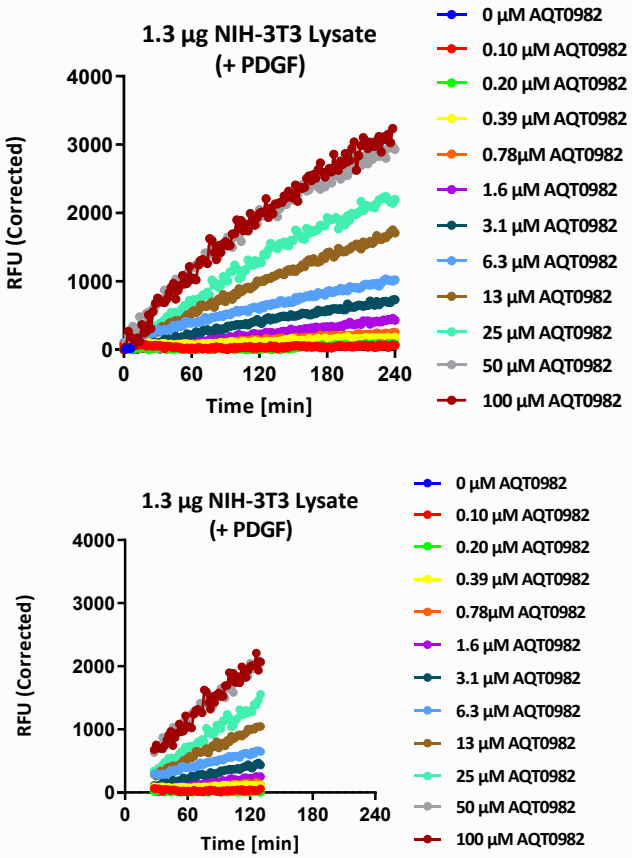
Notes:

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.

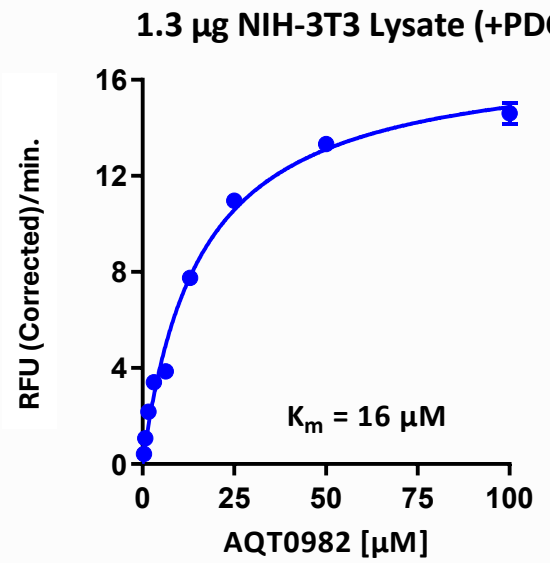
Sensor Peptide K_m Determination

Titration Curves and K_m Plot and Table

Sensor Peptide Titration Curves



Sensor Peptide K_m Plot



Nonlin fit Table of results	A rfu/min
Michaelis-Menten	
Best-fit values	
Vmax	17.19
Km	15.60
95% CI (profile likelihood)	
Vmax	15.55 to 19.08
Km	11.56 to 21.07
Goodness of Fit	
Degrees of Freedom	7
R squared	0.9909
Sum of Squares	2.144
Sy.x	0.5534
Constraints	
Km	Km > 0
Number of points	
# of X values	9
# Y values analyzed	9

Sensor Peptide K_m Table

	AQT0982 K_m (µM)
NIH-3T3 (50 ng/mL stimulated) Lysate	16
Recombinant FL AKT1 (Sino, A16-10G	17
Recombinant FL AKT2 (Sino, A17-10G)	12
Recombinant FL AKT3 (Sino, A18-10G)	115

The K_m value for AQT0982 is 16 µM, similar to the K_m values observed for recombinant AKT1 and 2, which are much lower than AKT3.

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

0.54 mM EGTA

10 mM $MgCl_2$

15 µM AQT0982

2.0 µg/well NIH-3T3 crude cell lysate (from cells activated with 50 ng/mL PDGF)

0-10% DMSO

Reaction Set Up:

2.5 µL 10X DMSO Titration
17.5 µL Reaction Mix with CSx Substrate, ATP & DTT
15 minutes incubation at 30°C
5 µL Enzyme dilution buffer (EDB) (1x) or Lysate (5x in EDB)
25 µL Final reaction volume

Reaction was 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.

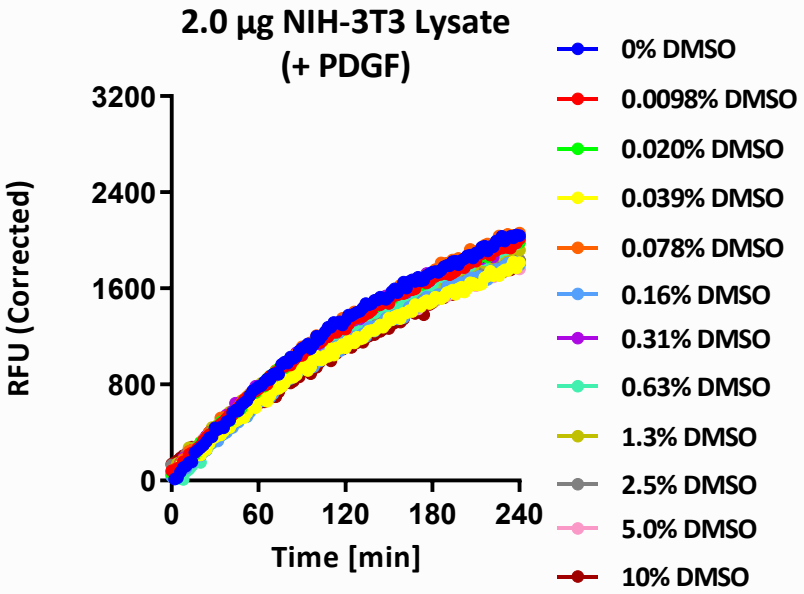
Notes:

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.

