***PhosphoSens*® Cell Lysate Activity Assay Format**

DNA-PK Assay Validation Using the AQ0440 Selective Sensor Peptide

HGNC Name: PRKDC (DNA-PK)
Long Names: DNA-dependent protein kinase catalytic subunit

INTRODUCING A NEW INITIATIVE FROM OUR DIRECT-TO-PHASE II NIH/NCI SBIR AWARD ($2M):

PhosphoSens-Lysate

PhosphoSens-Lysate assays are here!

Experience the Benefits of Running Kinase Activity Assays in Complex Samples

Study Kinase Enzyme Activity Where Biology Happens

Simply Lyse Cells & Read
No Fractionation Needed

PhosphoSens-Lysate Assays transform kinase activity measurement by enabling continuous, real-time kinetic analysis directly in cell and tissue lysates. This innovative approach provides key insights into your target's native environment throughout the drug discovery pipeline.

NEW PRODUCTS – Available Now for ERK1/2, AKT1/2/3, GSK3α/β, JNK1-3, and DNA-PK!

COMING SOON – From our active R&D pipeline of selective sensor peptide substrates for:
p38A/B/G/D, CDK1-3/5, CDK4/6, MEK1/2, SGK1/2/3, and PIM1/3.

PhosphoSens-Lysate Assays

A Simple & Powerful Solution to a Complex Problem

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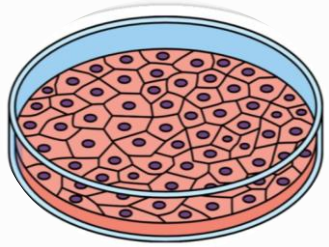
1. Grow Cells

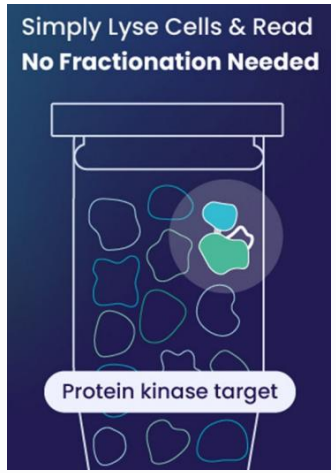
(+/- Pathway Activation)

or Access Tissues



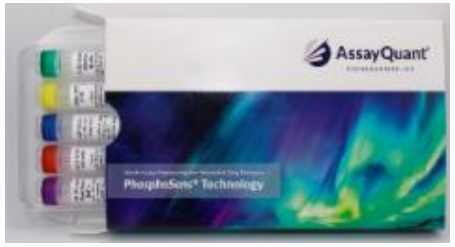
2. Harvest

ADD *PhosphoPreserve*
Lysis Buffer + Protease & Phosphatase Inhibitors



Study Kinase Enzyme Activity Where Biology Happens

We have combined innovative PhosphoSens detection technology, invented at MIT, with high-throughput peptide synthesis methods to design sensor peptide substrates that are highly selective for the target of interest. This development integrates the advantages of the PhosphoSens platform (activity-based, direct, homogeneous, continuous/kinetic, quantitative) with the enabling capability of measuring endogenous target kinase activity in unfractionated cell or tissue lysates.



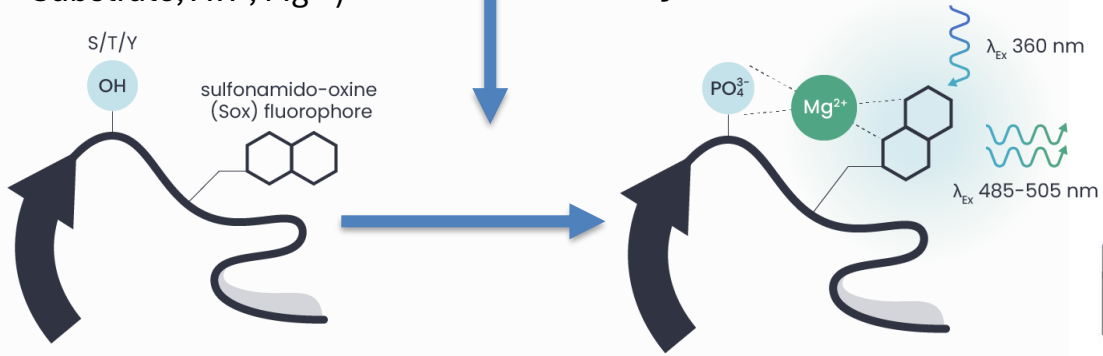
Easy-to-use Kits & Bulk Sensor Peptide

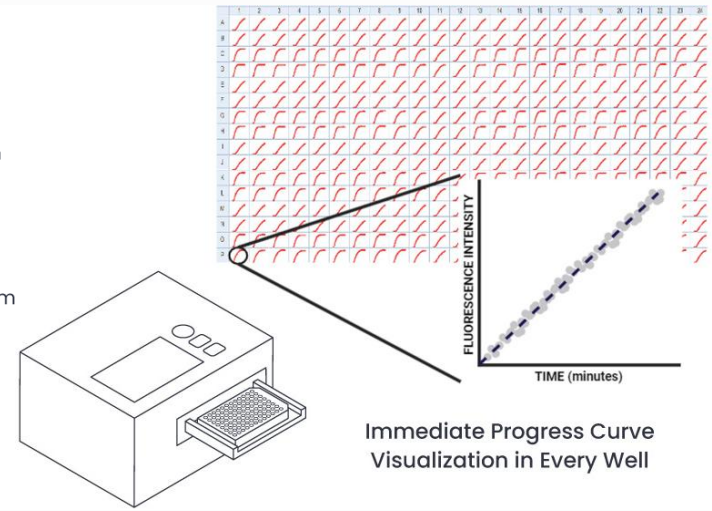
(Native substrates, stable, scalable, excellent lot consistency)

3. Prepare Reaction Mix

(PhosphoSens Sensor Peptide Substrate, ATP, Mg^{2+})

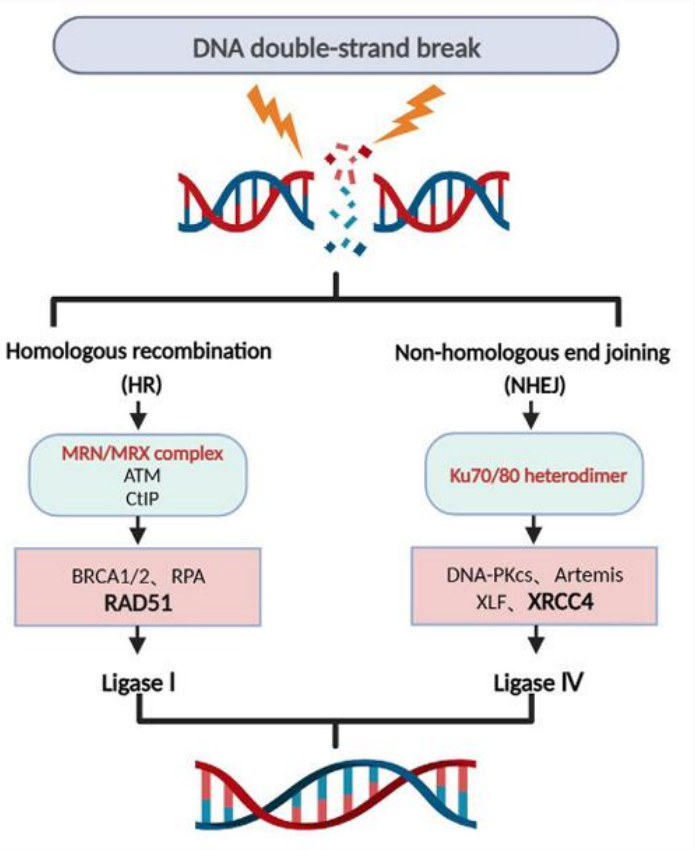
ADD Lysate & Read





Immediate Progress Curve Visualization in Every Well

DNA-PK Plays a Critical Role in the Repair of Double-strand DNA Breaks from Ionizing Radiation Used in Cancer Radiotherapy



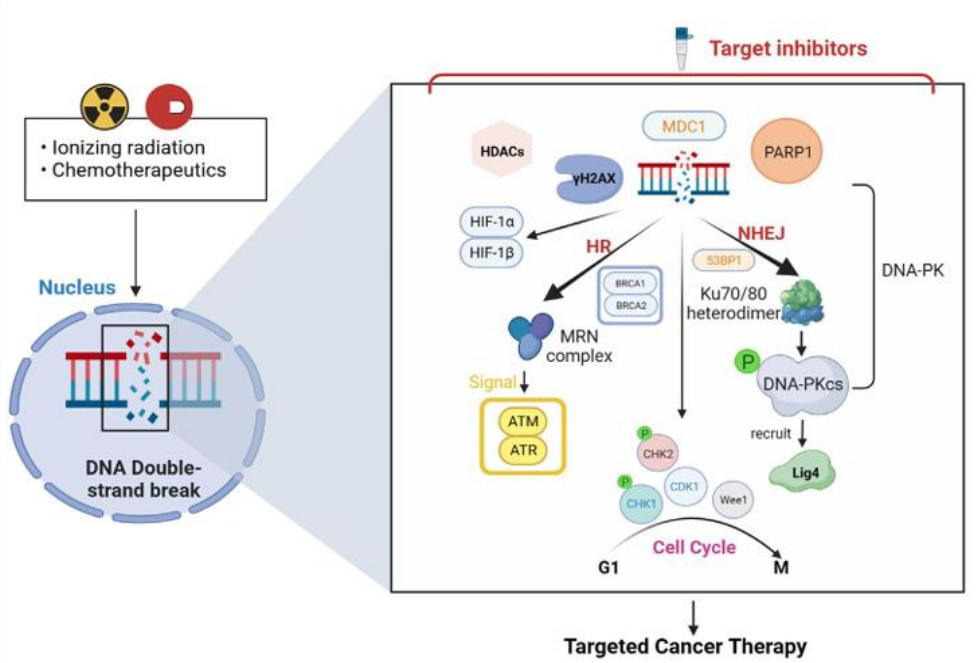


Fig. 1 of Jinpeng Tan, et al. 2023: Double-strand break (DSB), a significant DNA damage brought on by ionizing radiation, acts as an initiating signal in tumor radiotherapy, causing cancer cells death. The two primary pathways for DNA DSB repair in mammalian cells are nonhomologous end joining (NHEJ) and homologous recombination (HR), which cooperate and compete with one another to achieve effective repair.

