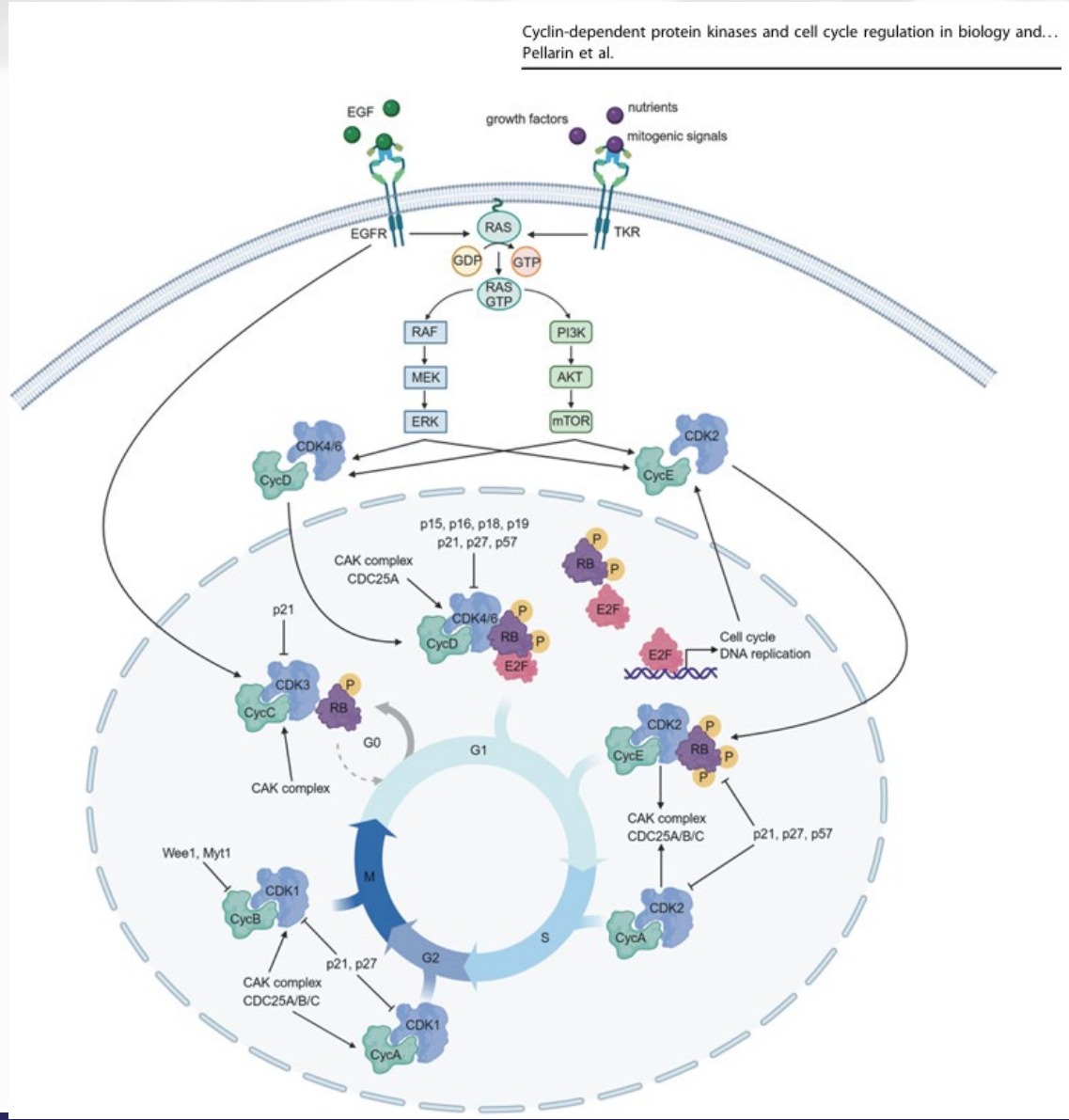
PhosphoSens® Cell Lysate Activity Assay Format

CDK1/2/3/5 Assay Validation Using Relevant Selective Cell Models with the AQT1271 Pan CDK1/2/3/5 Sensor Peptide

HGNC Name: CDK1 (CDC2), CDK2, CDK3, CDK5
Long Names: Cyclin-dependent kinase 1, 2, 3, & 5

Cyclin-Dependent Kinases (CDKs) in Cancer

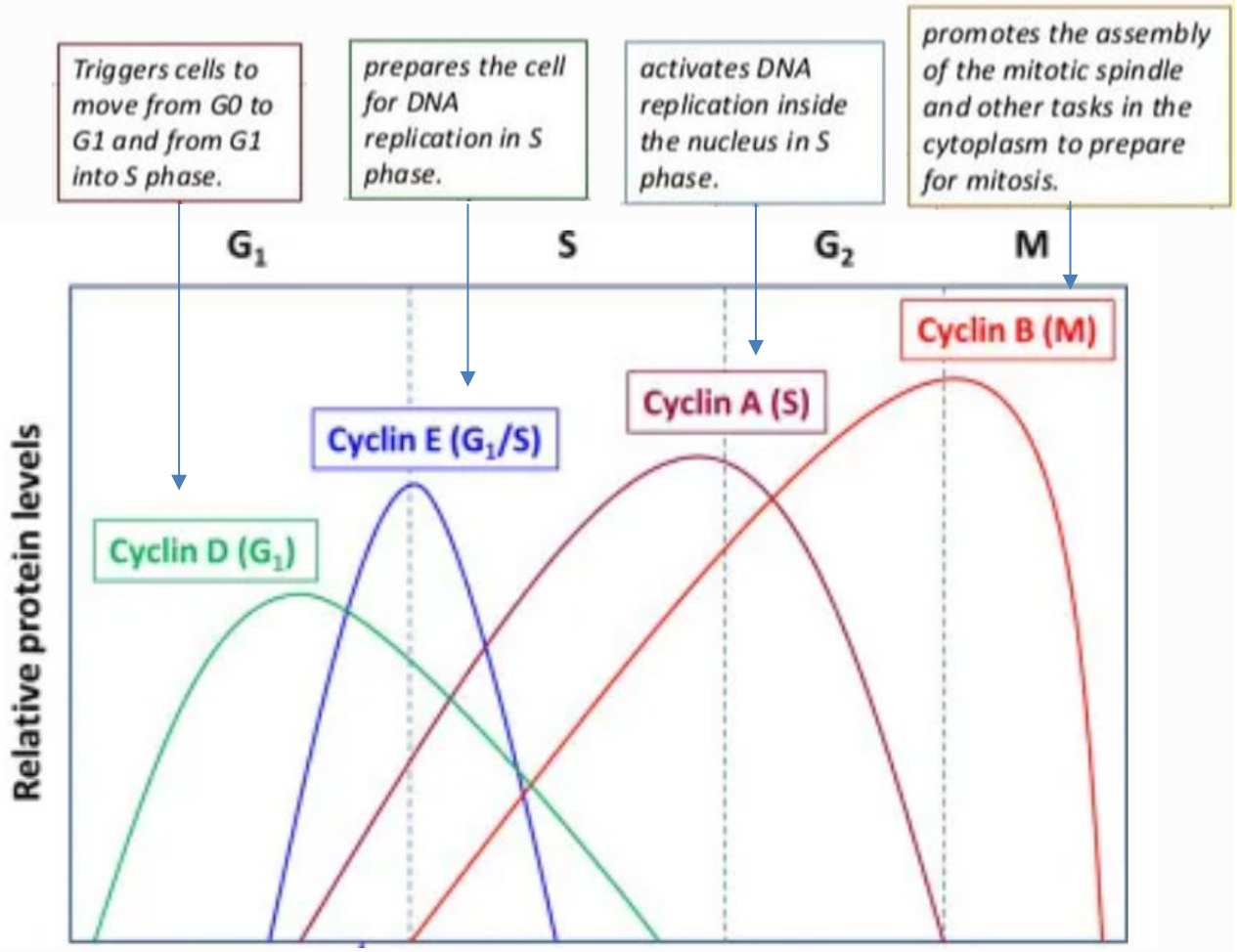


Cell Cycle Progression via EGFR and Receptor Tyrosine Kinase Signaling. EGFR stimulation promotes the activation of the CyclinC-CDK3 complex, enabling the cell to exit quiescence (G0) and enter the G1 phase by priming RB phosphorylation. Activation of various Receptor Tyrosine Kinases (RTKs) can similarly promote G1 progression through signal cascades involving RAS-GTP and its downstream RAF/MEK/ERK and PI3K/AKT/mTOR pathways. These signaling cascades lead to the formation and activation of CyclinD-CDK4/6 complexes and their translocation to the nucleus. In the nucleus, CyclinD-CDK4/6 complexes further phosphorylate RB. The inhibition of RB allows the accumulation of E2F on DNA, promoting the transcription of genes essential for cell cycle progression and DNA replication. Subsequently, the activation of the CyclinE-CDK2 complex drives the transition from G1 to S phase by hyper-phosphorylating RB, enabling cell cycle progression independently of growth factor stimuli (bypassing the restriction point). The accumulation of CyclinA and the displacement of CyclinE from the CyclinE-CDK2 complex facilitate the formation of the CyclinA-CDK2 complex, which drives S phase entry, progression, and DNA synthesis. Following DNA replication, the CyclinA-CDK1 complex triggers entry into mitosis. This is followed by the formation and activation of the CyclinB-CDK1 complex, which is necessary for the completion of proper cell division. The roles of activating proteins (such as CAK and CDC25A/B/C) and inhibitory proteins (such as CDK inhibitors, WEE1, and MYT1) on specific cyclin-CDK complexes are indicated by black arrows. Abbreviations used: CAK complex (CDK Activating Kinase complex) and CDC25 (Cell Division Cycle 25). Adapted from 1. "Cell Cycle Checkpoints", "RAS Pathway", by BioRender.com, 2024. 2. Pellarin, Dall'Acqua, Favero, et al. Cyclin-dependent protein kinases and cell cycle regulation in biology and disease. *Nature: Signal Transduction and Targeted Therapy* **10**:11, 1-62 (2025).

CDK1, CDK2, CDK4, and CDK6 are all high priority targets for lysate assays.

Regulation of Cyclin Levels During the Cell Cycle Control CDK Activity

Thymidine is a DNA synthesis inhibitor that arrests cells at the G1/S boundary, prior to DNA replication. Colchicine is a DNA synthesis inhibitor that arrests cells at G2/M boundary.