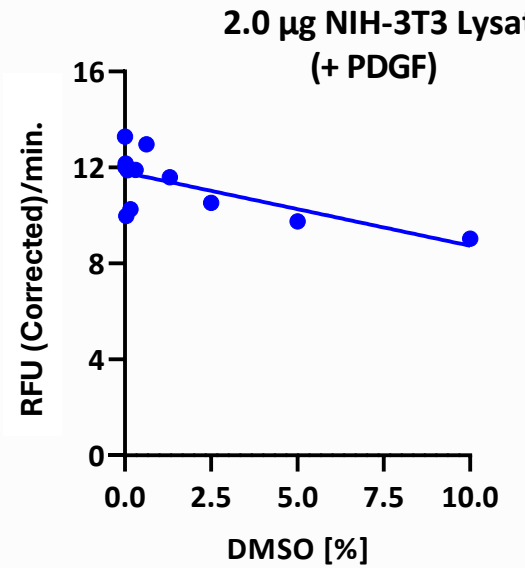
DMSO Tolerance Test

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No significant loss in enzyme activity was observed up to 10% DMSO.
We assessed compound potency using 2% DMSO final.

IC_{50} Determination

Reaction Conditions and Set Up

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Reaction Conditions:

54 mM HEPES, pH 7.5
1.0 mM ATP
1.2 mM DTT
0.012% Brij-35
1% glycerol
0.2 mg/ml BSA
0.54 mM EGTA
10 mM $MgCl_2$
15 µM AQT0982 sensor peptide substrate

Compounds:

- 0-10 µM Rizavasertib (A-443654) and Vevorisertib trihydrochloride (ARQ 751 trihydrochloride) were diluted in 100% DMSO at 50X the final concentrations and diluted 50-fold into the assay for a final concentration of 2% DMSO.

AKT Enzyme:

- Lysate: 1.5 µg/well NIH-3T3 crude cell lysate (from cells activated with 50 ng/mL PDGF)
- Recombinant AKT1-3: 0.5 nM of full-length AKT. These are partially activated; to fully activate, we included in the reaction 0.05 nM PDK1, 0.025 nM MK2, and 1 µM PI(3,4,5)P3 (18:1)/10 µM DOPC/DOPS 1:1, wt %).

Reaction Set Up:

0.5 µL 50X Rizavasertib and/or 0.5 µL 50X Vevorisertib dilution in 100% DMSO
19 µL Reaction Mix with CSx Substrate, ATP & DTT
5 µL Enzyme dilution buffer (EDB) (1x), Lysate or AKT/PDK1/MK2/Lipid (5x in EDB)
25 µL Final reaction volume

Reaction was 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.

IC_{50} Determination with PDGF stimulated NIH-3T3 Lysate using Rizavasertib (A-443654)

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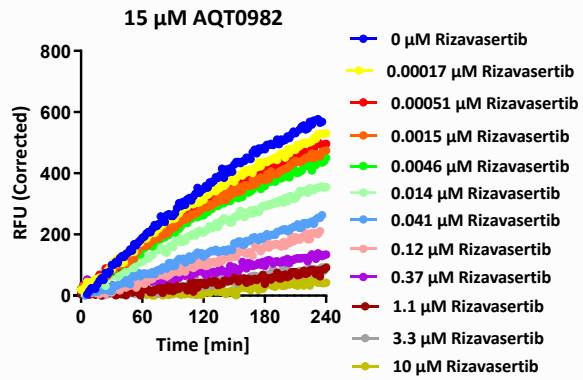
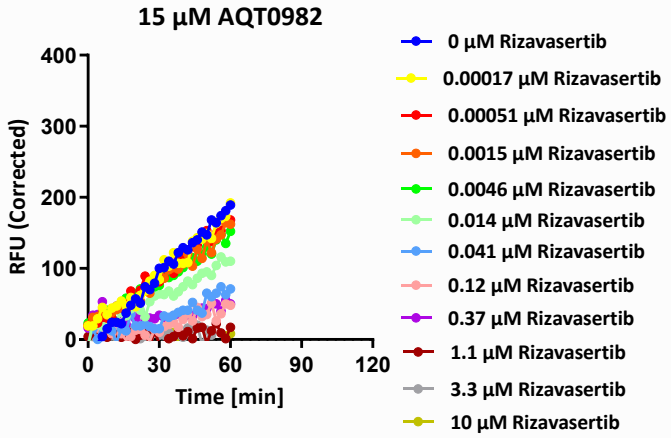
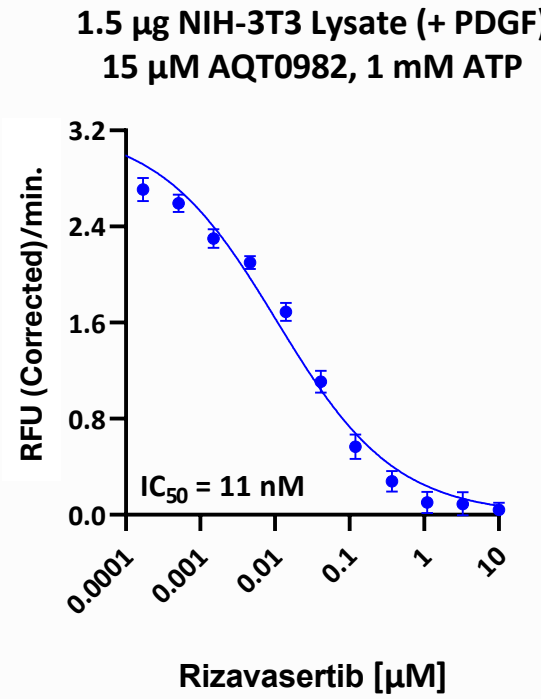
Full Progress Curves



Progress Curves (Linear Region)