Fig. 9 of Jinpeng Tan, et al. 2023: HR and NHEJ are the two primary repair procedures in the DSB damage repair pathway. DNA double-strand breaks can be harmed by radiation or toxins. Pathway proteins that are important in DSB damage repair include the MRN complex, BRCA1/2, ATM/ATR, Ku70/80 heterodimer, DNA–PKcs, Lig4, MDC1, 53BP1, HDACs, HIF-1, PARP1, and H2AX. The creation of inhibitors to enable tailored therapy for the relevant tumors might conceivably use these proteins as targets.

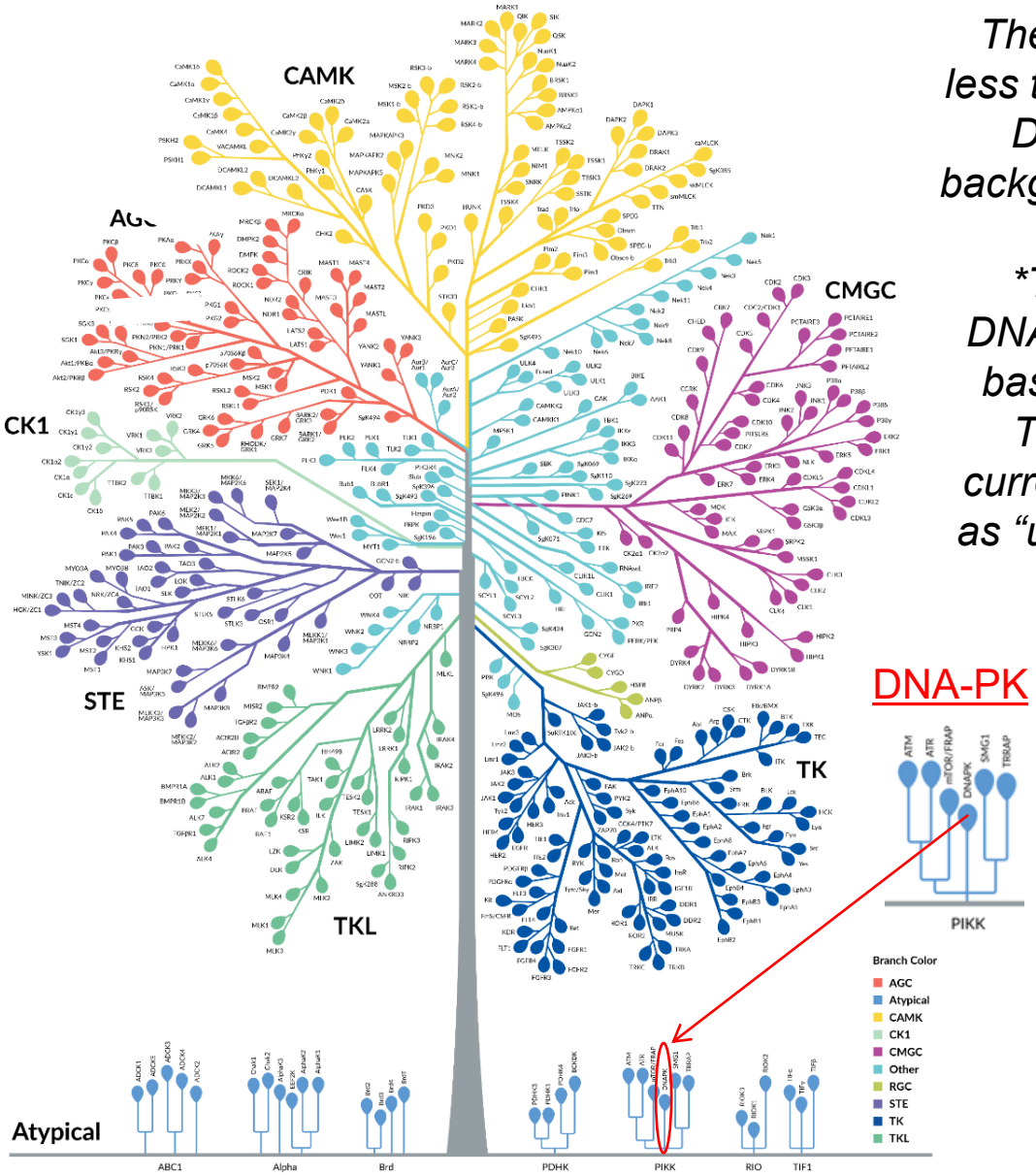
Chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://pmc.ncbi.nlm.nih.gov/articles/PMC10556206/pdf/MCO2-4-e388.pdf

DNA-PK, ATM, ATR, CDK4/6, CDK1, WEE1, and CHK1/2 are all high priority targets for lysate assays

High Selectivity for DNA-PK with the AQT0440 Sensor Peptide

Top 25 Hits with AQT0440 in Kinome Profiling with 411 Wild-type Kinases

Kinase target	Enzyme conc.	Average (RFU/pmol/min)	Rank	Selectivity Ratio	% Activity
DNA-PK	10	2176*	1	1	100
CAMK1B	1.0	140	2	16	6.42
YES	1.0	125	3	17	5.73
CK1G1	0.80	92	4	24	4.22
CK1G2	0.80	71	5	31	3.24
CK1G3	0.80	64	6	34	2.95
TSSK2	2.0	53	7	41	2.45
SMG1	8.3	42	8	51	1.95
RSK4	1.0	40	9	54	1.85
CAMK1G	2.7	37	10	59	1.69
SRC	0.32	35	11	63	1.59
FAM20C	10	27	12	81	1.23
MARK4	1.0	23	13	94	1.07
SRPK2	2.0	23	14	96	1.04
TBK1	0.50	23	15	96	1.04
ATR-ATRIP	10	20	16	111	0.90
FGR	5.0	18	17	123	0.82
ZAP70	0.60	16	18	139	0.72
IKKB	8.0	15	19	148	0.67
ATM	10	14	20	151	0.66
CDK15/B1	4.1	13	21	162	0.62
GLK/KHS2	2.0	13	22	163	0.61
P70S6KB	4.6	11	23	200	0.50
TLK1	15	9.32	24	234	0.43
CDK9/T2	1.8	9.30	25	234	0.43



The off-target kinases have less than 6.5% of the activity of DNA-PK, which is in the background range (see slide 6).

**The nM concentration of DNA-PK has been estimated based on a previous batch. The amount of DNA-PK currently provided is reported as "units" rather than "mg/mL" by the vendor.*

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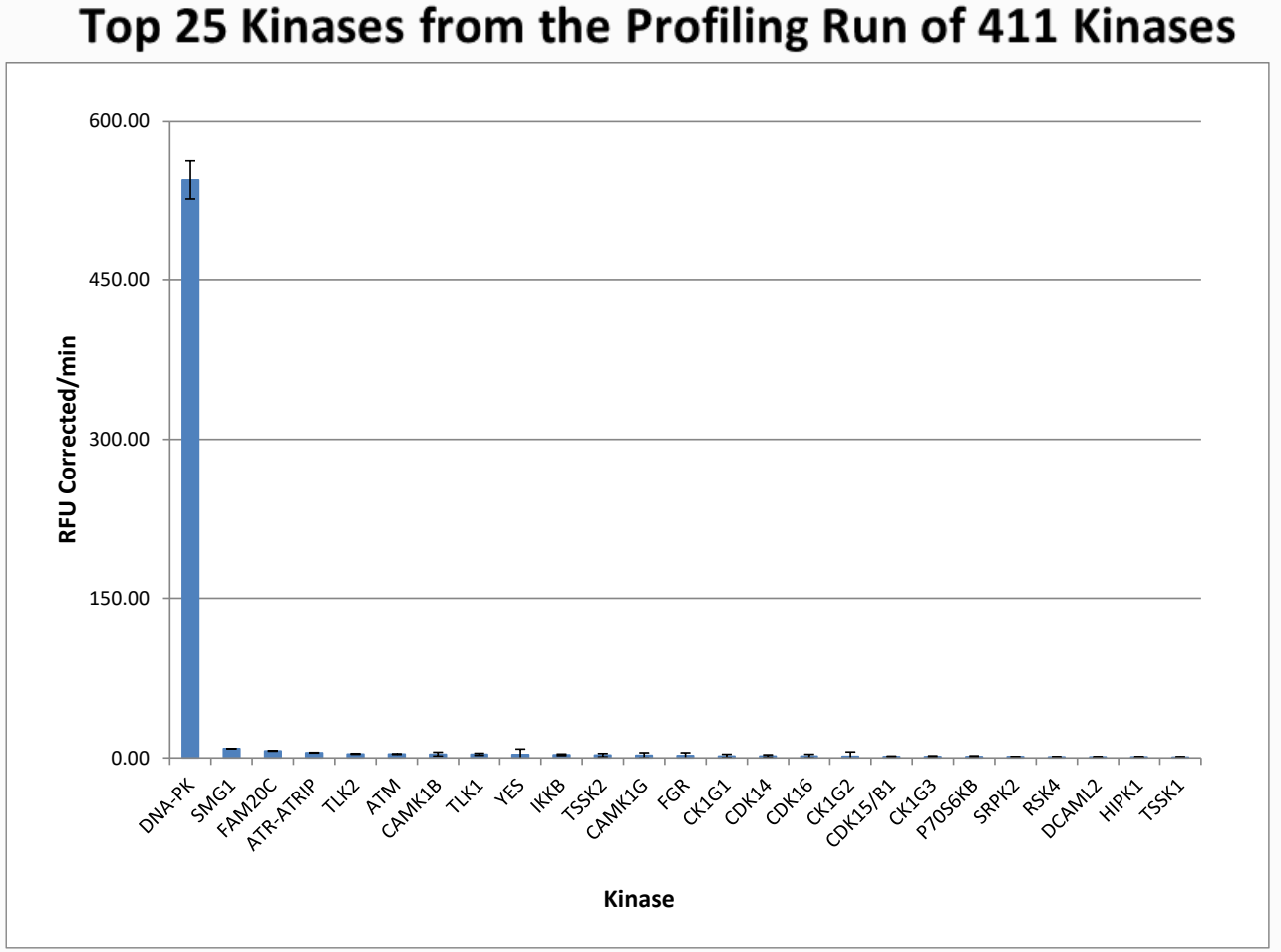
Selectivity of AQT0440 Retested Manually with the Top 25 Kinases from AQT's Kinome Profiling



High-throughput Milestone Optimization led to the identification of the AQT0440 sensor peptide substrate.