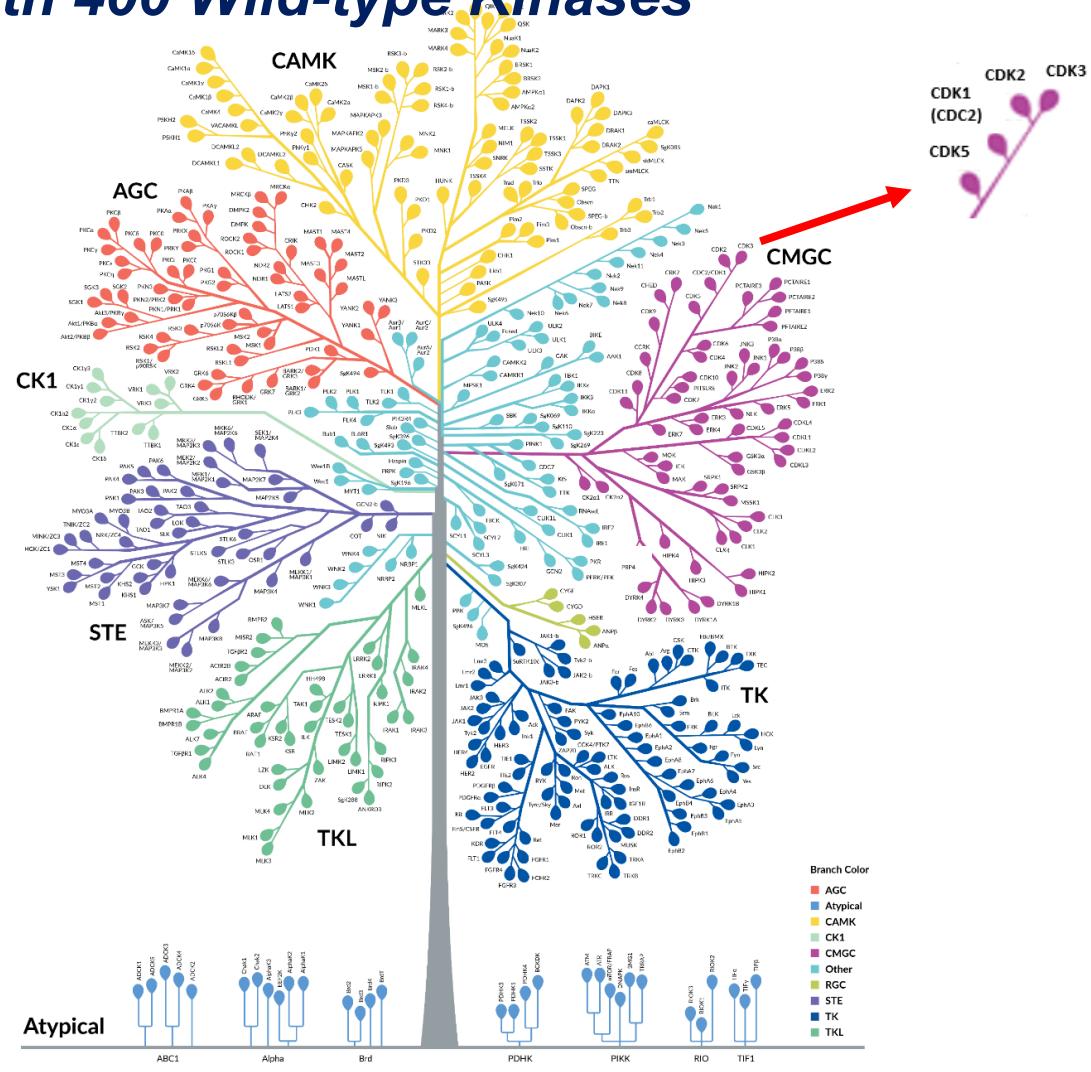


The cell cycle is driven by cyclin-cyclin-dependent kinase (CDK) complexes, which are regulated by the relative protein levels of the four cyclins, since they undergo a cycle of synthesis and degradation in each phase of the cell cycle. The G1/S checkpoint (also called the G1 checkpoint, restriction or R point) occurs near the end of the G1 phase, just before the entry into S phase. In mammalian cells, this is a point at which cells typically arrest the cell cycle if environmental conditions are unfavorable for cell division, such as the presence of DNA damage or a lack of growth factors. The G1 checkpoint is controlled by the inhibition of CDK4 & 6 by INK4 and CDK2 by Cip/Kip families of CDK Inhibitors. Degradation of INK4 and activation of CDK4/6-Cyclin D complexes synthesis of new proteins, including cyclin E. The rise in cyclin E (a G 1 /S cyclin) levels and the activity of CDK2/CyclinE drive the cell past a restriction point, with irreversible commitment to advancing to DNA synthesis. Entry from G2 into M phase allows formation of the mitotic spindle essential for separating chromosomes during cell division. Colchicine prevents spindle fibers from forming thus preventing chromosomes from separating.
Adapted from: https://abdominalkey.com/the-cell-cycle/

High Selectivity for CDK1/2/3/5 with the AQT1271 Sensor Peptide

Top 18 Hits with AQT1271 in Kinome Profiling with 400 Wild-type Kinases

	Kinase target	Enzyme conc. (nM)	Average (RFU/pmol/min)	Selectivity Ratio (RFU/pmol/	% Activity (RFU/pmol/min)
1	CDK2/E1	0.50	4495	1.0	100
2	CDK3/E2	0.50	1472	3.1	33
3	CDK2/A1	0.75	1190	3.8	26
4	CDK5/P25	2.0	827	5.4	18
5	CDK3/E1	3.0	826	5.4	18
6	CDK1/B1	3.0	818	5.5	18
7	CDK2/A2	2.0	718	6.3	16
8	CDK1/E1	1.0	517	8.7	12
9	CDK2/E2	2.0	497	9.0	11
10	CDK5/P35	3.0	384	12	8.5
11	CDK1/A2	5.0	203	22	4.5
12	CDK6/D3	20	41	109	0.92
13	CDK15/B1	20	32	143	0.70
14	CDK2/O	20	20	224	0.45
15	CDK9/T2	8	17	266	0.38
16	CDK4/D3	20	15	308	0.32
17	CDK9/T1	15	10	430	0.23
18	CDK9/K	15	8.8	513	0.19



The 18 kinases with the highest Reaction Rates (RFU/min) in Kinome Profiling for AQT1271 were determined and then sorted by RFU/pmole/min, which adjusts for enzyme concentration. The kinases with the highest Reaction Rates (RFU/pmole/min) were CDK2, CDK3, CDK5, and CDK1. The closest off-target kinases had less than 1% of the activity of CDK2-CyclinE1, which is in the background range.

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Selectivity of AQT1271 with the Top 25 Kinases from AQT's Kinome Profiling

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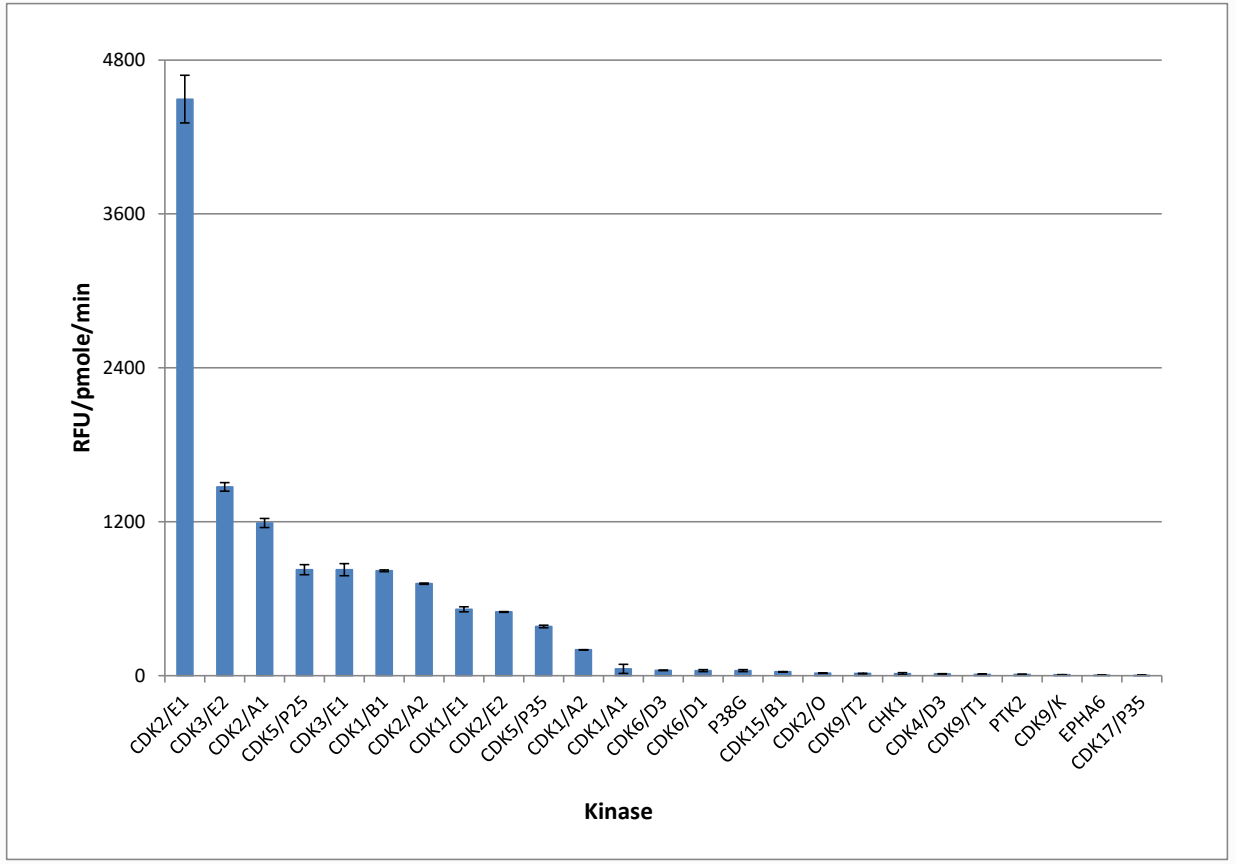
Top 25 Kinases from the Profiling Run of 400 Kinases



High-throughput Milestone Optimization led to the identification of the AQT1271 sensor peptide substrate.