IC_{50} Curve



IC_{50} Table

[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	3.223
IC50	0.01060
HillSlope	-0.5458
logIC50	-1.975
Span	3.223
95% CI (profile likelihood)	
Top	2.877 to 3.633
IC50	0.004771 to 0.02124
HillSlope	-0.7475 to -0.4192
logIC50	-2.321 to -1.673
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9858
Sum of Squares	0.2250
Sy.x	0.1581
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

Rizavasertib IC_{50} in lysate is 11 nM

IC_{50} Determination with PDGF stimulated NIH-3T3 Lysate using Vevorisertib (ARQ 751)

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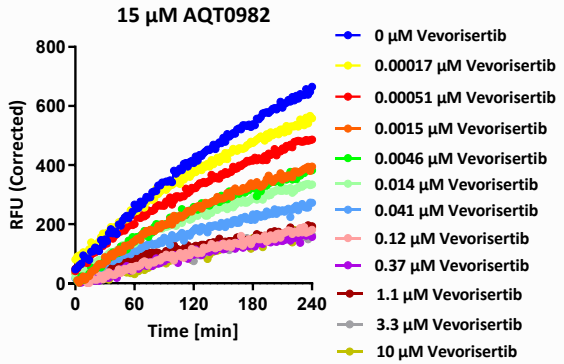
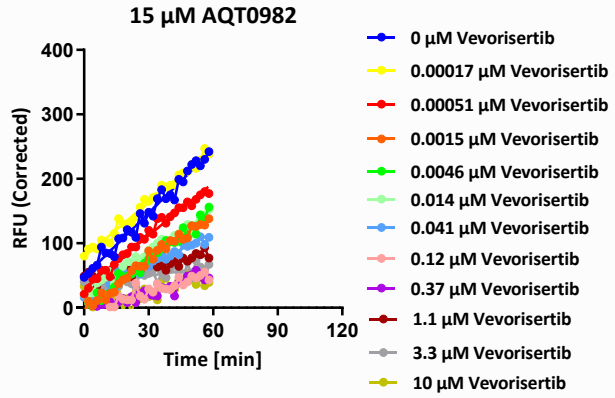
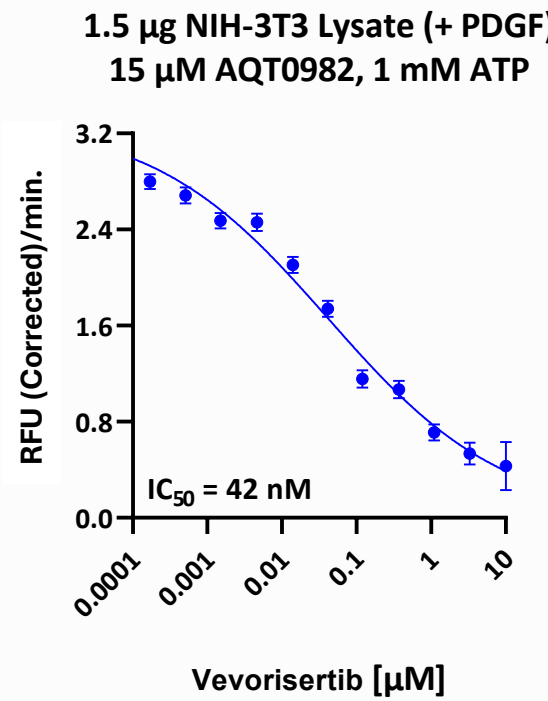
Full Progress Curves



Progress Curves (Linear Region)



IC_{50} Curve



IC_{50} Table

[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	3.315
IC50	0.04177
HillSlope	-0.3683
logIC50	-1.379
Span	3.315
95% CI (profile likelihood)	
Top	3.040 to 3.655
IC50	0.01835 to 0.08305
HillSlope	-0.4559 to -0.2986
logIC50	-1.736 to -1.081
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9879
Sum of Squares	0.1313
Sy.x	0.1208
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

Vevorisertib IC_{50} in lysate is 42 nM

IC_{50} Determination with PDGF stimulated NIH-3T3 Lysate for Rizavasertib (A-443654) & Vevorisertib (ARQ 751)

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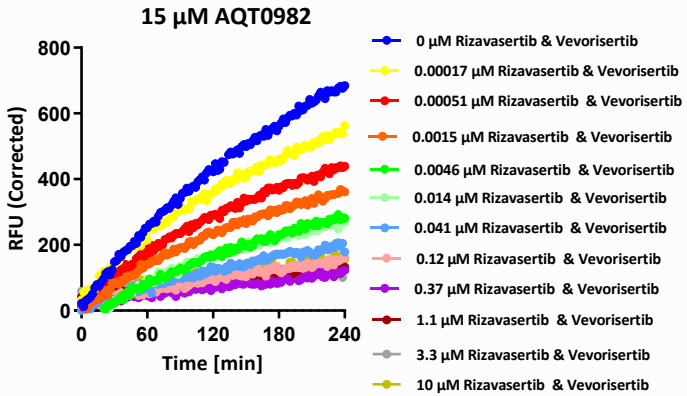
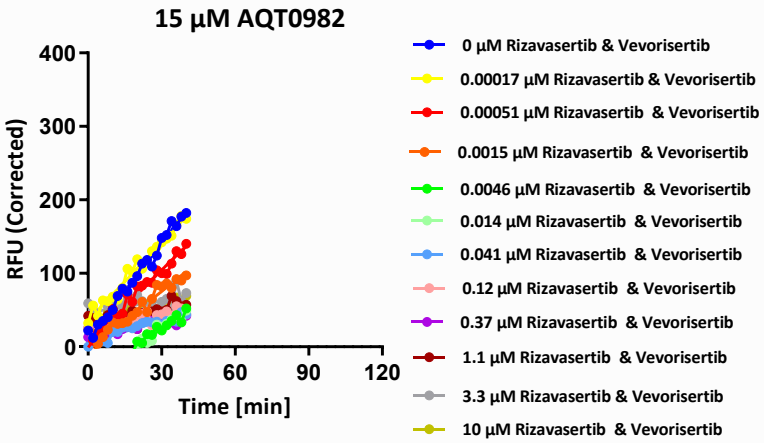
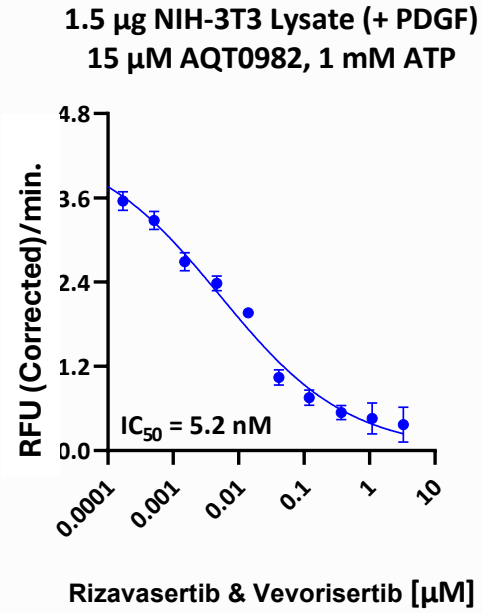
Full Progress Curves