The top 25 off-target kinases were reassessed manually and showed only 1.7% or less of the DNA-PK signal.

AQT0440 shows exquisite selectivity for the target kinase, a critical requirement for accurately measuring the activity of DNA-PK in crude lysates

Outline for this Study

PhosphoSens-Lysate Assay Validation

Lysate Source:

- HCT116 Cells +/- 0.50 to 1.0 µg/mL Neocarzinostatin (MedChemExpress, HY-111183; resuspended in 100% DMSO) for 1 hour or 100 µM Cobalt Chloride (Alfa Aesar; resuspended in water) for 48 hours

Reference Compound Information:

- DNA-PK Inhibitor AZD-7648 (Cayman Chemical Co., 28598; resuspended in 100% DMSO)

Experimental Validation at AssayQuant:

- Treated HCT116 cell lysate titration
- AQT0440 substrate K_m determination
- DMSO Tolerance Test
- Reference Compound IC_{50} Determination
- Full-length DNA-PK with Ku domain (Promega, V5811), to serve as a positive control
- Phosphopeptide control (AQT0442)
- Assessment of DNA-PK Activity in Multiple Cell Lines +/- Neocarzinostatin or Cobalt Treatment

Preparation of Crude Cell Lysates from HCT116 Cells Treated ±Neocarzinostatin or Cobalt to Promote Double-Stand DNA Breaks

AssayQuant TECHNOLOGIES INC.

1) HCT116 Cells (passage 3) were plated in T-175 flasks and grown to 75% confluency over 48 hours at 37°C in McCoy's 5A Medium (ATCC, 30-2007) with 10% FBS (ATCC, 30-2020) and 1% PenStrep (30-2300) 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 1 hour with or without 0.50 to 1.0 µg/mL Neocarzinostatin (MedChemExpress, HY-111183; resuspended in 100% DMSO) to promote double-strand DNA breaks. Alternatively, after 48 hours of normal cell growth, cells were incubated with 100 µM Cobalt Chloride (Alfa Aesar, 10692; resuspended in water) for 48 hours to generate reactive oxygen species (ROS), which also promote double-strand DNA breaks. Cells were placed on ice, then washed with cold PBS and lysed with 500 µL of cold Cell Extraction Buffer (CEB) as follows:

Cell Extraction Buffer (CEB) with Protease and Phosphatase Inhibitors

- 50 mM HEPES, pH 7.4
- 150 mM NaCl
- 2 mM EGTA
- 1 mM DTT
- 1% Triton X-100
- 0.2 mM $Na_4P_2O_7$
- 100 µM Na_3VO4
- 0.2 mM NaF
- 50 mM β-glycerophosphate
- Protease Inhibitor Cocktail diluted 60-fold into lysis buffer
- Phosphatase Inhibitor Cocktail diluted 60-fold into lysis buffer

2) Cells were triturated to ensure solubilization and then lysates were 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, with each aliquot **used only once (one freeze/thaw cycle)**. Long-term storage of DNA-PK Lysates is not recommended, *as a loss in activity was observed over time.*

Note: The DNA-PK complex is a holoenzyme composed of the Ku heterodimer (Ku70 and Ku80) and a DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs), a serine/threonine protein kinase. Ku binds to the ends of DNA double-strand breaks and recruits and activates DNA-PKcs to carry out its function in DNA repair. Because of the large size of this complex, measuring endogenous DNA-PK activity in lysates provides many advantages.

Lysate Titration with the AQT0440 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

1 µg/mL calf thymus DNA

10 mM $MgCl_2$

15 µM AQT0440 sensor peptide substrate

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 HCT116 cells treated with 0.5 µg/mL Neocarzinostatin

Reaction Set Up:

20 µL Reaction Mix with AQT0440, ATP, & DTT
Seal plate and incubate at 30 °C for 15 minutes to equilibrate
5 µL Enzyme dilution buffer (EDB) with DNA-PK Lysate Buffer (1x) or DNA-PK 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-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.

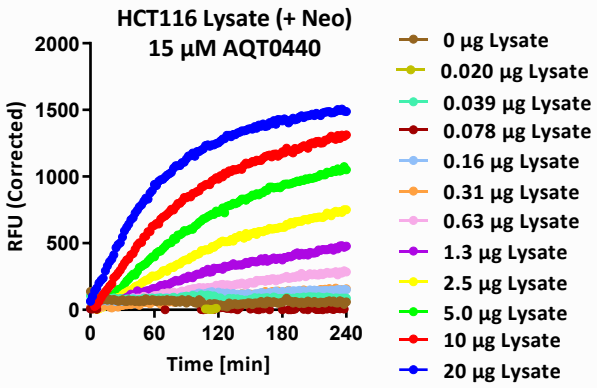
Notes:

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.

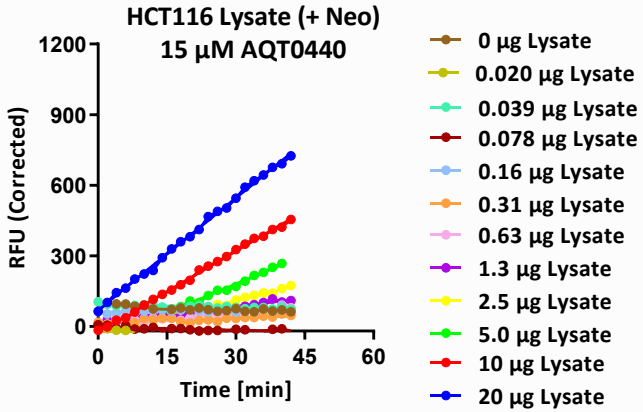
Lysate Titration with the AQT0440 Sensor Peptide

Progress Curves

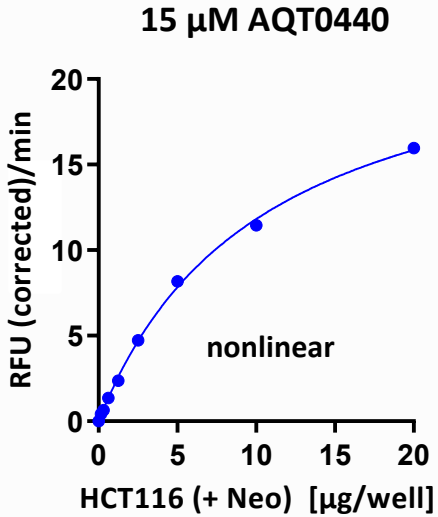
Complete Progress Curves



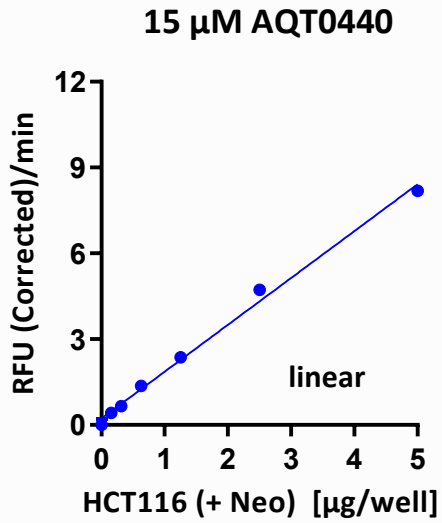
Linear Region of Progress Curves



Nonlinear Reaction



Linear Reaction



The DNA-PK Lysate Assay is linear from 0.078 – 5.0 µg/well of lysate (64-fold)

DNA-PK 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

1 µg/mL calf thymus DNA

10 mM $MgCl_2$

0, 0.20, 0.39, 0.78, 1.6, 3.1, 6.3, 13, 25, 50, and 100 µM **AQT0440 sensor peptide substrate**

4.0 µg/well NIH-3T3 crude cell lysate (from cells treated with 0.5 µg/mL Neocarzinostatin)

Reaction Set Up:

2.5 µl 10X AQT0440 Substrate dilutions
17.5 µL Reaction Mix with ATP & DTT
Seal plate and incubate at 30 °C for 15 minutes to equilibrate
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-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 for AQT0440

Titration Curves and K_m Plot and Table

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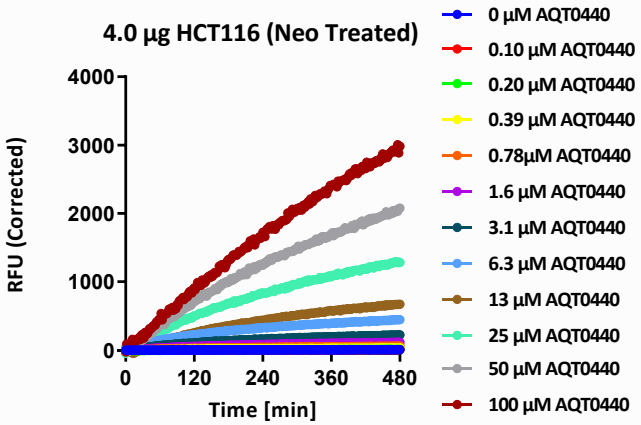
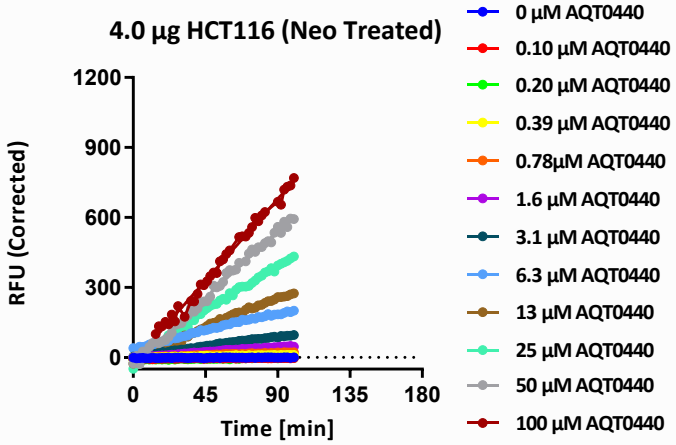
Sensor Peptide Titration Progress Curves