AQT1271 shows selectivity for CDK2, CDK3, CDK5, and CDK1, a critical requirement for accurately measuring the activity of CDK1/2/3/5 in crude lysates

Outline for PhosphoSens-Lysate Assay Validation

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Lysate Source:

- SK-BR-3, NIH-OVCAR3, T98G, M059K, and U-87 MG cell lines, untreated or treated with thymidine (Sigma-Aldrich, T1895-1G) or colchicine (Cayman Chemical, 9000760) to synchronize the cells to a particular growth phase (see Slide 5, 10 and 28 for further details)

Reference Compound Information:

- Ro-3306 (Cayman, 15149), Avotaciclib (BEY1107) (MedChemExpress, HY-137432), CDK2-IN-23 (MedChemExpress, HY-162255), CDK5-IN-3 (MedChemExpress, HY-145694), CDK1/2 Inhibitor III (K00546) (Cayman, 18859), Dinaciclib (Cayman, 14707), and PD0332991 (Palbociclib) (Cayman, 16273)

Experimental Validation at AssayQuant:

- Cell lysate titrations
- AQT1271 substrate K_m determinations
- DMSO Tolerance Tests
- Reference Compound IC_{50} determinations
- CDK1-CycA1 (AQT-C22-18BG), CDK2-CycA1 (AQT-C29-10BG), CDK3-CycE1 (AQT-C30-10G), and CDK5-p25 (AQT-C33-10G) to serve as positive recombinant protein controls
- Phosphopeptide control (AQT1277)
- Assessment of CDK Activity in Multiple Cell Lines +/- Cell Phase Synchronization
- Western blot comparison (in progress)

Summary of CDK 1/2/3/5 Cell Cycle Regulation

- **CDK3** activation enables the cell to exit quiescence (GO) and enter G1 Phase by priming RB phosphorylation. Blocking cells at G1 Phase would be expected to elevate CDK3 levels.
- CDK4/6 complex formation and activation results in further phosphorylation and inactivation of RB, and gene transcription essential for cell cycle progression and DNA replication.
- **CDK2-CycE1** is activated, which drives the transition from G1 Phase to S Phase by hyper-phosphorylating RB and enabling cell cycle progression independent of growth factor stimuli.
- **CDK2-CycA** formation from CDK2 with displaced CycE drives S Phase entry and progression resulting in DNA replication. Blocking cells at S Phase would be expected to elevate CDK2 levels.
- **CDK1-CycA** triggers entry into mitosis, followed by **CDK1-CycB** formation to complete cell division during G2 Phase. Blocking cells at G2 Phase elevates CDK1 levels.
- **CDK5** is a neuronal CDK that promotes neuron migration and the maturation of excitatory synapses. There is also evidence to support its involvement in circadian rhythm, immune response, glucose homeostasis, and angiogenesis.

Pellarin, Dall'Acqua, Favero, et al. Cyclin-dependent protein kinases and cell cycle regulation in biology and disease. *Nature: Signal Transduction and Targeted Therapy* **10**:11, 1-62 (2025).

Summary of Cell Lines Used for CDK 1/2/3/5 Lysate Activity Assays

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Cell Line Information							
#	Cell line	Origin	Stage or Disease	Tissue	Morphology	Growth Properties	Comments
1	NIH-OVCAR-3	Human	Adenocarcinoma	Ovary	Epithelial	Adherent	CDK2-dependent
2	SK-BR-3	Human	Adenocarcinoma	Breast; Mammary gland	Epithelial	Adherent	CDK1-dependent
3	M059K	Human	Glioblastoma, Malignant	Brain	Fibroblast	Adherent	CDK5-dependent
4	T98G	Human	Glioblastoma	Brain	Fibroblast-like	Adherent	CDK3-dependent, High CDK5
5	U87-MG	Human	Glioblastoma	Brain	Epithelial	Adherent	Mod-High CDK1/2/3/5

Preparation of Crude Cell Lysates from SK-BR-3, NIH-OVCAR3, T98G, M059K, and U-87 MG Cells ± Cell Cycle Synchronization

SK-BR-3, NIH-OVCAR3, T98G, M059K, and U-87 MG Cells (passage 3-8) were plated in T-75 flasks and grown to 75% confluency over 48 hours at 37 °C in McCoy's 5A Medium (SK-BR-3), RPMI-1640 Medium (NIH-OVCAR3), DMEM:F12 (M059K), or EMEM (U-87 MG, T98G), with 10% FBS and 1% PenStrep (30-2300) in an atmosphere of 5% CO_2. SK-BR-3, M059K, and U-87 MG cells were given fresh media. NIH-OVCAR3 and T98G cells were incubated with 2.5 mM thymidine for 24 hours (**first block**). After 24 hours, NIH-OVCAR3 and T98G media were removed and replaced with fresh media for 4 hours (**first release**) and then incubated a second time with 2.5 mM thymidine for 24 hours (**second block**). After these 48 hours, the media was removed from the NIH-OVCAR3, M059K, and U-87 MG cells. The cells were then washed once with PBS with calcium and magnesium salts, scraped into 6 mL of this buffer, and then centrifuged for 5 minutes at 2,500 x g. Liquid was removed, and the pellets were placed on ice. The medium was removed from the T98G cells and replaced with fresh media for 4 hours. These cells were then washed, scraped, centrifuged, and the pellets were placed on ice as above. SK-BR-3 cells were incubated with 1 µg/mL colchicine for 7 hours (**first block**). They were then also washed, scraped, centrifuged, and the pellets were placed on ice as above. Cells placed on ice were immediately lysed with 100-300 µL of cold Cell Extraction Buffer (CEB) containing protease (but not phosphatase) inhibitors (see below), triturated to ensure solubilization, and then centrifuged for 5 minutes at 12,000 x g to pellet cellular debris. Lysate supernatants were removed, aliquoted, supplemented with 10% glycerol, and used immediately or frozen at –80 °C; each aliquot was **used only once (one freeze/thaw cycle)**. Long-term storage of lysates is not recommended, *as activity may decline over time.*

Note: SK-BR-3 cells were treated with colchicine to synchronize them to G2/M Phase (CDK1). NIH-OVCAR3 cells were treated with a double thymidine block to synchronize them to G1/S Phase (CDK2), T98G cells were treated with thymidine followed by growth medium with 10% FBS to synchronize to G1 Phase (CDK3).

Cell Extraction Buffer (CEB) with protease inhibitors:

Note: No phosphatase inhibitors are added to the CEB B Buffer because there are phosphatases that activate some of the CDKs.

- 50 mM HEPES, pH 7.4
- 150 mM NaCl
- 2 mM EGTA
- 1 mM DTT
- 1% Triton X-100
- Protease Inhibitor Cocktail diluted 60-fold into lysis buffer

Lysate Titration with the AQT1271 Sensor Peptide

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 AQT1271 sensor peptide substrate

0, 0.010, 0.020, 0.039, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, 5.0, 10 µg/well **crude cell lysate** from **SK-BR-3, NIH-OVCAR3, T98G, M059K, and U-87 MG Cells**

Reaction Set Up:

20 µL Reaction Mix with AQT1271, ATP, & DTT
Seal plate and incubate at 30 °C for 15 minutes to equilibrate
5 µL Enzyme dilution buffer (EDB) with CEB Lysate Buffer (1x) or CDK Lysate (5x in EDB)
25 µL Final reaction volume

Reaction was run at 30˚C for 480 minutes in Corning, low volume 384-well, white flat-bottom polystyrene NBS microplates (Cat. #3824) at 25 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.

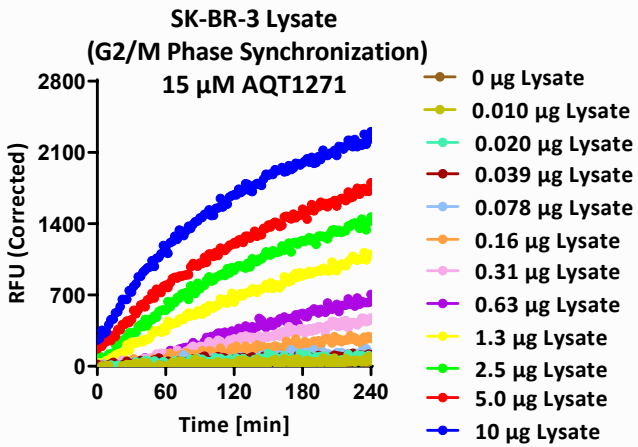
Notes:

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.

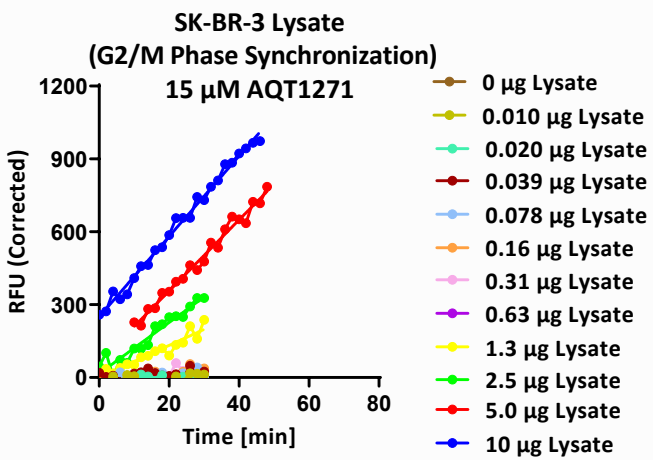
SK-BR-3 Lysate Titration with the AQT1271 Sensor Peptide

Progress Curves

Complete Progress Curves



Linear Region of Progress Curves



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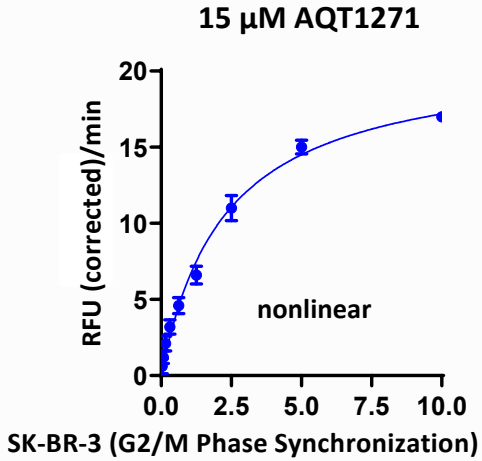
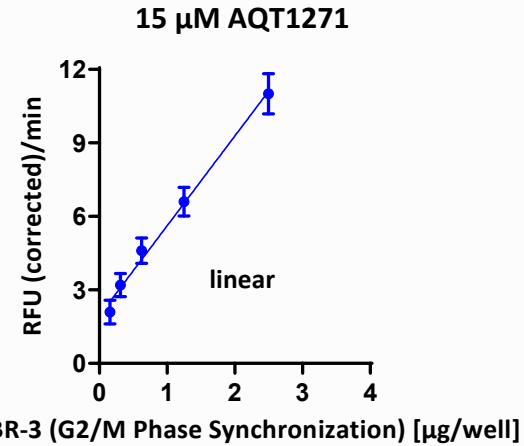