Progress Curves (Linear Region)



IC_{50} Curve



IC_{50} in lysate with both inhibitors is 5.2 nM (2-fold lower than Rizavasertib alone). These two inhibitors are being used together to increase coverage across cell lines.

IC_{50} Table

[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	4.416
IC50	0.005163
HillSlope	-0.4422
logIC50	-2.287
Span	4.416
95% CI (profile likelihood)	
Top	4.046 to 4.839
IC50	0.002715 to 0.009175
HillSlope	-0.5351 to -0.3672
logIC50	-2.566 to -2.037
Goodness of Fit	
Degrees of Freedom	8
R squared	0.9912
Sum of Squares	0.1736
Sy.x	0.1473
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	11
# Y values analyzed	11

IC_{50} Determination for Rizavasertib (A-443654) with Full-length Recombinant & Activated AKT 1-3

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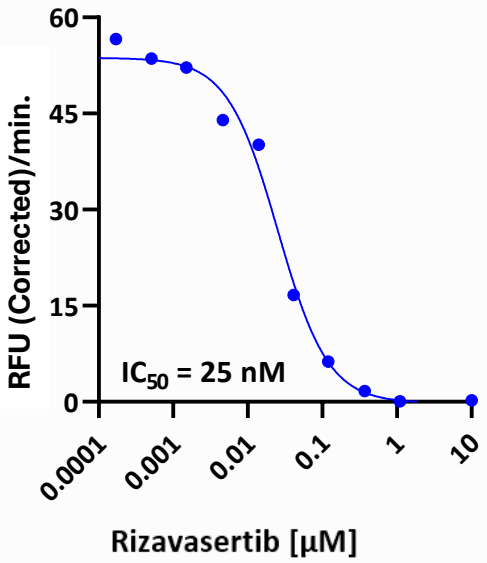
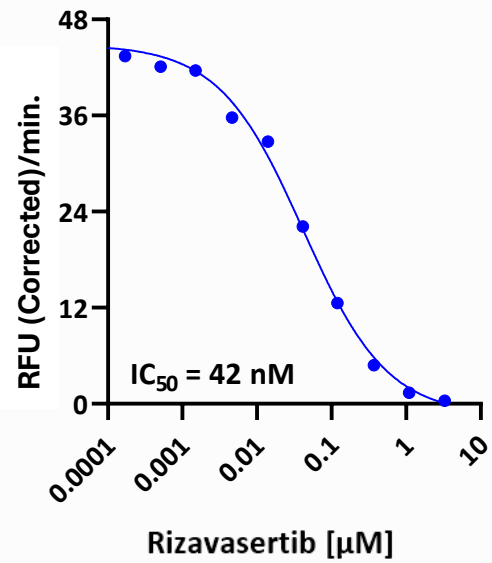
AKT1

2 nM AKT1 (1 mM ATP)



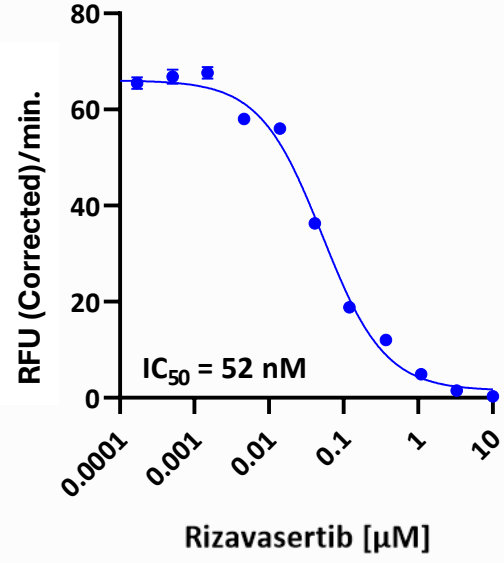
AKT2

2 nM AKT2 (1 mM ATP)



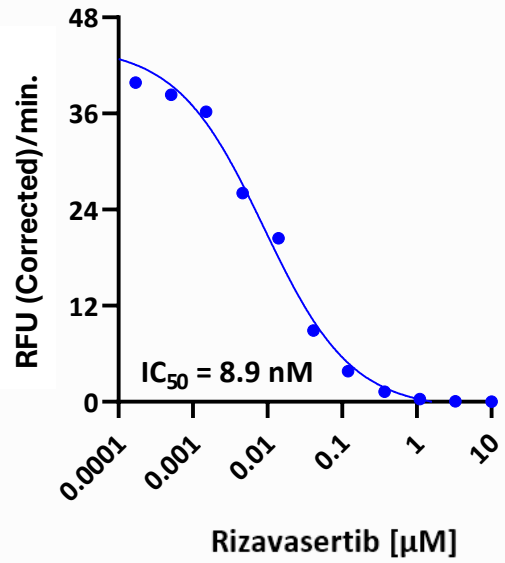
AKT3

2 nM AKT3 (1 mM ATP)