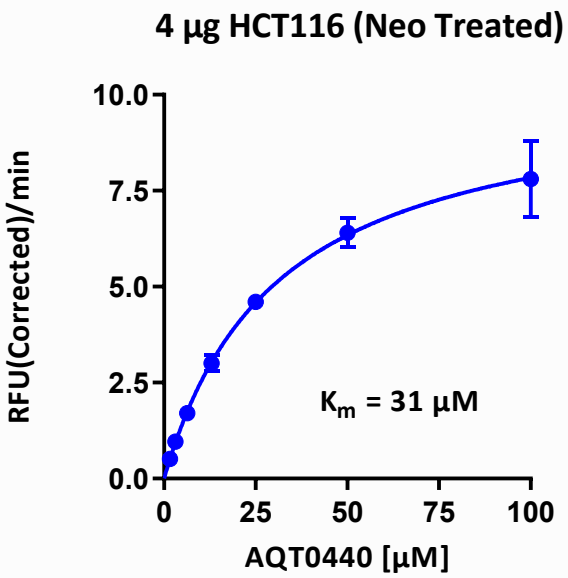
Complete Time Course



Linear Region



Sensor Peptide K_m Plot



Michaelis-Menten	
Best-fit values	
Vmax	10.26
Km	30.91
95% CI (profile likelihood)	
Vmax	10.03 to 10.49
Km	29.28 to 32.63
Goodness of Fit	
Degrees of Freedom	5
R squared	0.9998
Sum of Squares	0.008537
Sy.x	0.04132
Constraints	
Km	Km > 0
Number of points	
# of X values	7
# Y values analyzed	7

The K_m value for AQT0440 is 31 µM.

DMSO Tolerance Test with the AQT0440 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

1 µg/mL calf thymus DNA

10 mM $MgCl_2$

15 µM AQT0440 sensor peptide substrate

4.0 µg/well HCT116 crude cell lysate (from cells treated with 0.5 µg/mL Neocarzinostatin)

0-10% DMSO

Reaction Set Up:

2.5 µL 10X DMSO Titration
17.5 µL Reaction Mix with CSx Substrate, ATP & DTT
Seal plate and incubate at 30 °C for 15 minutes to equilibrate
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-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.

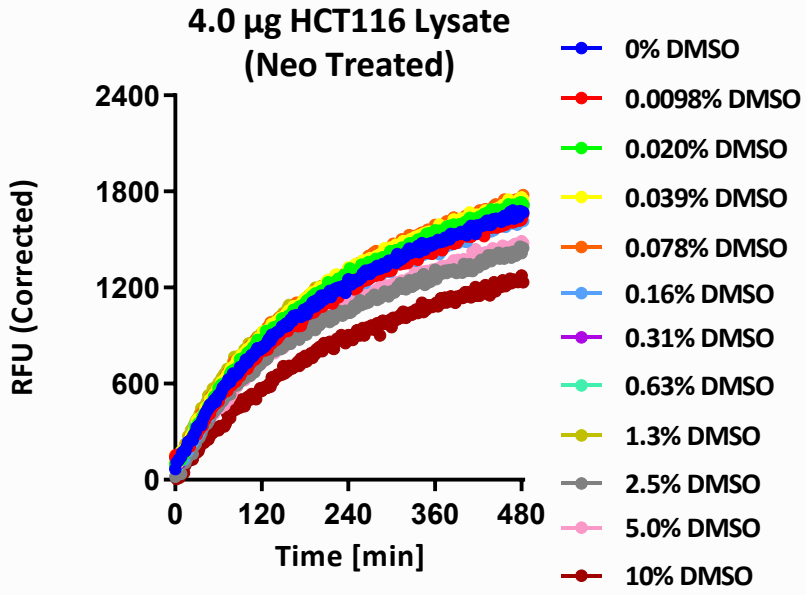
Notes:

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.

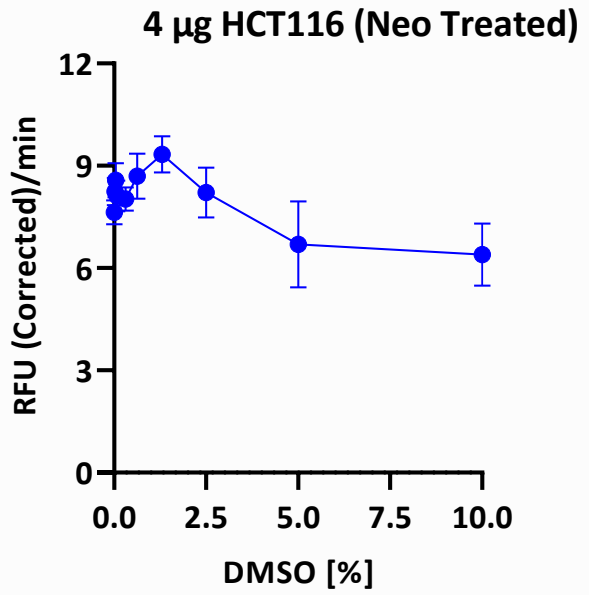
DMSO Tolerance Test with the AQT0440 Sensor Peptide

Titration Curves and Inhibition Plot

Complete Progress Curves



Reaction Rate vs [DMSO] Plot



No significant loss in enzyme activity was observed up to 2.5% DMSO. There was a 13% loss in signal at 5% DMSO, and a 25% loss in signal at 10% DMSO. We assessed compound potency using 2% DMSO final.

IC_{50} Determination with the AQT0440 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
1 µg/mL calf thymus DNA (for recombinant DNA-PK only)
10 mM $MgCl_2$
15 µM AQT0440 sensor peptide substrate

Compounds:

- AZD-7648 was 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.

DNA-PK Enzyme:

- **Cell Lysate for IC_{50} determination:**
  - 2.0 µg/well untreated HCT116 crude cell lysate
- **Recombinant DNA-PK for AQT1196 phosphorylation:**
  - 20 Units/well Full-length DNA-PK protein with the Ku domain (Promega, V5811)

Reaction Set Up:

0.5 µL 50X AZD-648 diluted in 100% DMSO or DMSO alone
19.5 µL Reaction Mix with CSx Substrate, ATP & DTT
Seal plate and incubate at 30 °C for 15 minutes to equilibrate
5 µL Enzyme dilution buffer (EDB) (1x), Lysate Buffer or DNA-PK 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-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:

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.

IC_{50} Determination with Untreated HCT116 Lysate using AZD-7648 and the AQT0440 Sensor Peptide

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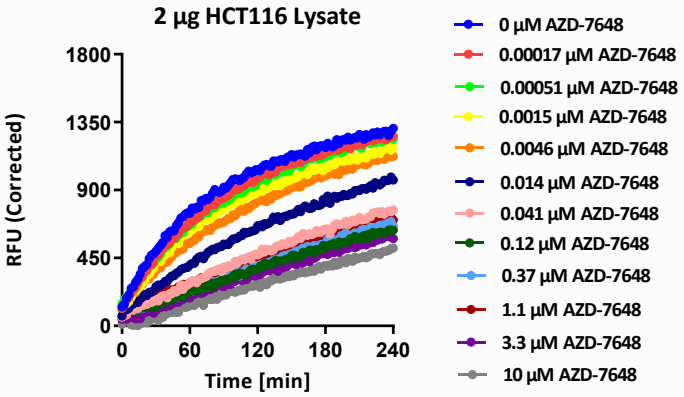
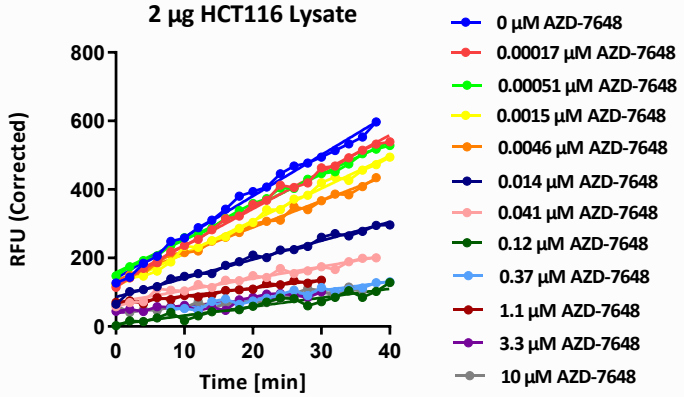
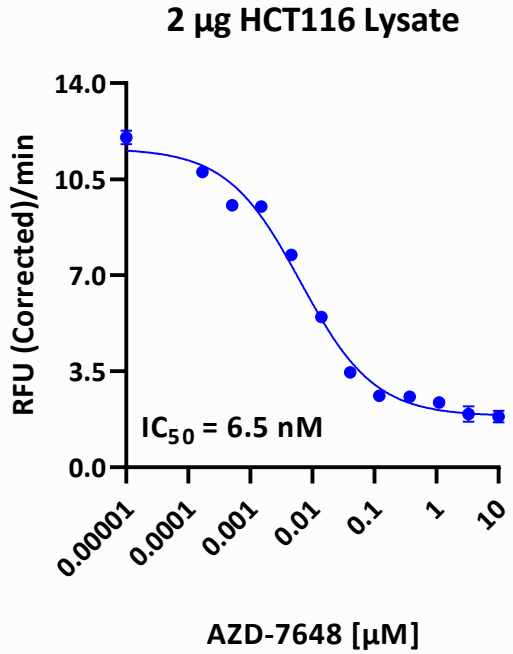
Full Progress Curves