Linear Reaction

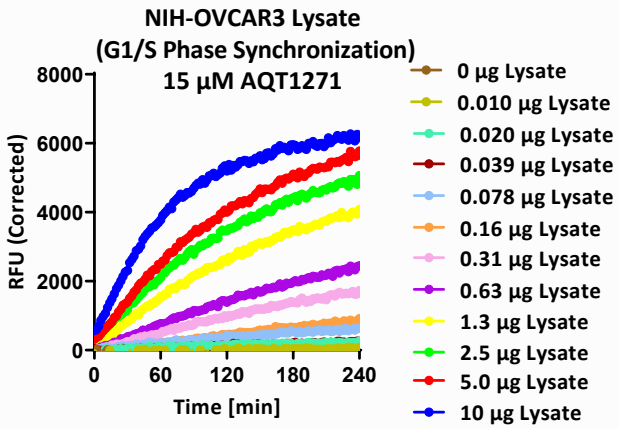


CDK1 Lysate Assay is linear from 0.16 – 2.5 µg/well of lysate (16-fold)

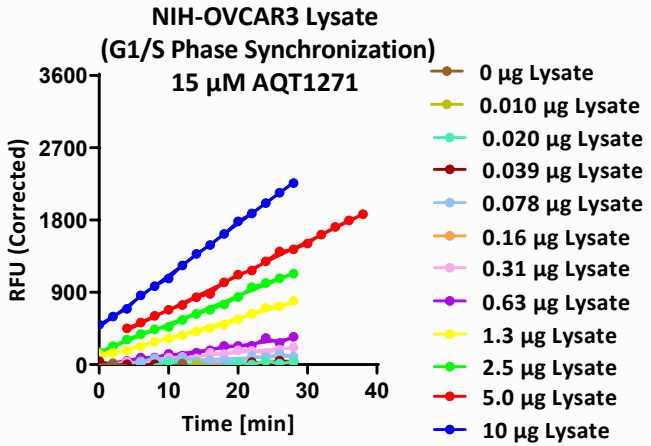
NIH-OVCAR3 Lysate Titration with the AQT1271 Sensor Peptide

Progress Curves

Complete Progress Curves



Linear Region of Progress Curves



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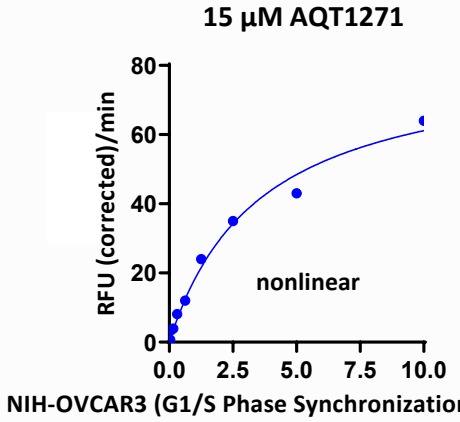
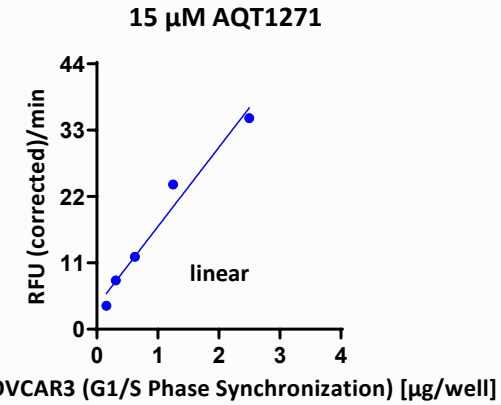


Linear Reaction

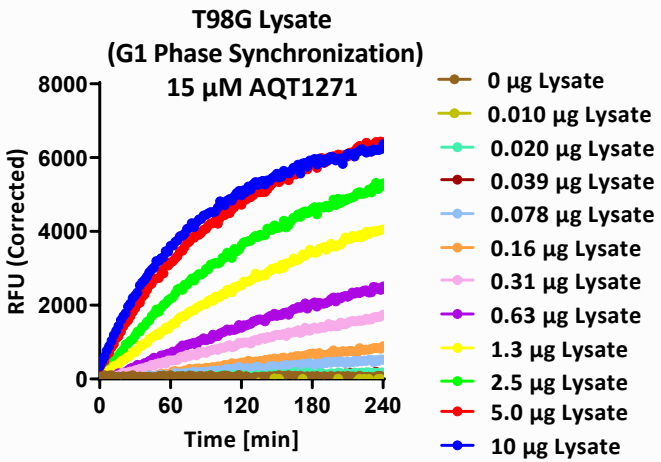


CDK2 Lysate Assay is linear from 0.16 – 2.5 µg/well of lysate (16-fold)

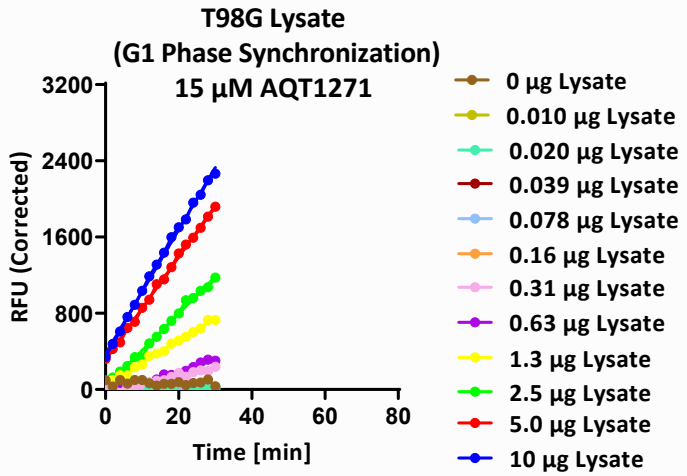
T98G Lysate Titration with the AQT1271 Sensor Peptide

Progress Curves

Complete Progress Curves



Linear Region of Progress Curves



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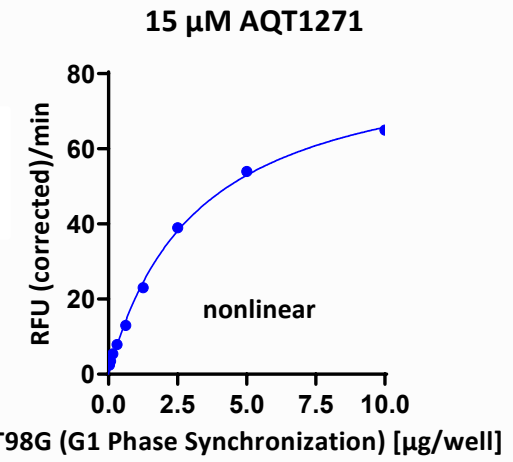
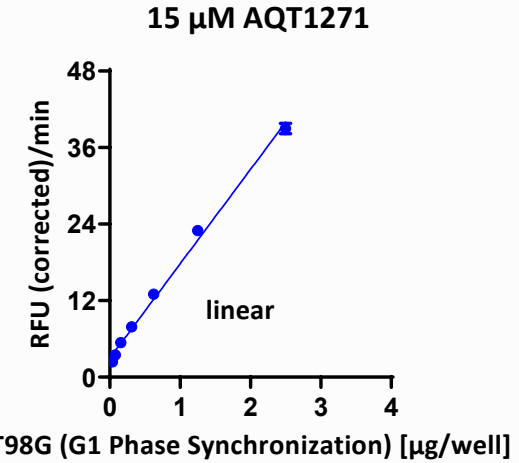


Linear Reaction

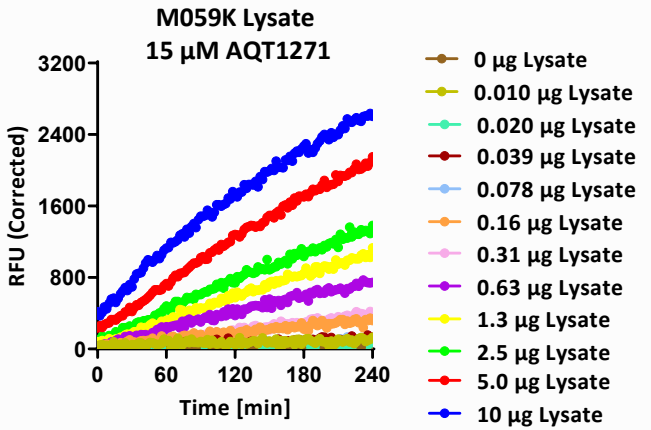


CDK3 Lysate Assay is linear from 0.038 – 2.5 µg/well of lysate (66-fold)

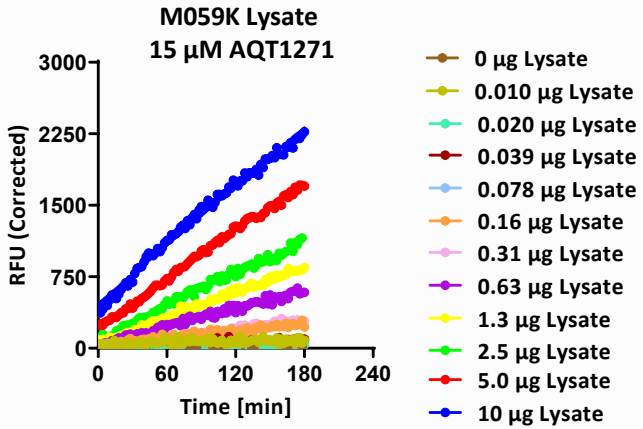
MO59K Lysate Titration with the AQT1271 Sensor Peptide

Progress Curves and Linearity Plots with Lysate from M059K Cells

Complete Progress Curves



Linear Region of Progress Curves



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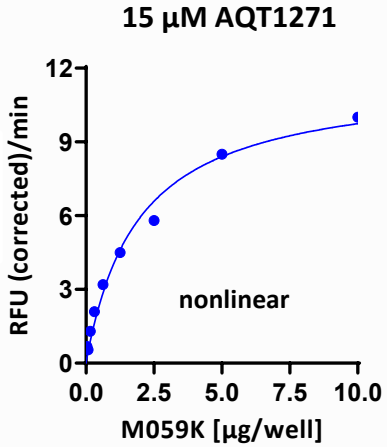
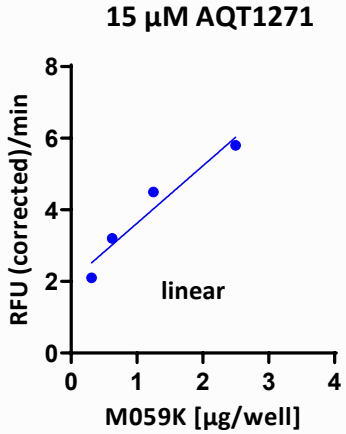