AKT1 (E17K)

2 nM AKT1 (E17K) (1 mM ATP)



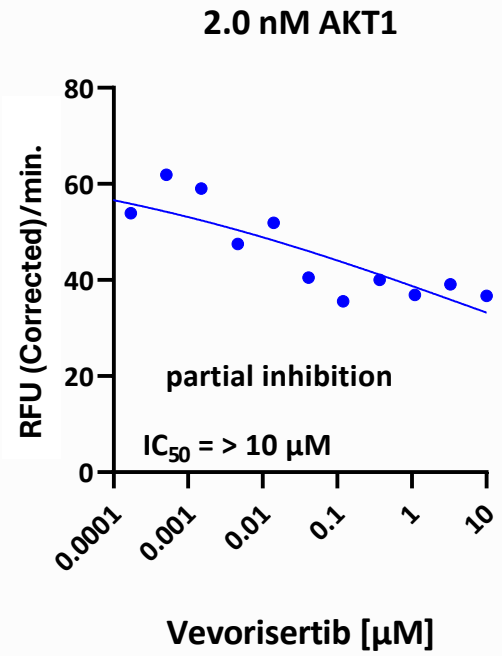
Rank order of potency with Rizavasertib: AKT1 (E17K) > AKT1 > AKT2 ~AKT3.

Notes: Rizavasertib is an ATP-competitive inhibitor that blocks all forms of AKT1-3. The IC_{50} values will be higher (lower potency) at 1 mM ATP than at the ATP K_m. The AKT1 E17K mutation is a specific genetic alteration in the AKT1 gene that is found in various cancers, including breast, colorectal, ovarian, and lung cancers. This mutation is located in the pleckstrin homology (PH) domain of AKT1 and is known to constitutively activate the PI3K/AKT/mTOR pathway. The mutation has been shown to sensitize cancer cells to AKT inhibitors, suggesting a potential therapeutic strategy. See Wu et al., 2020, Int J Clin Exp Pathol. 2020 Mar 1;13(3):332–346. https://pubmed.ncbi.nlm.nih.gov/32269671/

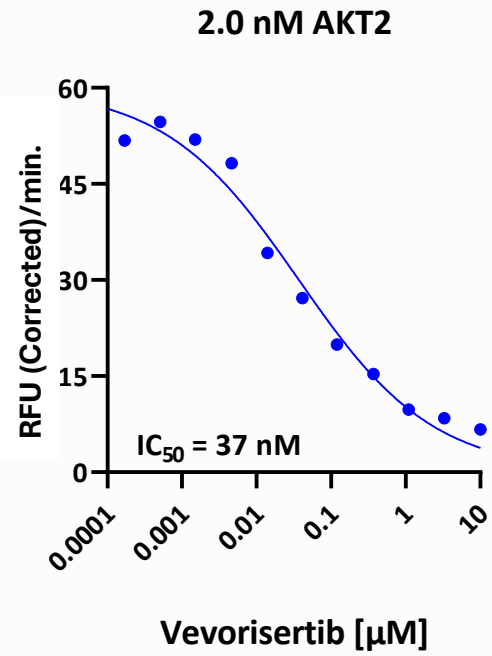
IC_{50} Determination for Vevorisertib (ARQ 751) with Full-length Recombinant & Activated AKT 1-3

AssayQuant TECHNOLOGIES INC.

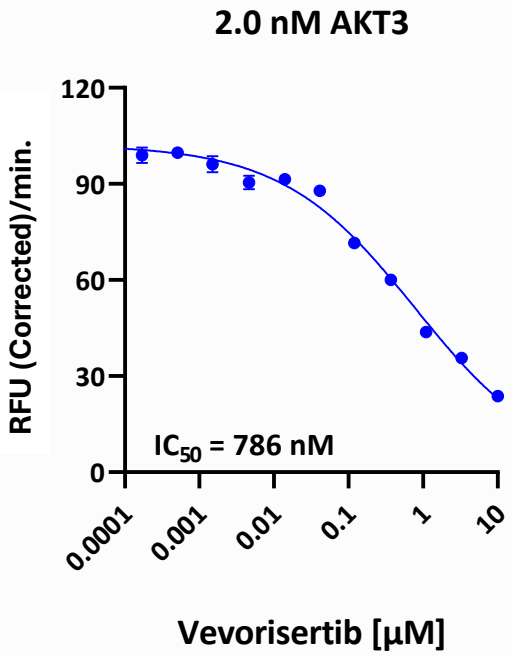
AKT1



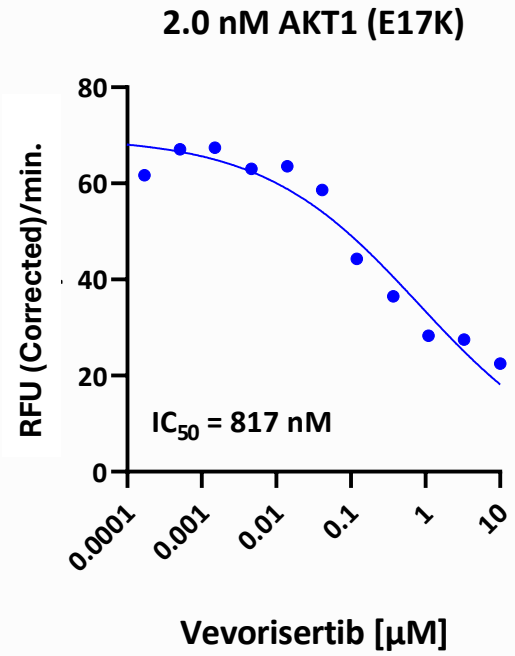
AKT2



AKT3



AKT1 (E17K)



Rank order of potency with Vevorisertib: AKT2 >>AKT3 ~ AKT1 (E17K) >> AKT1

Note that Vevorisertib binds to the unactive form of AKT1-3, so it is less potent with these activated AKTs. It has very low potency with wild-type AKT1. See slide 19 for the relevance of the AKT1 E17K mutation.