Progress Curves for Linear Region



IC_{50} Curve



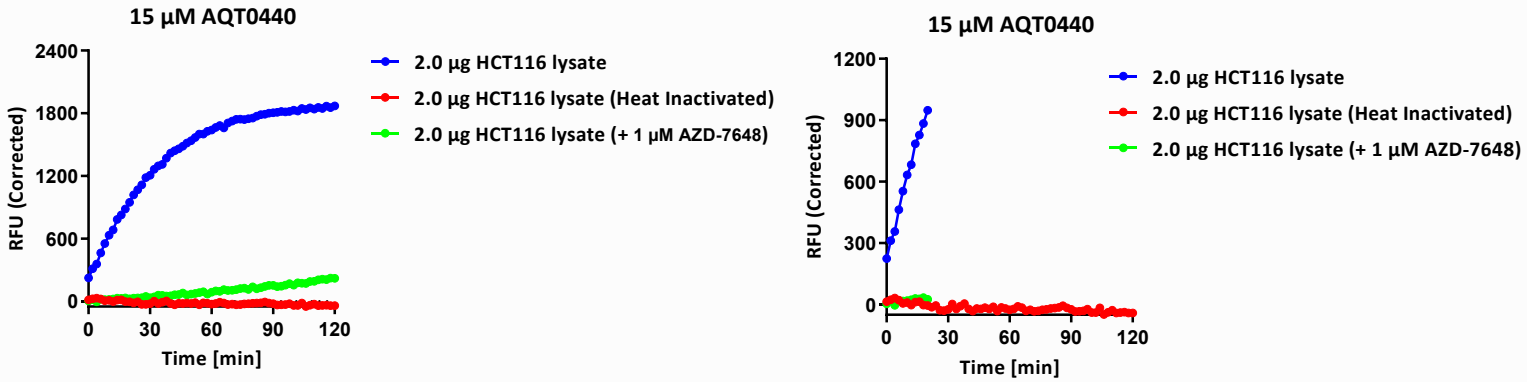
[Inhibitor] vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	1.865
Top	11.62
IC50	0.006506
HillSlope	-0.7331
logIC50	-2.187
Span	9.760
95% CI (profile likelihood)	
Bottom	1.116 to 2.455
Top	10.68 to 12.90
IC50	0.003635 to 0.01069
HillSlope	-1.105 to -0.5001
logIC50	-2.440 to -1.971
Goodness of Fit	
Degrees of Freedom	8
R squared	0.9906
Sum of Squares	1.534
Sy.x	0.4379
Constraints	
IC50	IC50 > 0
Number of points	
# of X values	12
# Y values analyzed	12

The IC_{50} value for AZD-7648 in HCT116 Cell Lysate is 6.5 nM.

DNA-PK Lysate Activity Assay Using the AQT0440 Sensor Peptide

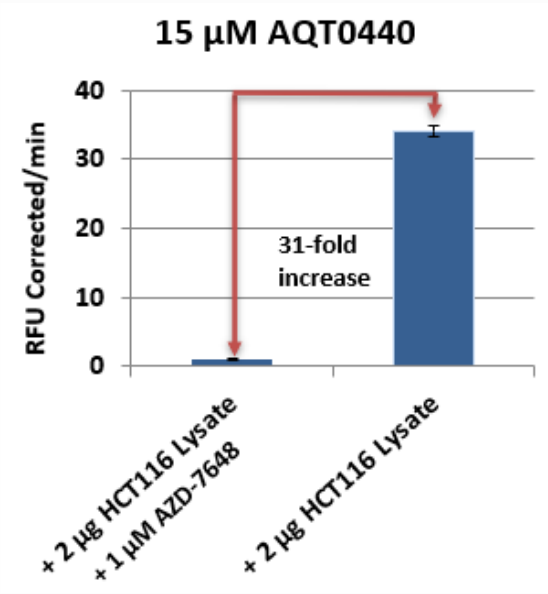
AssayQuant TECHNOLOGIES INC.

1) Full Time Course (0-120 min.) & Linear Range (0-40 min.) with 2.0 µg/well



2) Reaction Rates to Assess Change

	Reaction Rate (RFU/min)	Change
2.0 µg HCT116 lysate (no treatment)	34 ± 0.82	
2.0 µg HCT116 lysate, Heat Inactivated	0 ± 0.34	100% inhibition DNA-PK Activity
2.0 µg HCT116 lysate, + 1.0 µM AZD-7648	1.1 ± 0.17	97% inhibition DNA-PK Activity



Crude lysate samples: The AQT0440 sensor peptide (15 µM) was used to generate **1)** Full progress curve time course and 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 **2)**, highlighting complete elimination of signal with heat inactivation and by AZD-7648, a DNA-PK inhibitor.

Note: The amount of activation depends on several factors, including cell type and passage number, serum deprivation pretreatment used to make cells quiescent, and the nature, concentration, and duration of the exposure to DNA-damaging agents. With HCT116 Cell Lysates, we saw little to no effect on activity with 0.5 µg/mL Neocarzinostatin treatment for 1 hour. These conditions can be varied to determine the effect on DNA-PK activity. The total amount of DNA-PK 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.

DNA-PK Recombinant Activity Assay with AQT0440 Sensor Peptide and Phosphocontrol AQT0442

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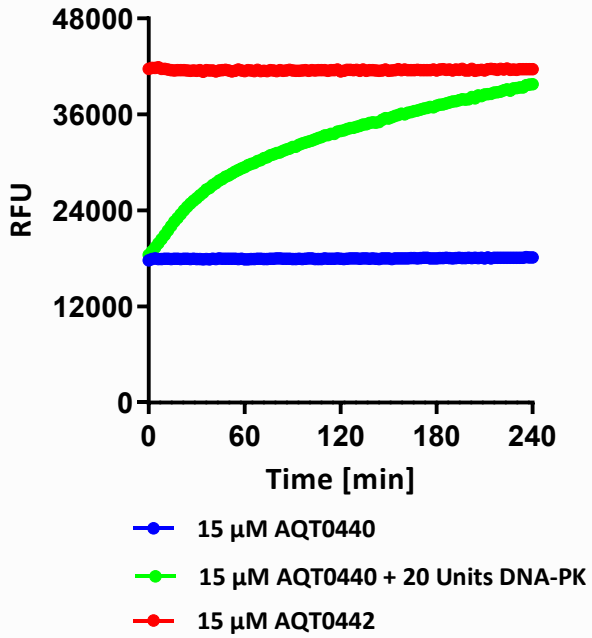
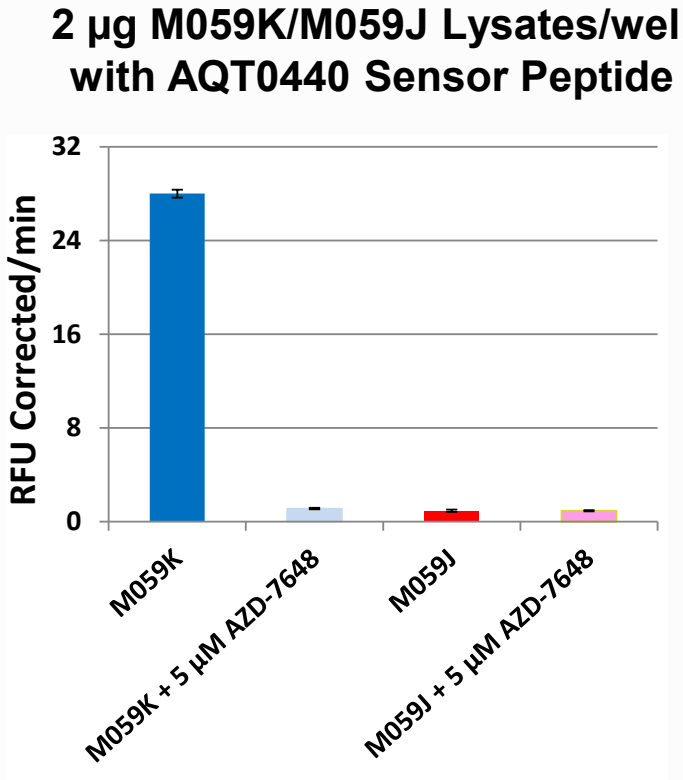
Full Time Course (0-4 hours)



Time course for phosphorylation of the AQT0440 sensor peptide substrate (15 µM) ± DNA-PK recombinant enzyme, compared to the signal with the AQT0442 phosphopeptide control (15 µM): Full-length DNA-PK protein with the Ku domain (20 Units, Promega, V5811) fully phosphorylated the AQT0440 sensor peptide substrate by 4 hours, as shown by convergence with the signal obtained with the AQT0442 phosphopeptide positive control (a flat horizontal line defining the maximum RFU with this sensor peptide). It was necessary to add calf thymus DNA to this reaction since recombinant DNA-PK was used rather than a cell lysate. The signal with AQT0442 is used to convert RFU (Corrected) values to nmoles of phosphopeptide product/minute.