Linear Reaction

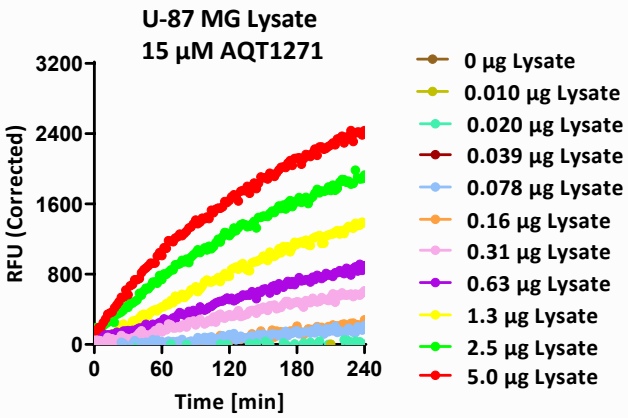


CDK5 Lysate Assay is linear from 0.31 – 2.5 µg/well of lysate (8-fold)

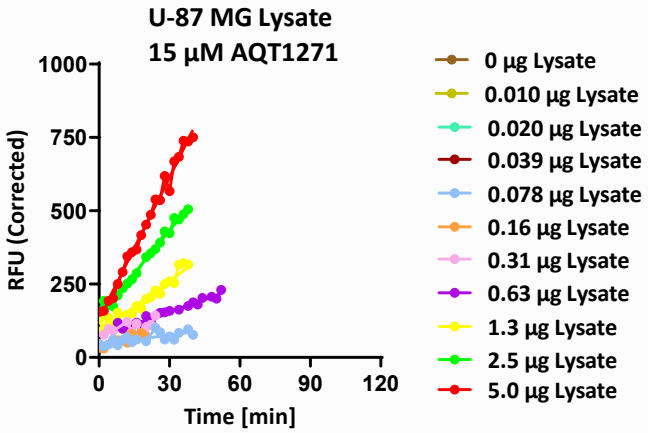
U-87 MG Lysate Titration with the AQT1271 Sensor Peptide

Progress Curves and Linearity Plots with Lysate from U-87 MG Cells

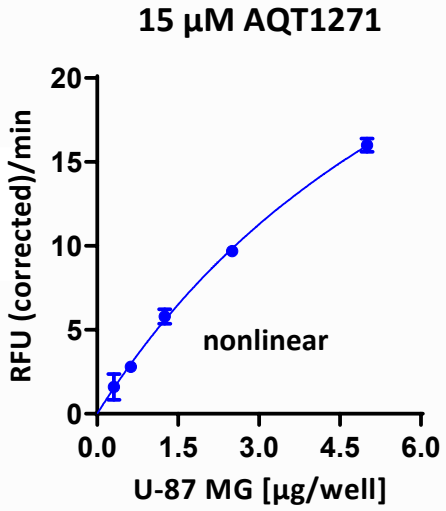
Complete Progress Curves



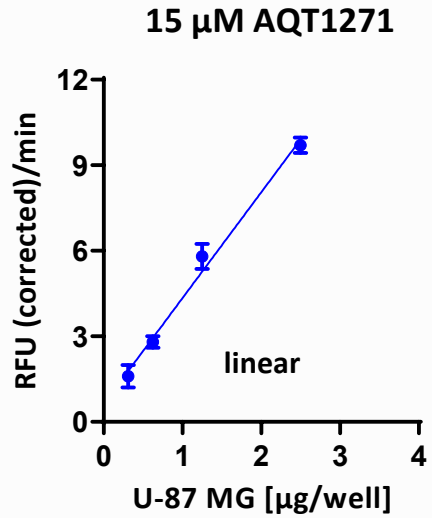
Linear Region of Progress Curves



Nonlinear Reaction



Linear Reaction



CDK 1/2/3/5 Lysate Assay is linear from 0.31 – 2.5 µg/well of lysate (8-fold)

IC_{50} Determination with the AQT1271 Sensor Peptide

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 AQT1271 sensor peptide substrate

Compounds:

- Dinaciclib, K00546, Ro-3306, Avotaciclib, CDK2-IN-23, and CDK5-IN-3 were titrated with 3-fold dilutions in 100% DMSO at 50X the final concentrations and then diluted 50-fold into the assay for final concentrations from 0-10 µM in 2% DMSO.

Cell Lysate for IC_{50} determination:

2.0 µg/well U-87 MG, SK-BR-3, NIH-OVCAR3, T98G, and M059K Cell Lysate

Reaction Set Up:

0.5 µL 50X Inhibitor diluted in 100% DMSO or DMSO alone
19.5 µL Reaction Mix with AQT1271, ATP & DTT
Seal plate and incubate at 30 °C for 15 minutes to equilibrate
5 µL Enzyme dilution buffer (EDB) with CEB Lysate Buffer (1x) or CDK Lysate (5x in EDB) 25 µL Final reaction volume

Reaction was run at 30˚C for 480 minutes in Corning, low volume 384-well, white flat-bottom polystyrene NBS microplates (Cat. #3824) at 25 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:

1X Enzyme Dilution Buffer (EDB) is used to dilute enzyme and for the blank. Composition is 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.

Ro-3306 IC_{50} Determination with SK-BR-3 Lysate from Cells Synchronized to G2/M Phase and using the AQT1271 Sensor Peptide

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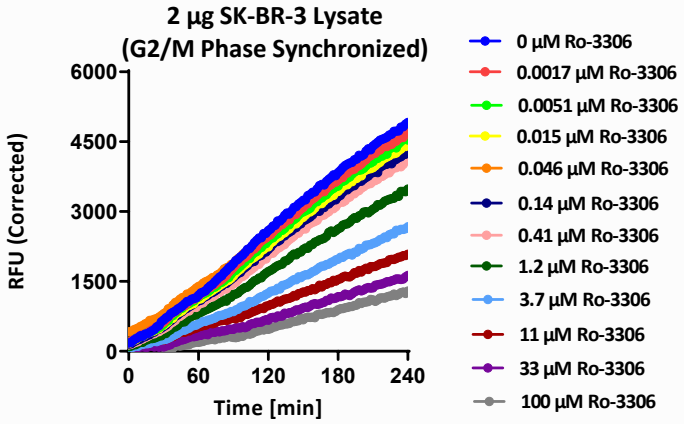
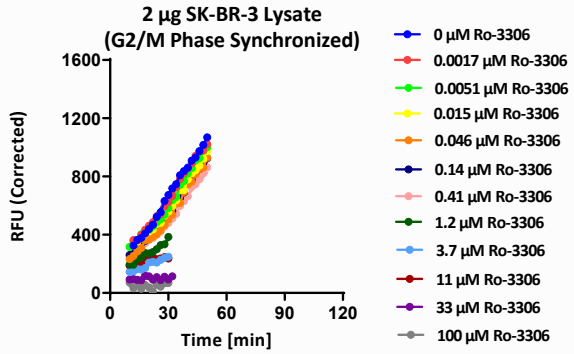
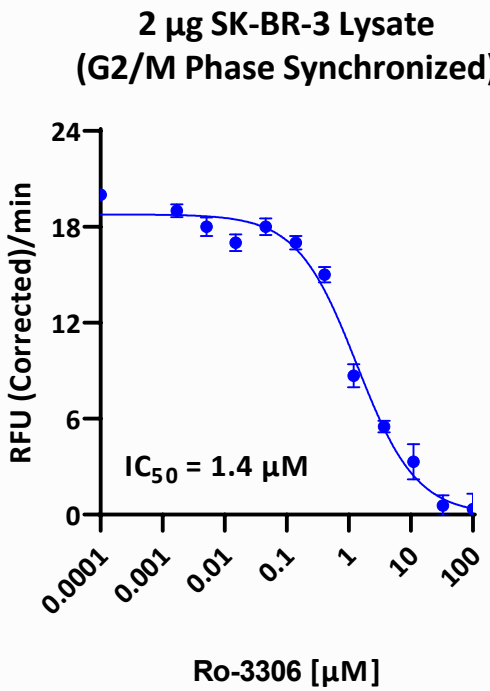
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



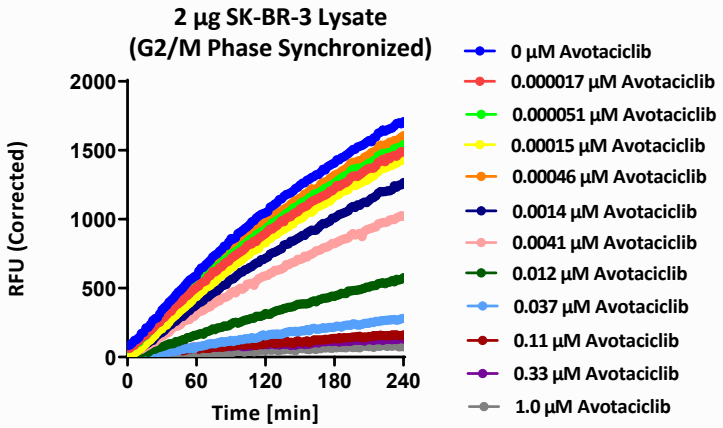
[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	18.78
IC50	1.350
HillSlope	-0.9034
logIC50	0.1305
Span	18.78
95% CI (profile likelihood)	
Top	17.78 to 19.88
IC50	0.9477 to 1.907
HillSlope	-1.214 to -0.6888
logIC50	-0.02334 to 0.2804
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9881
Sum of Squares	7.588
Sy.x	0.9182
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

IC_{50} value for Ro-3306 in SK-BR-3 Cell Lysate from cells synchronized G2/M phase *is 1.4 µM.*

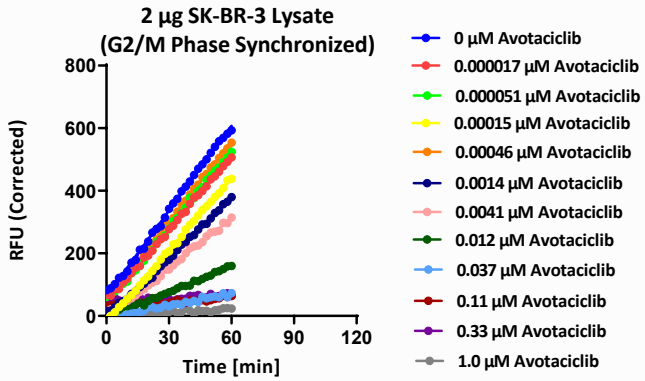
Avotaciclib IC_{50} Determination with SK-BR-3 Lysate from Cells Synchronized to G2/M Phase and Using the AQT1271 Sensor Peptide

AssayQuant TECHNOLOGIES INC.