Summary of Rizavasertib and Vevorisertib IC_{50} Values for PDGF Stimulated NIH-3T3 AKT Lysate or Activated Recombinant AKT1-3 with AQT0982

AssayQuant TECHNOLOGIES INC.

Kinase Source Sample Description	Rizavasertib	Vevorisertib
	IC_{50} (nM) @ 1 mM ATP	IC_{50} (nM) @ 1 mM ATP
Recombinant full length AKT1	25	> 10,000
Recombinant full length AKT2	42	37
Recombinant full length AKT3	52	786
Recombinant full length AKT1 (E17K)	8.9	817
NIH-3T3 PDGF Stimulated Lysate	11	42

The IC_{50} values for Rizavasertib and Vevorisertib were determined at AQT with the full-length recombinant and active forms of AKT 1-3 or lysate from NIH3T3 cells treated with PDGF, and all reactions used 1 mM ATP as described (slide 14).

Reported Potency Values:

Rizavasertib (A-443654): *In vitro* potency against AKT is reported by the supplier as 160 pM (K_i) for AKT1, AKT2, and AKT3. In cellular assays, IC_{50} values for inhibiting AKT phosphorylation in HEK-293T cells ranged from 2.5 nM to 51 nM (Okuzumi *et al.*, 2009: https://pubmed.ncbi.nlm.nih.gov/19465931/)

Vevorisertib (ARQ 751): https://pubmed.ncbi.nlm.nih.gov/26469692/
Yu *et al.* (2015) reported IC_{50} values of 0.55 nM, 0.81 nM, and 1.3 nM for AKT1, AKT2, and AKT3, respectively, measured in a biochemical format with the full-length inactive forms of AKT1-3 and 10 µM ATP. Their much lower values emphasize the nature of Vevorisertib, which binds to the unactive form of AKT and prevents activation. In contrast, they determined the IC_{50} for the phosphorylation of an AKT substrate, pPRAS40[T246], using AN3CA endometrial cancer cells to be 49 nM, which agrees with our assessment of 42 nM using lysates from PDGF-treated NIH3T3 cells.

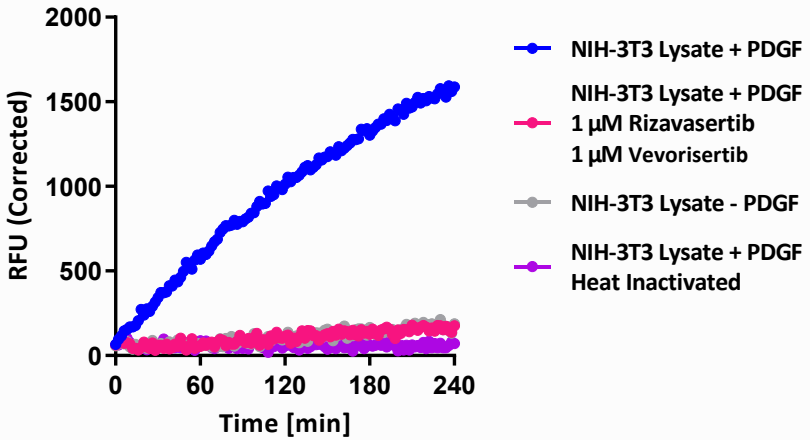
Since Rizavasertib is ATP-competitive, the potency will be lower at 1 mM ATP than if performed at ATP K_m as reported by Okuzumi et al., 2009. With Vevorisertib, the potency will be lower with activated AKT1-3 than if performed with unactive forms (Yu et al., 2015). AQT's IC_{50} values with crude lysates for Rizavasertib (11 nM) and Vevorisertib (42 nM) are consistent with values with cells reported by Okuzumi et al., 2009 for Rizavasertib (2.5 nM to 51 nM) and Yu et al., 2015 for Vevorisertib (49 nM).

AKT1/2/3 Lysate Activity Assay Using the AQT0982 Sensor Peptide (15 µM)

AssayQuant TECHNOLOGIES INC.

A. Crude Lysate Samples (2.0 µg/well)

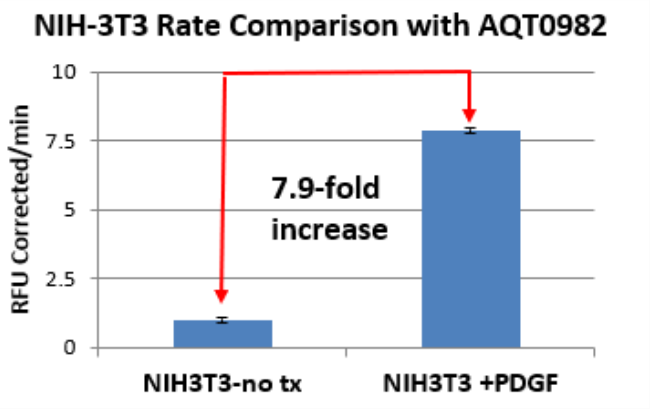
1) Full Time Course and Linear Range (0-240 min.)



2) Reaction Rates to Assess Change

Description of Conditions for Crude Cell Lysates (2 µg/well)	Reaction Rate (RFU/min)	Change
NIH-3T3 Control lysate (-PDGF)	1.0 ± 0.07	
NIH-3T3 lysate (+50 ng/mL PDGF)	7.9 ± 0.10	8-fold increase above control
NIH-3T3 lysate (+50 ng/mL PDGF), Heat Inactivated	0	100% inhibition of PDGF Activation
NIH-3T3 lysate (+50 ng/mL PDGF) + 1.0 µM Rizavasertib & 1.0 µM Vevorisertib	0.41 ± 0.08	100% inhibition of PDGF Activation