AQT0442 is a chemically synthesized phosphopeptide control for AQT0440

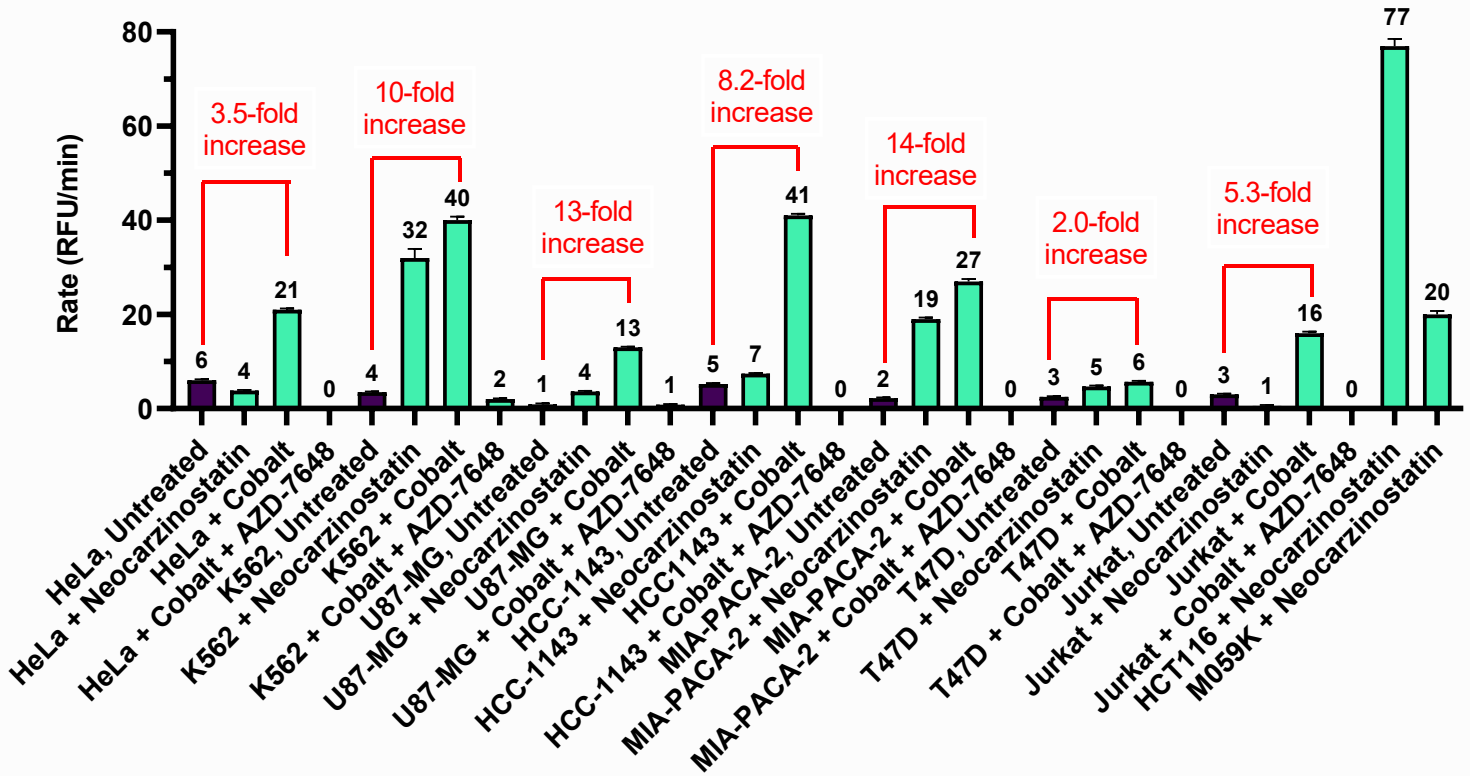
DNA-PK Lysate Activity Assay Using AQT0440 with a Paired Set of M059 Glioblastoma Cell Lines



Background: M059K and M059J were originally isolated concurrently from the same tumor specimen from a 33-year-old male patient with untreated glioblastoma. M059K cells express normal levels of DNA-dependent protein kinase (DNA-PK), while M059J cells lack DNA-PK protein and activity. M059K cells are approximately 30-fold less sensitive to ionizing radiation than M059J cells due to the ability of DNA-PK to promote double-strand DNA break repair. M059K cells are also less sensitive than M059J cells to the cytotoxic effects of bleomycin, N, N-bis(2-chloroethyl)-N-nitrosourea and nitrogen mustard. M059K cells are DNA double-strand break repair proficient, while M059J cells are deficient in the repair of DNA double-strand breaks. 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. Reaction rates (RFU Corrected/min) were determined from the slopes using the linear portion of each progress curve. Values are the average of triplicate reactions +/- standard deviation. In M059K cell lysates vs M059J cell lysates, there was a 25-fold increase in the rate. This activity was inhibited by 5 µM AZD-4648, a selective DNA-PK inhibitor.

Demonstrates detection of endogenous DNA-PK activity in M059K cells and full inhibition with the DNA-PK inhibitor AZD-7648. As expected, no DNA-PK activity was observed with the MO59J Control cell line.

DNA-PK Lysate Activity Assay Using the AQT0440 Sensor Peptide Across a Variety of Cell Types



Description of cell lines where lysates had the highest DNA-PK activity (and/or highest increases with treatment):

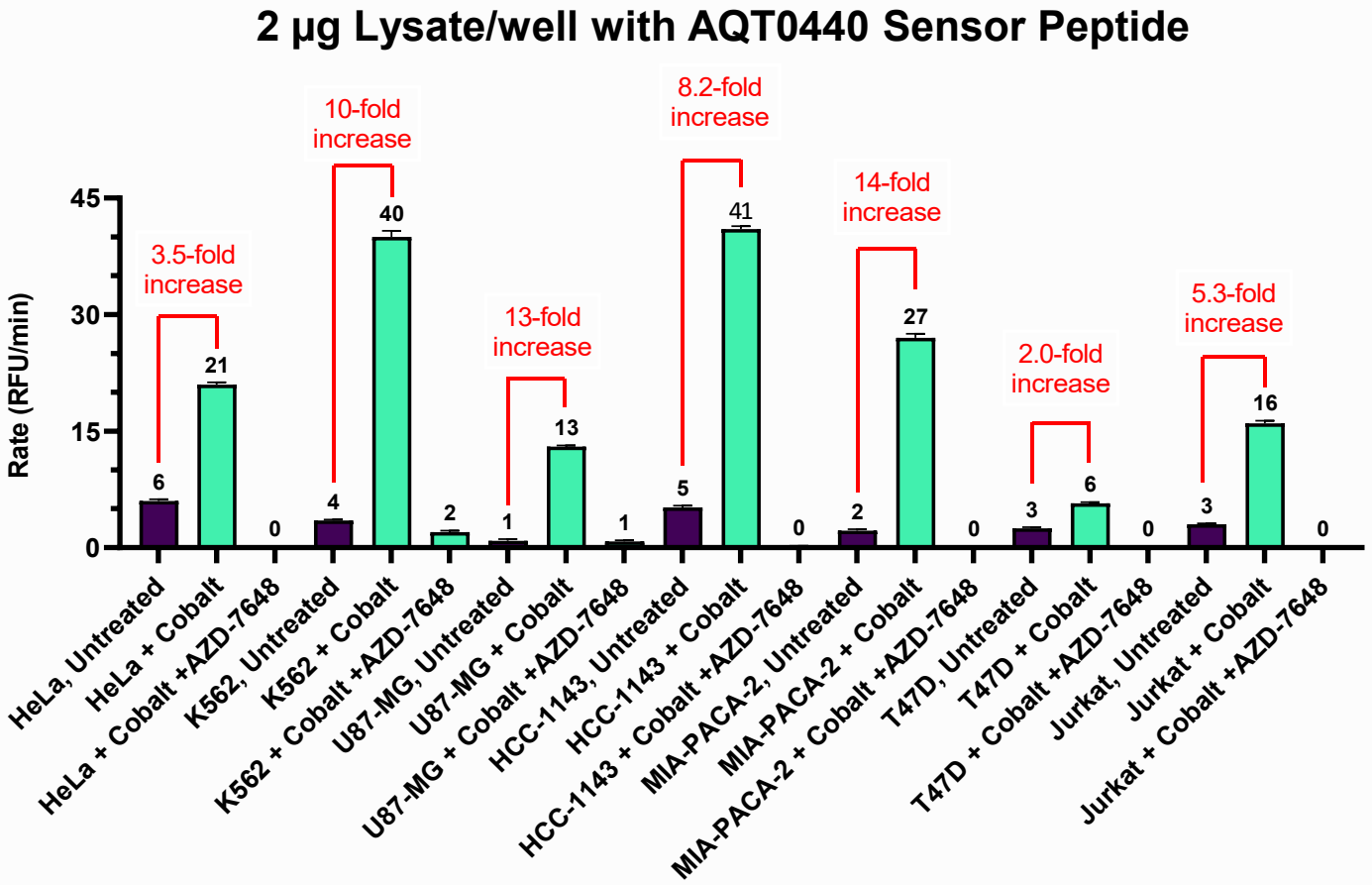
- **K562 cells** have a hematopoietic morphology and were isolated from the bone marrow of a 53-year-old female with chronic myeloid leukemia (CML) at blast crisis.
- **U87-MG cells** have an epithelial morphology and were isolated from malignant gliomas from a male patient, likely with Glioblastoma.
- **HCC1143 cells** are a triple negative (ER-/PR-/HER2-) mammary gland breast cancer line isolated from a 54-year-old female patient with ductal carcinoma TMN Stage IIA, grade 3.
- **MIA-PaCa-2 cells** are an adherent epithelial cell line isolated from the pancreas of a 65-year-old male with carcinoma.
- **HCT116 cells** are an adherent cell line isolated from the colon of a patient with colon cancer. There is a mutation in codon 13 of the ras-proto-oncogene. This cell line is near-diploid and has a relatively stable genetic profile.

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. Reaction rates (RFU Corrected/min) were determined from the slopes using the linear portion of each progress curve. Values are the average of duplicate reactions +/- standard error.

Results – Cobalt was more effective than Neocarzinostatin in increasing reaction rates, likely because it is more effective in causing double-strand DNA breaks. No or very low DNA-PK activity was observed with A549 and MCF-7 cells, and no significant increase in rate was observed with either treatment (data not shown).

Demonstrates detection of high DNA-PK activity in HCT116 (Colorectal), HCC1143 (triple negative breast), K562 (CML), and MIA-PACA-2 (Pancreatic) cell lysates after treatment with Cobalt or Neocarzinostatin and inhibition with the selective DNA-PK inhibitor AZD-7648 .

DNA-PK Lysate Activity Assay Using the AQT0440 Sensor Peptide Across a Variety of Cell Types Following Treatment with Cobalt



Cell Lysates with Cobalt Treatment ± AZD-7648

HCC1143, K562, U87-MG, and MIA-PACA-2 cells are described on slide 20. Lysates from these cells had the largest increase in DNA-PK activity with Cobalt treatment vs the untreated controls with increases in rate of 8-fold for HCC1143, 10-fold for K562, 13-fold for U87-MG, and 14-fold for MIA-PACA-2. AZD-7648 inhibited all DNA-PK activity in these lysates for 30 minutes. Cobalt treatment results in an increase in reactive oxygen species, which can cause DNA double-strand breaks that activates DNA-PK catalytic kinase activity that is measured selectively with the AQT0440 sensor peptide.

Demonstrates activation of DNA-PK activity in all cells (2-14-fold) by cobalt treatment with inhibition by the selective DNA-PK inhibitor AZD-7648.