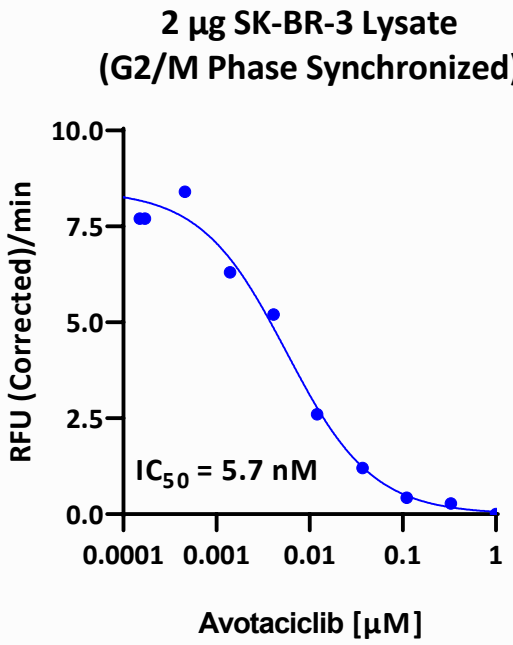
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



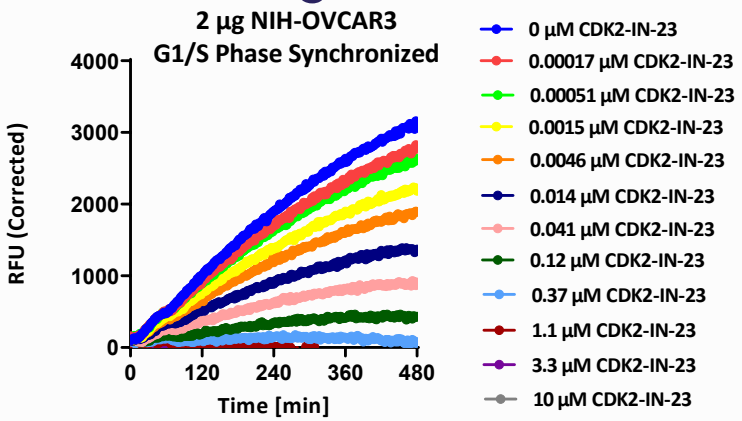
[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	8.443
IC50	0.005654
HillSlope	-0.9427
logIC50	-2.248
Span	8.443
95% CI (profile likelihood)	
Top	7.884 to 9.125
IC50	0.003722 to 0.008194
HillSlope	??? to -2.429
logIC50	2.087
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9882
Sum of Squares	1.681
Sy.x	0.4322
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

IC_{50} value for Avotaciclib in SK-BR-3 Cell Lysate from cells synchronized G2/M phase *is 5.7 nM.*

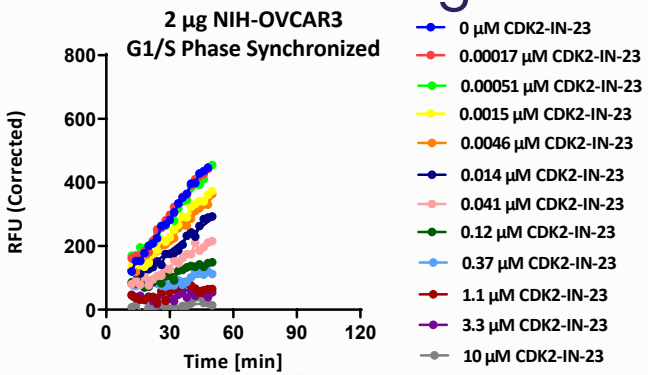
CDK2-IN-23 IC_{50} Determination with NIH-OVCAR3 Lysate from Cells Synchronized to G1/S Phase and Using the AQT1271 Sensor Peptide

AssayQuant TECHNOLOGIES INC.

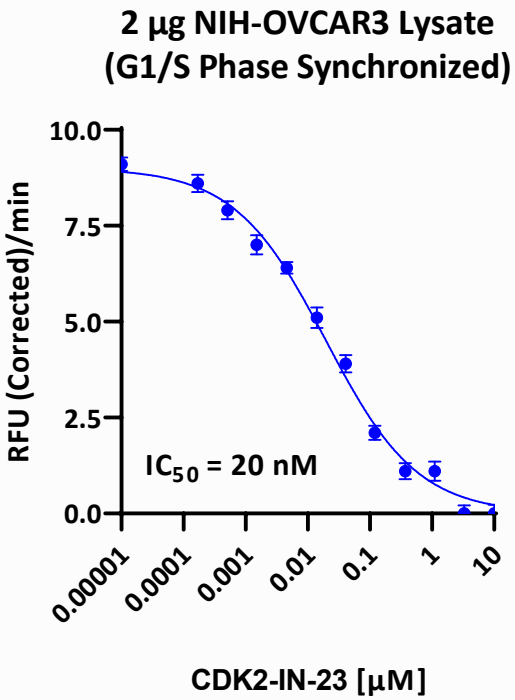
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



[Inhibitor] vs. response -- Variable slope (four parameters)

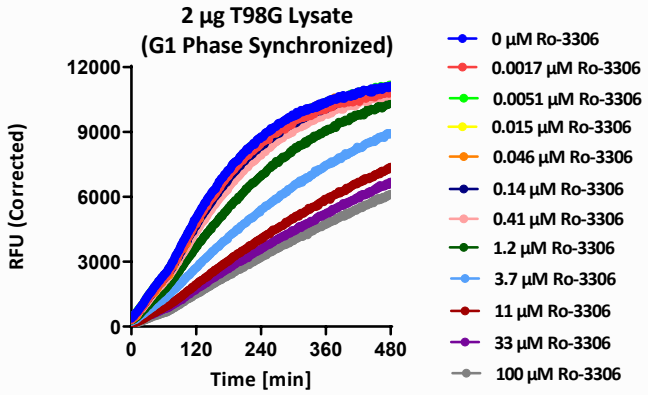
Best-fit values	
Bottom	= 0.000
Top	9.007
IC50	0.01971
HillSlope	-0.5902
logIC50	-1.705
Span	9.007
95% CI (profile likelihood)	
Top	8.431 to 9.684
IC50	0.01246 to 0.02961
HillSlope	-0.7155 to -0.4923
logIC50	-1.904 to -1.529
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9936
Sum of Squares	0.8270
Sy.x	0.3031
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

IC_{50} value for CDK2-IN-23 in NIH-OVCAR3 Cell Lysate from cells synchronized G1/S phase *is 20 nM.*

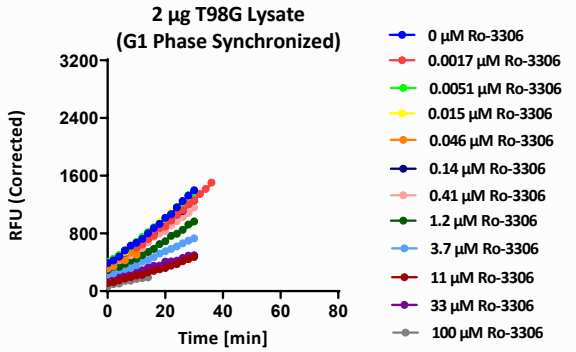
Ro-3306 IC_{50} Determination with T98G Lysate from Cells Synchronized to G1 Phase and Using the AQT1271 Sensor Peptide

AssayQuant TECHNOLOGIES INC.

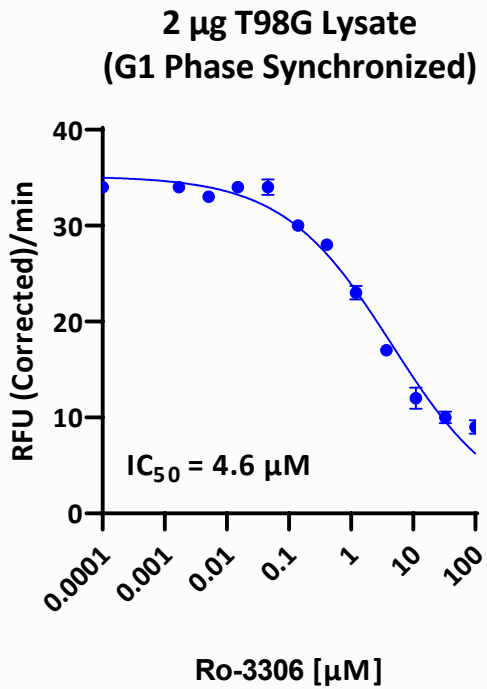
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



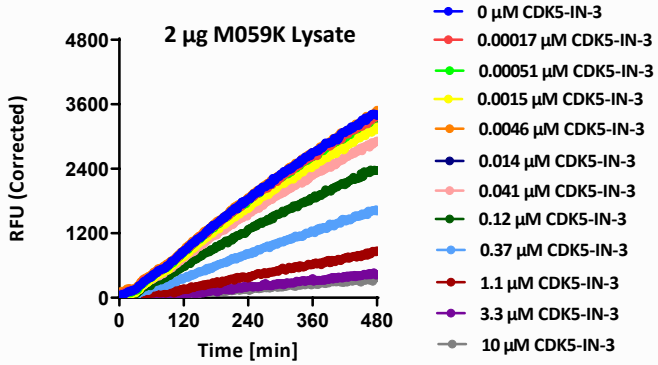
[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	35.18
IC50	4.555
HillSlope	-0.4965
logIC50	0.6585
Span	35.18
95% CI (profile likelihood)	
Top	33.17 to 37.56
IC50	2.730 to 7.421
HillSlope	-0.6286 to -0.3888
logIC50	0.4361 to 0.8705
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9811
Sum of Squares	21.51
Sy.x	1.546
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

IC_{50} value for Ro-3306 in T98G Cell Lysate from cells synchronized G1 phase *is 4.6 µM.*

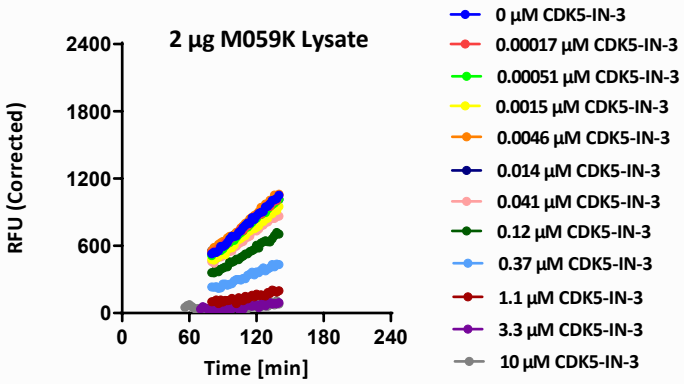
CDK5-IN-3 IC_{50} Determination with M059K Cell Lysate Using the AQT1271 Sensor Peptide