3) Histogram of Results

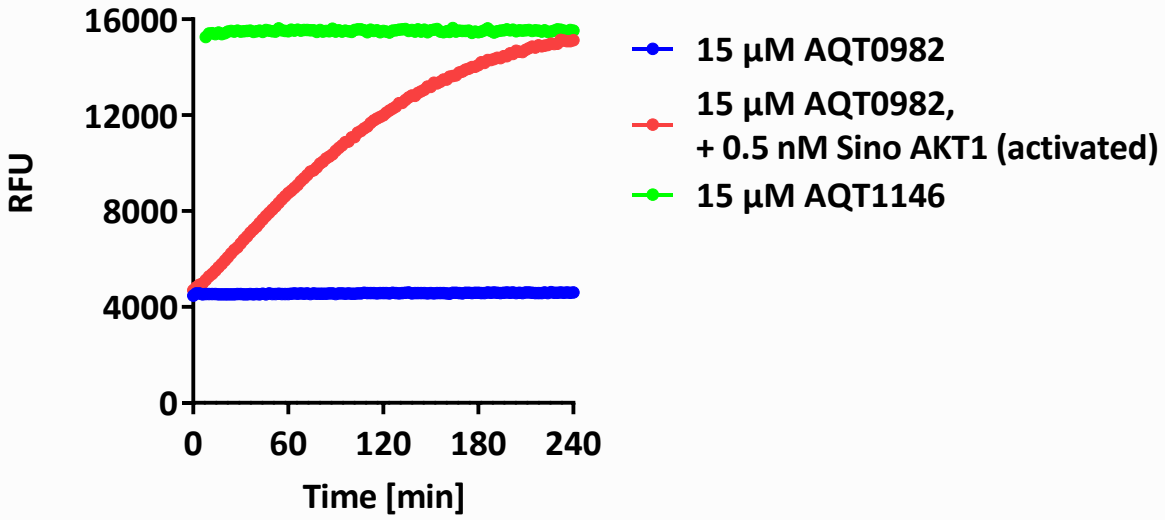


A. Crude lysate samples: The AQT0982 sensor peptide (15 µM) was used to generate **1)** Full progress curve time course (0-240 min.), which also corresponds to the Linear range. The reaction rates (RFU Corrected values [Total – Background]/min. +/- standard deviations) are the slope of the linear region of each progress curve, which are presented in the table in **2)** and as a histogram in **3)**, highlighting a 7.9-fold activation of AKT kinase activity in lysates from NIH3T3 cells treated with PDGF. The signal was eliminated by heat inactivation of the lysate or by adding the selective AKT inhibitors Rizavasertib (ATP Competitive) and Vevorisertib (Allosteric) to the reactions. **Note:** The amount of activation depends on several factors, including cell type, the serum deprivation pretreatment used to make cells quiescent, as well as the nature, concentration, and duration of the activating stimulus (in this experiment, we used (50 ng/mL PDGF for 15 minutes) as described on slide 8. These conditions can be varied to determine the effect on AKT activity. The total amount of AKT protein can be determined by Western Blotting or an ELISA; however, with the short stimulation times typically used, these levels are not expected to change.

AKT1/2/3 Lysate Activity Assay Using the AQT0982 Sensor Peptide (15 µM)

B. AQT0982 ± recombinant AKT1 & AQT1146 Phosphopeptide Control

1) Full Time Course (0-4 hours)



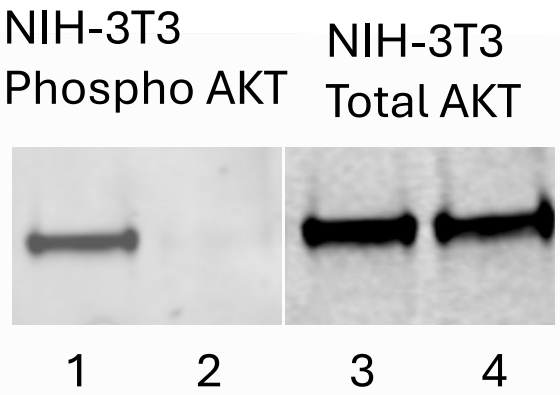
AQT1146 is a chemically synthesized phosphopeptide control for AQT0982

B.1. 15 µM AQT0982 ± 0.50 nM activated AKT1 & 15 µM AQT1146 Phospho Control: Activated AKT1 protein (0.50 nM) fully phosphorylated the AQT0982 sensor peptide substrate by 4 hours, as shown by convergence with the signal obtained with the AQT1146 phosphopeptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). The signal with AQT1146 is used to convert RFU (Corrected) values to nmoles of Phosphopeptide product.

Note: Recombinant active AKT1 is provided in the kit (from Sino but then fully pre-activated by AQT prior to shipment to serve as a positive control). If running a recombinant assay "in house", it is highly recommended to use unactive AKT (Sino A16-14G) to observe the effects of allosteric inhibitors, as the enzyme is activated over time.

AKT Lysate Western Blots

A Commonly Used Method to Assess AKT Activation



Lane Description:

1. NIH-3T3 Cells (+ 50 ng/mL PDGF)
2. NIH-3T3 Cells (untreated)
3. NIH-3T3 Cells (+ 50 ng/mL PDGF)
4. NIH-3T3 Cells (untreated)

Method: Western blots were developed with antibodies from Cell Signaling Technology diluted 1000-fold for total AKT (pan) (2920) or phospho-AKT pS473 (9271), and then LI-COR, IR Dye goat anti-mouse (800CW 926-32210) or donkey anti-rabbit antibody (680RD 926-68073) antibodies diluted 20,000-fold, followed by imaging in a LI-COR Odyssey.

Shows PDGF-induced phosphorylation of the Ser473 site in the C-terminal regulatory domain of AKT1-3 (lanes 1 & 2), as a surrogate measure for AKT activity, while total AKT levels remain the same (lanes 3 & 4).