Summary

- The PhosphoSens-Lysate Assay for DNA-PK using the AQT0440 selective sensor peptide demonstrates a robust, sensitive, and physiologically relevant assay that selectively measures endogenous DNA-PK activity with all the cellular components and signaling complexes using only 2 µg of crude cell lysate per test.
- Results include:
  - Treatment with Neocarzinostatin or Cobalt resulted in up to 10-fold and 14-fold increase in DNA-PK activity, respectively, with activation observed across multiple cell lines. Both chemicals result in DNA double-strand breaks. The highest DNA-PK activity was with HCT116 cells treated with Neocarzinostatin, resulting in a rate that was almost 2-fold higher than any other cell line. This lysate was used for further validation studies.
  - DNA-PK activity with lysates from Neocarzinostatin-treated HCT116 cells was linear from 0.078 to 5.0 µg/well, a 64-fold linear range.
  - The DNA-PK lysate activity was inhibited by 97% with 1 µM of AZD-7648 DNA-PK inhibitor reference compound.
  - The IC_{50} value for AZD-7648 with lysate from untreated HCT116 cells was 6.5 nM.
  - A K_m of 31 µM was determined for AQT0440 with the Neocarzinostatin-treated HCT116 cell lysate.
- These DNA-PK activity measurements are direct, highly quantitative, and in an easy-to-use format. This allows functional assessment of the native DNA-PK/Ku activity in complex samples using crude cell lysates or tissue homogenates, thereby providing a more physiological and economical approach to study DNA-PK.

Company & Technology Supporting Slides

AQT Senior Scientific Team

Experienced & Diverse Team from Leading Institutions

AssayQuant TECHNOLOGIES INC.

Dr. Bill Radany
CEO & Head of Business Development

Dr. Erik Schaefer
Co-Founder, CSO

Dr. Barbara Imperiali
Co-Founder & CTO, MIT Faculty

Dr. Earl May
Senior Director, Discovery Technologies (DT)

Dr. Eric Berg
Director of Peptide Operations

Dr. Daniel Urul
Senior Scientist, DT

Dr. Venky Nemmara
Senior Scientist, DT

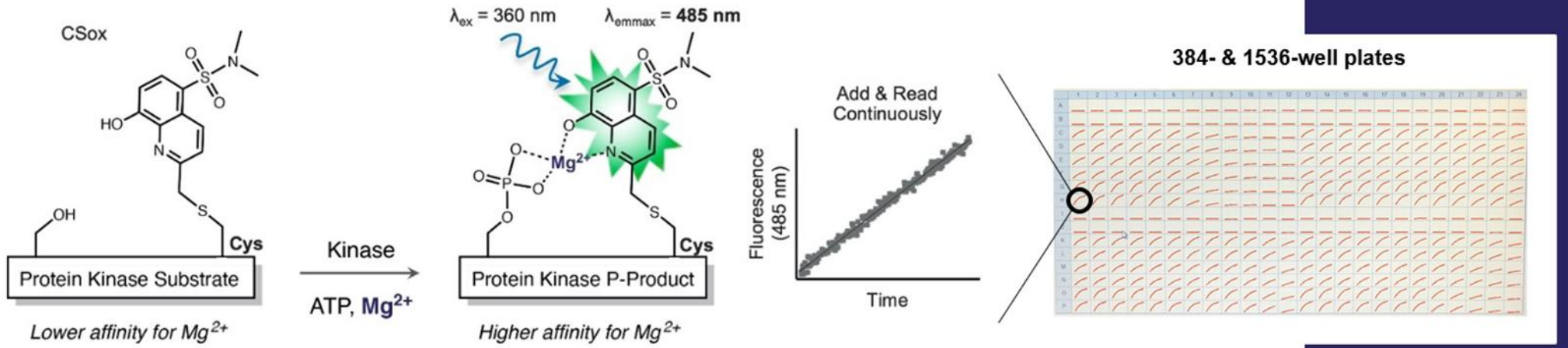
- We apply our >200 years of collective experience in kinase biology, drug or assay development, chemical-biology and automation to solve critical problems in target biology and drug development.
- AQT services allow us to evaluate drugs in diverse ways, delivering high-quality data combined with strong communication to enable discovery

Susan Cornell-Kennon
Senior Scientist, DT

Dr. Satish Pimrale
Business Development Leader

gsk | Pfizer | astellas | Bristol Myers Squibb | BIACORE | danaher | Caliper LifeSciences | PerkinElmer | ThermoFisher SCIENTIFIC | Merck KGaA Darmstadt, Germany | Promega

Sensor Peptide Substrates for Continuous (Kinetic) Monitoring of Protein Kinase Activity



- Uses chelation-enhanced fluorescence via sulfonamido-oxine (Sox) chromophore, invented by Barbara Imperiali (MIT).
- 10 patents with 1 pending, exclusively licensed from MIT. Rich trade secret portfolio
- Sox is small, minimally hydrophobic, and neutral
- Assay is direct, homogeneous, and kinetic (continuous)
- Can use a wide range of ATP concentrations (Low µM, ATP K_m or mM [physiological])
- Runs on commonly available readers with any plate type (96-, 384- or 1536-well)

Simple, Powerful & Flexible!

AssayQuant® TECHNOLOGIES INC.

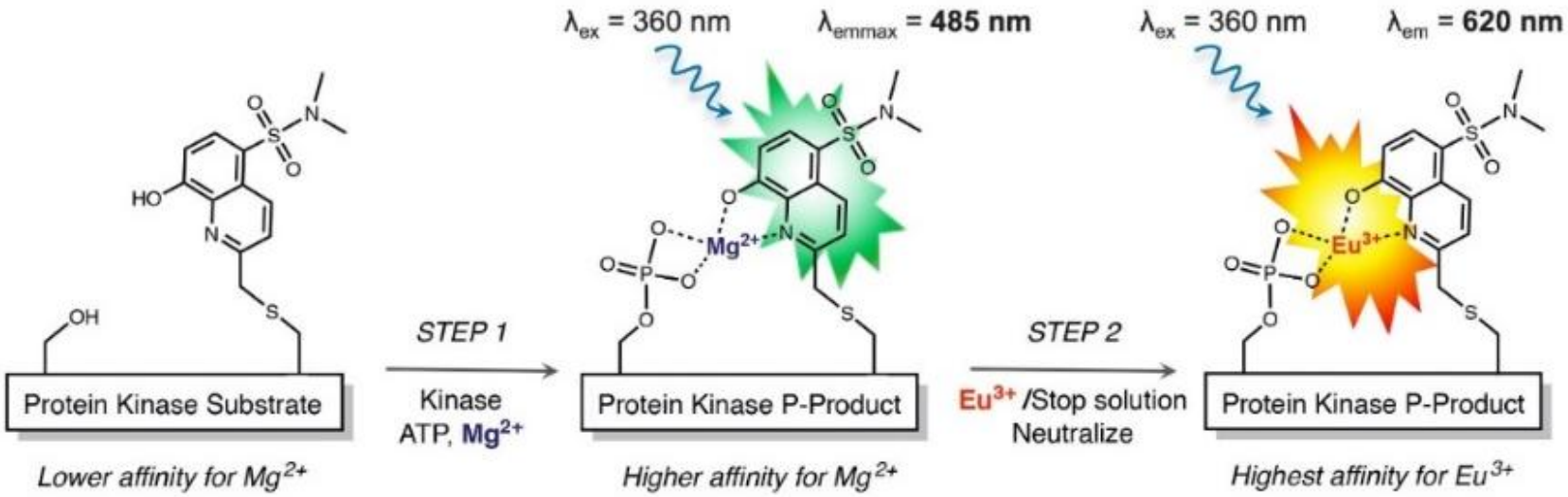
a full progress curve in every well

Ideal for quantitative assessments including Profiling, Potency & MOA

PhosphoSens®

Replacing Mg^{2+} with Eu^{3+} Create

PhosphoSens®- Red



- Sox sensitizes Europium (Eu^{3+}) luminescence for time-resolved fluorescence (TRF) with 620 nm emission and data acquisition delay, which eliminates compound interference
- Run as an Endpoint format; ideal for high-numbers of tests for HTS and SAR
- Patent approved in 2020

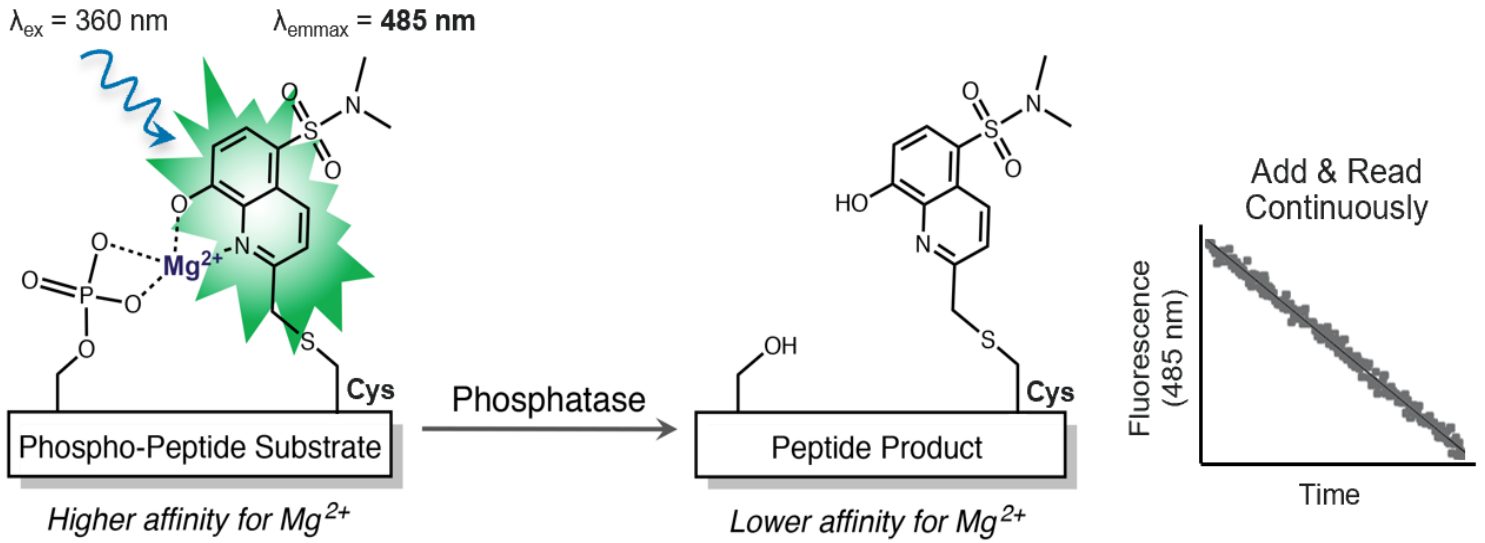
Eliminates autofluorescence while using the same small fluorophore

AssayQuant® TECHNOLOGIES INC.