Titration Curves and Inhibition Plot with Lysate from M059K Cells

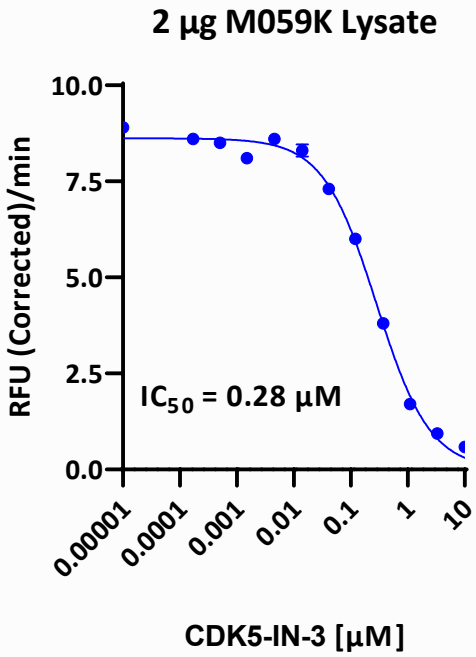
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	8.621
IC50	0.2836
HillSlope	-0.9259
logIC50	-0.5473
Span	8.621
95% CI (profile likelihood)	
Top	8.376 to 8.875
IC50	0.2343 to 0.3426
HillSlope	-1.089 to -0.7941
logIC50	-0.6303 to -0.4652
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9957
Sum of Squares	0.5083
Sy.x	0.2376
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

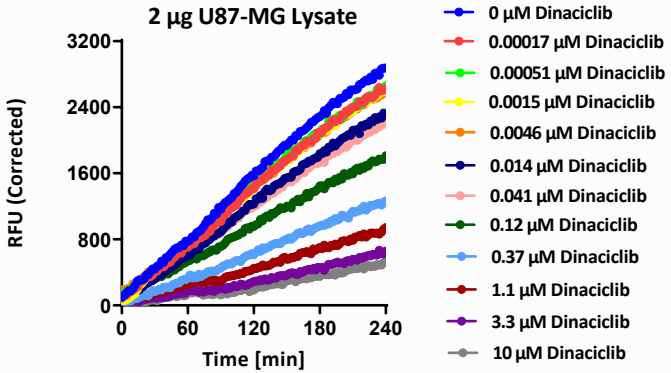
IC_{50} value for CDK5-IN-3 in M059K Cell Lysate is 280 nM

Dinaciclib IC_{50} Determination with U-87 MG Cell Lysate Using the AQT1271 Sensor Peptide

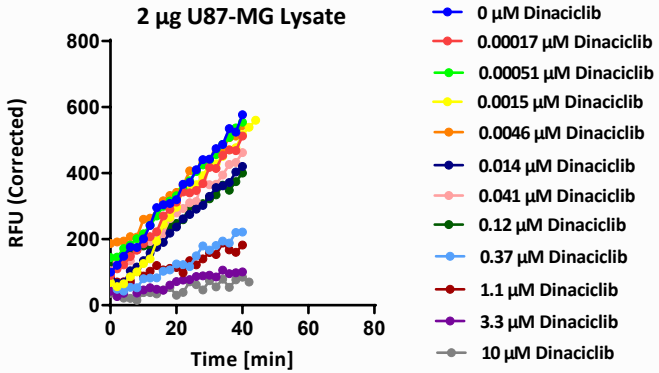
Titration Curves and Inhibition Plot with Lysate from U-87 MG Cells

AssayQuant TECHNOLOGIES INC.

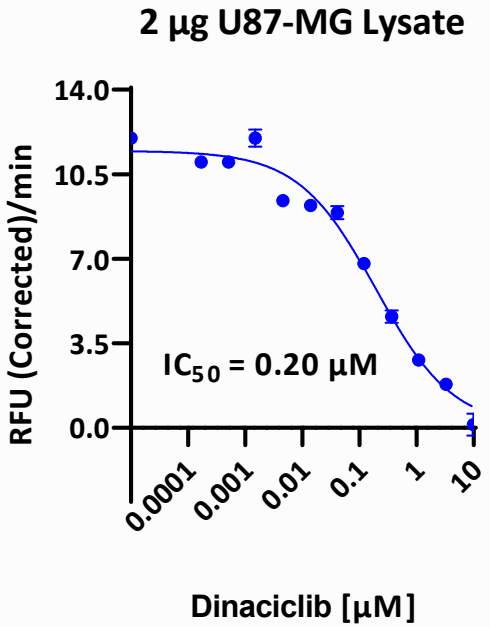
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.000
Top	11.47
IC50	0.1954
HillSlope	-0.6405
logIC50	-0.7092
Span	11.47
95% CI (profile likelihood)	
Top	10.64 to 12.47
IC50	0.1065 to 0.3343
HillSlope	-0.8855 to -0.4724
logIC50	-0.9725 to -0.4759
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9799
Sum of Squares	3.854
Sy.x	0.6544
Constraints	
Bottom	Bottom = 0
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

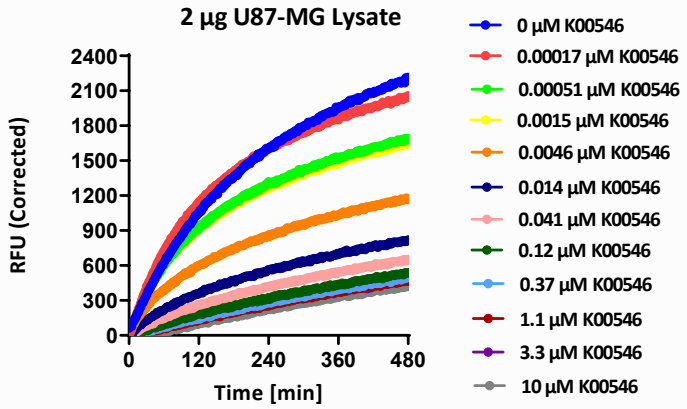
IC_{50} value for Dinaciclib in U-87 MG Cell Lysate is 0.20 µM.

K00546 IC_{50} Determination with U-87 MG Cell Lysate Using the AQT1271 Sensor Peptide

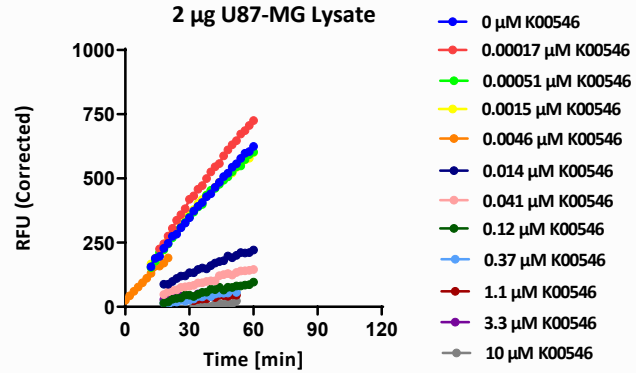
Titration Curves and Inhibition Plot with Lysate from U-87 MG Cells

AssayQuant TECHNOLOGIES INC.

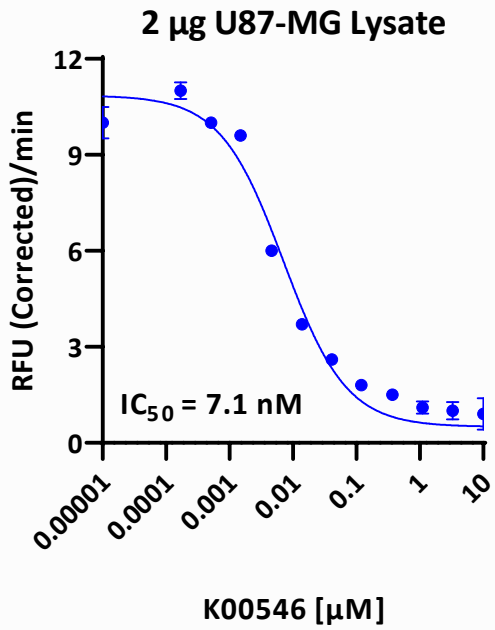
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



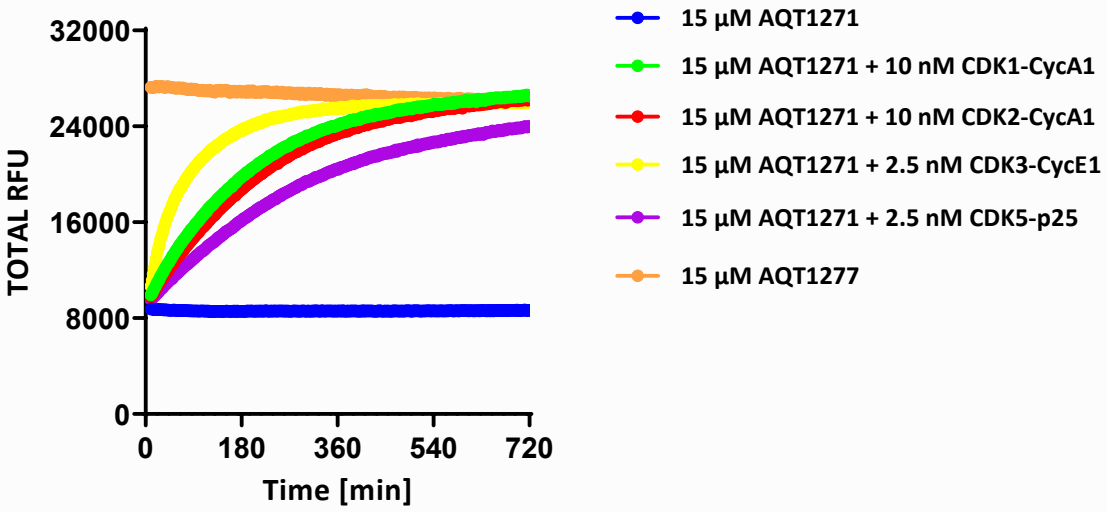
[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.5000
Top	10.86
IC50	0.007080
HillSlope	-0.8750
logIC50	-2.150
Span	10.36
95% CI (profile likelihood)	
Top	9.862 to 12.05
IC50	0.004254 to 0.01162
HillSlope	???
logIC50	-2.371 to -1.935
Goodness of Fit	
Degrees of Freedom	9
R squared	0.9800
Sum of Squares	3.712
Sy.x	0.6422
Constraints	
Bottom	Bottom = 0.5
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

IC_{50} value for K00546 in U-87 MG Cell Lysate is 7.1 nM.