Notes: Phospho-specific antibodies to pT308 and pS473 are commonly used to measure the active form of AKT; however, the AKT enzyme has at least 53 phosphorylation sites with some being activating (**Ser** 124, 129, 246, 378, 473; **Thr** 72, 195, 308, 378; **Tyr** 176, 215, 326, 474) or inhibitory (**Thr** 34 & 312; **Tyr** 315). In addition, kinase inhibitor compounds can block the access of phosphatases, resulting in elevated phosphospecific antibody signals as described for AKT (Lin *et al*., 2012: https://pubmed.ncbi.nlm.nih.gov/22569334/) and ERK1/2 MAPKs (Vaisar and Ahn, 2024: https://pubmed.ncbi.nlm.nih.gov/22569334/)

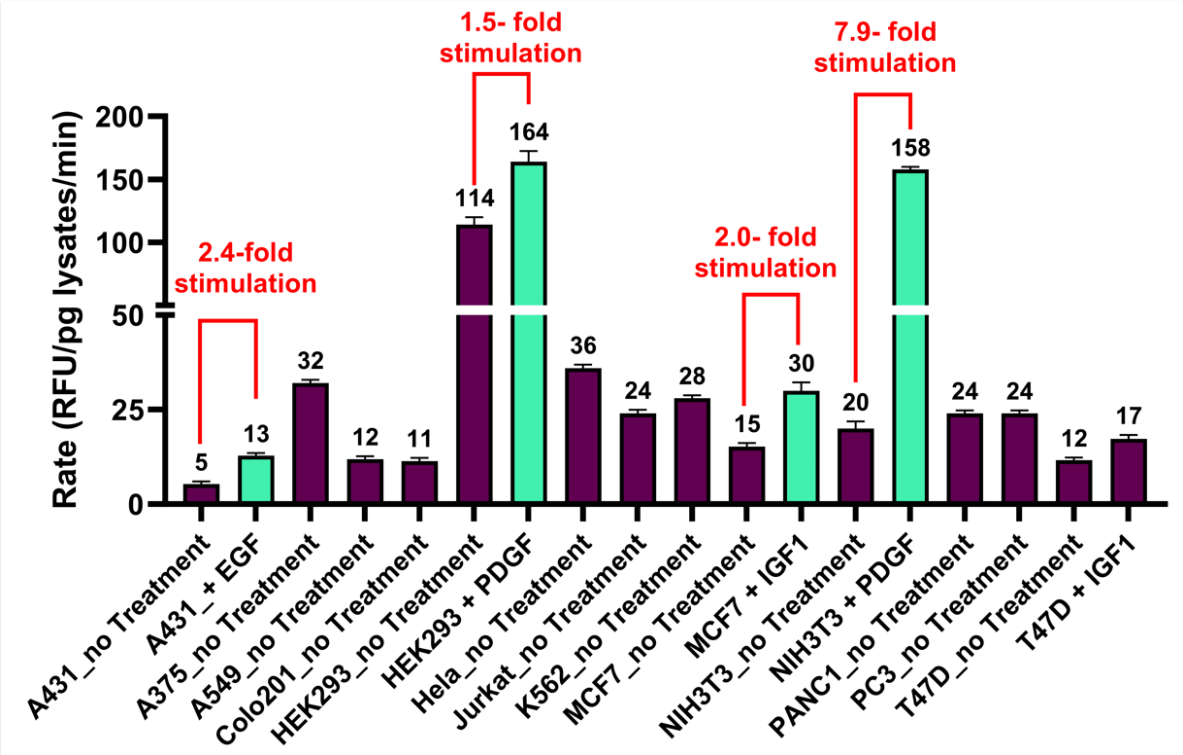
The solution: *Measure AKT1-3 activity directly with the PhosphoSens-Lysate assay!*

AKT1-3 Lysate Activity Assay Using the AQT0982 Sensor Peptide Across a Variety of Cell Types

AssayQuant TECHNOLOGIES INC.

Cell Lysate Panel with AQT0982

(2.0 µg total protein for each lysate/well)



The method described on slide 8 was used to prepare cell lysates for the cell lines in the histogram. Standard lysate assay conditions on slide 15 were used to run the assay with 2.0 µg total protein for each lysate. Reaction rates (RFU Corrected/pg of total lysate protein/min) were determined from the slopes using the linear portion of each progress curve. Values are the average of duplicate reactions +/- standard deviation. In four cell lines, AKT activity was stimulated by treatment (Green bars), with 8-fold stimulation seen with NIH3T3 cells +/- PDGF. Incorporation of the AKT-selective inhibitors, Rizavasertib (ATP Competitive) and Vevorisertib (Allosteric) @ 1 µM, blocked the signal with AQT0982 (see slide 22), highlighting the selectivity of the sensor peptide for evaluating AKT activity in these complex samples.

Demonstrates detection of endogenous AKT activity in multiple cell lines

Summary

- The PhosphoSens-Lysate Assay for AKT1/2/3 using the AQT0982 selective sensor peptide demonstrates a robust and physiologically relevant assay that measures endogenous AKT1/2/3 activity with all the cellular components and signaling complexes.
- Results include:
  - NIH-3T3 cell activation with 50 ng/mL PDGF-bb increases AKT kinase activity 3-fold compared to the unstimulated control. This can likely be improved further by varying conditions for the treatment of cells.
  - AKT activity with lysates from PDGF-stimulated NIH3T3 cells was linear from 0.078 to 1.5 µg/well, a 19-fold linear range.
  - The AKT lysate activity is completely inhibited by 1 µM of Rizavasertib (ATP competitive inhibitor) and 1 µM of Vevorisertib (allosteric inhibitor) reference compounds, demonstrating the selectivity of the assay.
  - The IC_{50} values for Rizavasertib and Vevorisertib with lysates from PDGF-stimulated NIH-3T3 cells were 11 nM and 42 nM, respectively, while using both inhibitors together resulted in an IC_{50} value of 5.2 nM.
  - Sensor peptide substrate AQT0982 has a K_m of 16 µM with the + PDGF stimulated cell lysate.
- These measurements are direct and highly quantitative, and in an easy-to-use format.
- Phosphorylation of AKT1/2/3, as a surrogate measure of activation, was also demonstrated and consistent, although this method is only semi-qualitative and doesn't consider other regulatory events.