CDK 1/2/3/5 Recombinant Activity Assay with AQT1271 Sensor Peptide and Phospho-control AQT1277

AssayQuant TECHNOLOGIES INC.

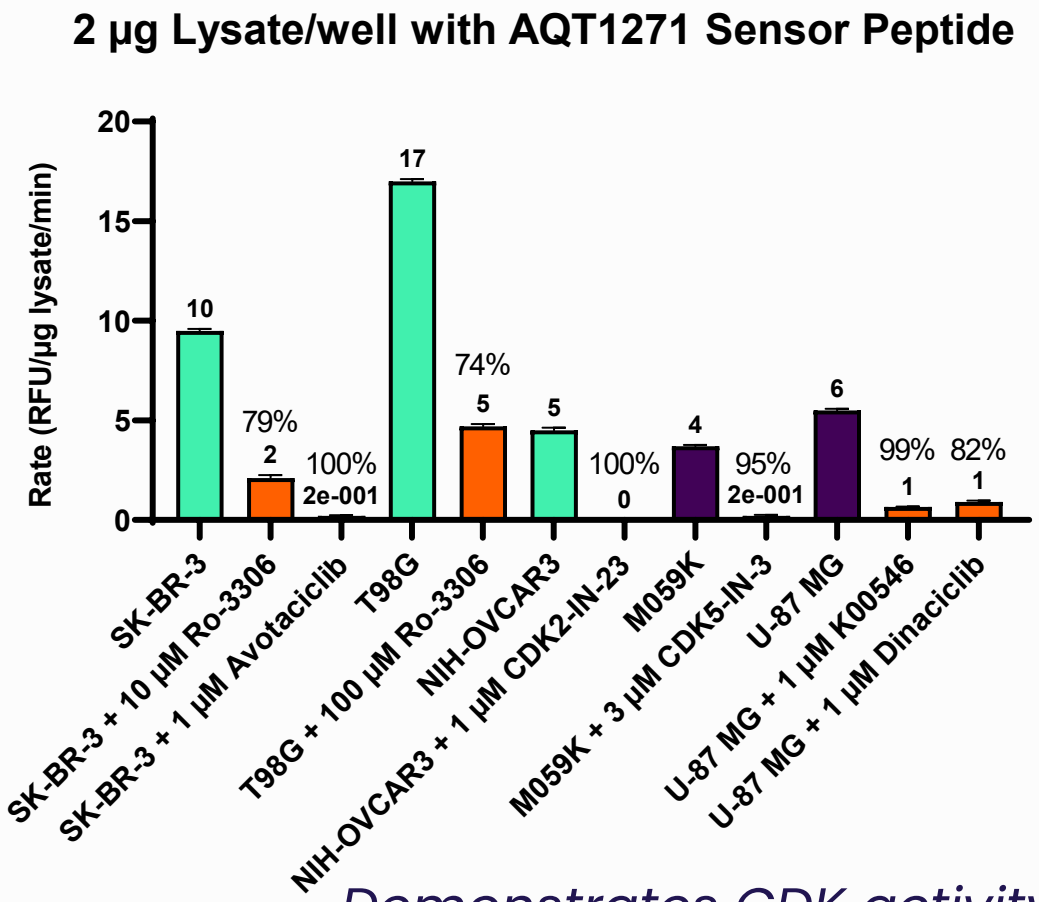
Full Time Course (0-12 hours)



Time course for phosphorylation of the AQT1271 sensor peptide substrate (15 µM) ± CDK1-CycA1, CDK2-CycA1, CDK3-CycE1, and CDK5-p25 recombinant enzymes, compared to the signal with the AQT1277 phospho-peptide control (15 µM): Full-length CDK-Cyclin proteins fully phosphorylated the AQT1271 sensor peptide substrate by 12 hours, as shown by convergence with the signal obtained with the AQT1277 phospho-peptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). CDK5-p25 has not quite reached maximum, but is approaching the others. The signal with AQT1277 is used to convert RFU (Corrected) values to nmoles of phospho-peptide product/minute.

AQT1277 is a chemically synthesized phospho-peptide control for AQT1271

CDK 1/2/3/5 Lysate Activity Assay Using the AQT1271 Sensor Peptide and Multiple CDK Isoform Dependent Cell Types with and without Cell Cycle Phase Synchronization



SK-BR-3 (CDK1-dependent), NIH-OVCAR3 (CDK2-dependent), T98G (CDK3-dependent), and M059K (CDK5-dependent) cells were treated as described on slide 10 to optimize the levels of specific CDKs through cell cycle synchronization (**Green bars**). CDK activity in lysates from these cells was measured using AQT1271 and CDK isoform-selective inhibitors (**Orange bars**) within the expected range, based on IC_{50} values for each recombinant CDK isoform.

Demonstrates CDK activity in CDK isoform-dependent cell lines, which is inhibited by CDK isoform-selective inhibitors

Summary Table – Assay Development

		CDK Assay Development & Related Values						
Cell Line	**CDK Dependency** (e.g., for proliferation, viability & tumorigenicity)	**Cell Cycle Synchronization (Method)**	**CDK Assay Target**	**Lysate/ Reaction**	**AQT1271, Final**	**Lysate Linear Range**	**AQT1271 K_m**	**DMSO Tolerance (final %)**
SK-BR-3	CDK1	Phase G2/M boundary (Colchicine)	CDK1	2.0 µg	15 µM	0.16 - 2.5 µg (16-fold)	14 µM	0-10
NIH-OVCAR3	CDK2	Phase G1/S boundary (Double Thymidine)	CDK2	2.0 µg	15 µM	0.16 - 2.5 µg (16-fold)	11 µM	0-10
T98G	CDK3	Phase G1 (Thymidine, then EMEM + 10% FBS)	CDK3	2.0 µg	15 µM	0.038 - 2.5 µg (66-fold)	17 µM	0-10
M059K	CDK5	None; High CDK5 levels	CDK5	2.0 µg	15 µM	0.31 - 2.5 µg (8-fold)	15 µM	0-5
U-87 MG	Defects in tumor suppressor pathways (e.g., pRB) constitutively activate multiple CDKs	None; Moderate-High levels CDK1-3 & 5	CDK1/2/ 3/5	2.0 µg	15 µM	0.31 - 2.5 µg (8-fold)	16 µM	0-10

Titrations for AQT1271 and DMSO with each individual CDK Lysate available upon request.

Summary Table – IC_{50} Values with Lysate vs Recombinant CDK

				CDK Inhibitor IC_{50}, nM, with Lysate or Recombinant Purified Enzyme								
Cell Line	**CDK Dependency** (e.g., for proliferation, viability & tumorigenicity)	**Cell Cycle Synchronization (Method)**	**CDK Assay Target**	**CDK Specific Control Inhibitor**	**Lysate**	**CDK1-CycA1**	**CDK1-CycB1**	**CDK2-CycA1**	**CDK2-CycE1**	**CDK3-CycE1**	**CDK5-p25**	**CDK5-p35**
SK-BR-3	CDK1	Phase G2/M boundary (Colchicine)	CDK1	Ro-3306 (CDK1/3) Avotaciclib (pan CDK)	1400 5.7	1653 9.7	2022 1.8	-	-	2757 -	-	-
NIH-OVCAR3	CDK2	Phase G1/S boundary (Double Thymidine)	CDK2	CDK-IN-23 (CDK2)	20	-	-	32	12	-	-	-
T98G	CDK3	Phase G1 (Thymidine, then EMEM + 10% FBS)	CDK3	Ro-3306 (CDK1/3)	4,600	-	-	-	-	2757	-	-
M059K	CDK5	None; High CDK5 levels	CDK5	CDK5-IN-3 (CDK5)	280	-	-	-	-	-	384	93
U-87 MG	Defects in tumor suppressor pathways (e.g., pRB) constitutively activate multiple CDKs	None; Moderate-High levels CDK1-3 & 5	CDK1/2/3/5	Dinaciclib (pan CDK) K00546 (pan CDK)	200 7.1	- 5.8	- -	- 8.4	- -	- 3.2	- 12	- -

IC_{50} values for the individual lysates are comparable (within 2-3-fold) to those obtained with recombinant enzyme. Individual IC_{50} determinations available upon request.

Summary for CDK 1/2/3/5 Lysate Activity Assays Using the AQT1271 Sensor Peptide

AssayQuant® TECHNOLOGIES INC.

- The PhosphoSens-Lysate Assay for CDK1/2/3/5 with the AQT1271 selective sensor peptide provides a robust, sensitive, and physiologically relevant assay to selectively measure endogenous CDK activity with cellular components/signaling complexes in 2 µg of crude lysate/test.
- Results include:
  - **CDK1-3:**
    - **CDK1** lysate activity is expected in SK-BR-3 cells (CDK1 dependent) synchronized to G2/M Phase of the cell cycle with colchicine, and this was entirely inhibited by Ro-3306, a specific CDK1/3 inhibitor, with an IC_{50} value of 1.4 µM. SK-BR-3 and T98G cell lysates were also potently inhibited by Avotaciclib (pan CDK inhibitor); however, this inhibitor is a potent, yet nonspecific, CDK1 inhibitor.
    - **CDK2** lysate activity is expected in NIH-OVCAR3 cells (CDK2 dependent) synchronized to G1/S Phase of the cell cycle with a double thymidine block, and this was entirely inhibited by CDK2-IN-23, a potent, specific CDK2 inhibitor, with an IC_{50} value of 20 nM.
    - **CDK3** lysate activity is expected in T98G cells (CDK3 dependent, Radiation-sensitive glioblastoma) synchronized to G1 Phase of the cell cycle with a double thymidine block followed by 10% FBS, and this was inhibited by Ro-3306, a specific CDK1/3 inhibitor, with an IC_{50} value of 4.6 µM (90% inhibition at 10 µM; the residual 10% may be due to CDK5 activity, which is low in G1, in these glioblastoma cells).
  - **CDK5:** Radiation-resistant Glioblastoma Lines: M059K cell lysate activity inhibited (95%) by 3 µM of CDK5-IN-3 (IC_{50} value of 280 nM), a specific CDK5 inhibitor. With lysates from U-87 MG cells, K00546, a CDK1/2/5 inhibitor, resulted in a 7.1 nM IC_{50} (87% inhibition).
  - **AQT1271 Sensor Peptide K_m:** Values for SK-BR-3, NIH-OVCAR3, T98G, M059K, and U-87 MG Lysates were 14, 11, 17, 15, and 16 µM, respectively.

These CDK activity measurements are direct, highly quantitative, and in an easy-to-use format. This enables the functional assessment of native CDK1/2/3/5 activity in complex samples using crude cell lysates or tissue homogenates, thereby providing a more physiological and economical approach to studying CDK1/2/3/5 kinases.