Ideal for:

- HTS
- SAR

Same Sox-based Detection Platform Enables Continuous Monitoring of **Protein Pho**



- Same mechanism - ChEF (Mg^{2+}) for kinetic assays and Eu^{3+}/TRF for Endpoint (Red), as for protein kinases
- Uses **CSox-based phosphopeptide substrates** derived from physiological targets to monitor loss of signal
- Covered by 10 patents (with 1 pending) and trade secret portfolio

Increasing requests for tyrosine & serine/threonine phosphatases

AssayQuant TECHNOLOGIES INC.

Kinetic and Endpoint

One Technology, Two Fit-for-Purpose Formats

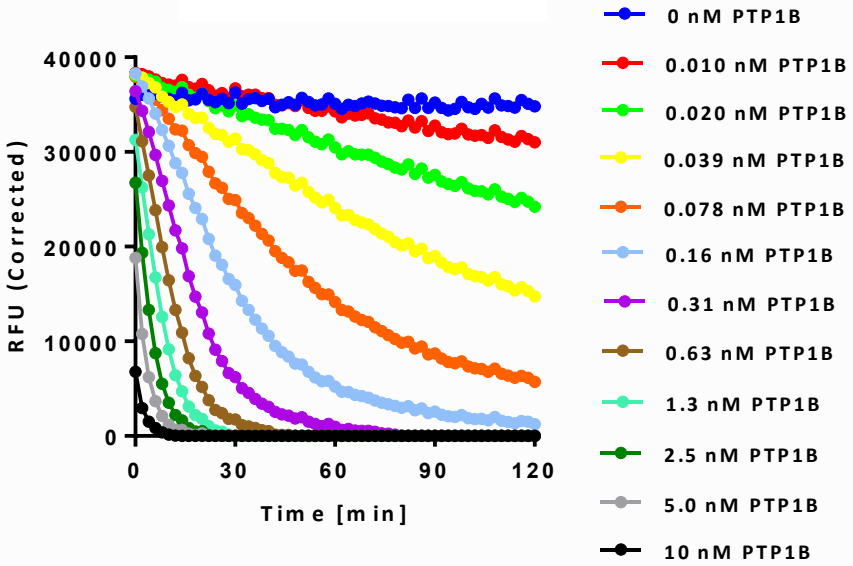
Expanding menu (currently 34 of the 190 Protein Phosphatases)

Analysis of **PTP1B** & **PTPN2** Tyrosine Phosphatases

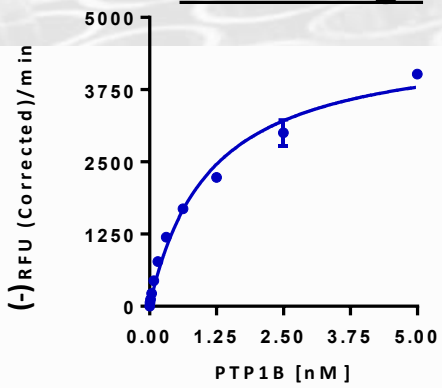
Optimized *PhosphoSens* Phosphopeptide Substrates

AssayQuant TECHNOLOGIES INC.

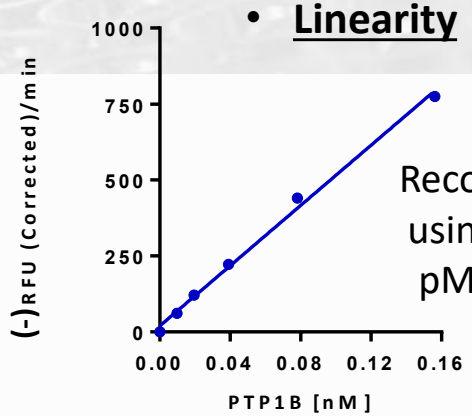
Progress Curves & Linearity for PTP1B with AQT0266 Sensor Peptide, Net Signal



Total Range

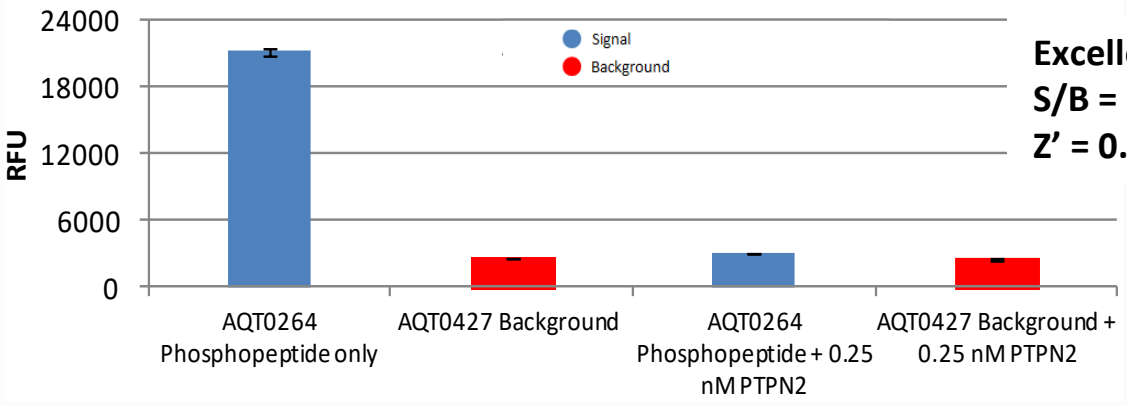


- **Linearity**



Recommend using 20-75 pM PTP1B

Total & Background for PTPN2 with AQT0264 in PhosphoSens®-Red



Excellent Assay
S/B = -7.1
Z' = 0.94 (n=3)

High-sensitivity & physiologically-relevant. Analysis of allosteric or substrate-competitive inhibitors

PhosphoSens® Platform

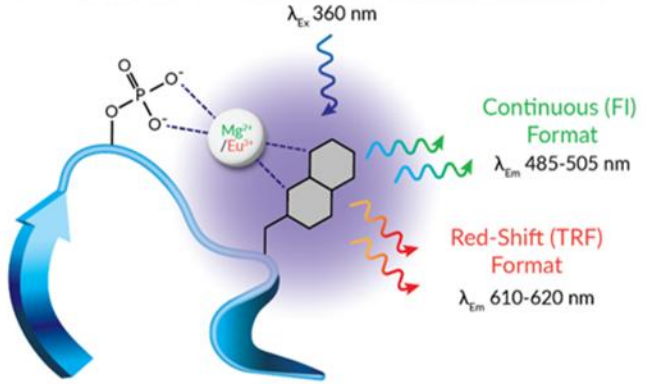
Enabling Analysis Across the Drug Development Workflow

AssayQuant TECHNOLOGIES INC.

96-, 384-, or 1536-well plates

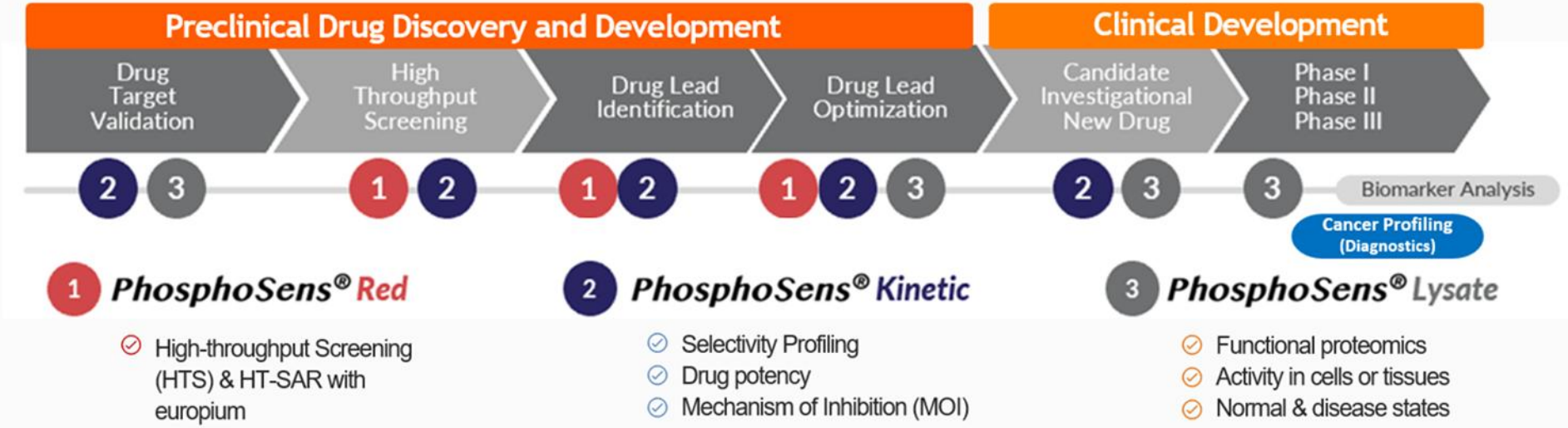
Any fluorescence microplate reader (kinetic mode)

Mix and read!



One Technology, Two Fit-for-Purpose Formats

Kinetic and Endpoint



- High-throughput Screening (HTS) & HT-SAR with europium
- Selectivity Profiling
- Drug potency
- Mechanism of Inhibition (MOI)
- Functional proteomics
- Activity in cells or tissues
- Normal & disease states

Accelerates progress and improves outcomes

Integration of Catalog Products & Services

Multiple entry & transition points to address your needs

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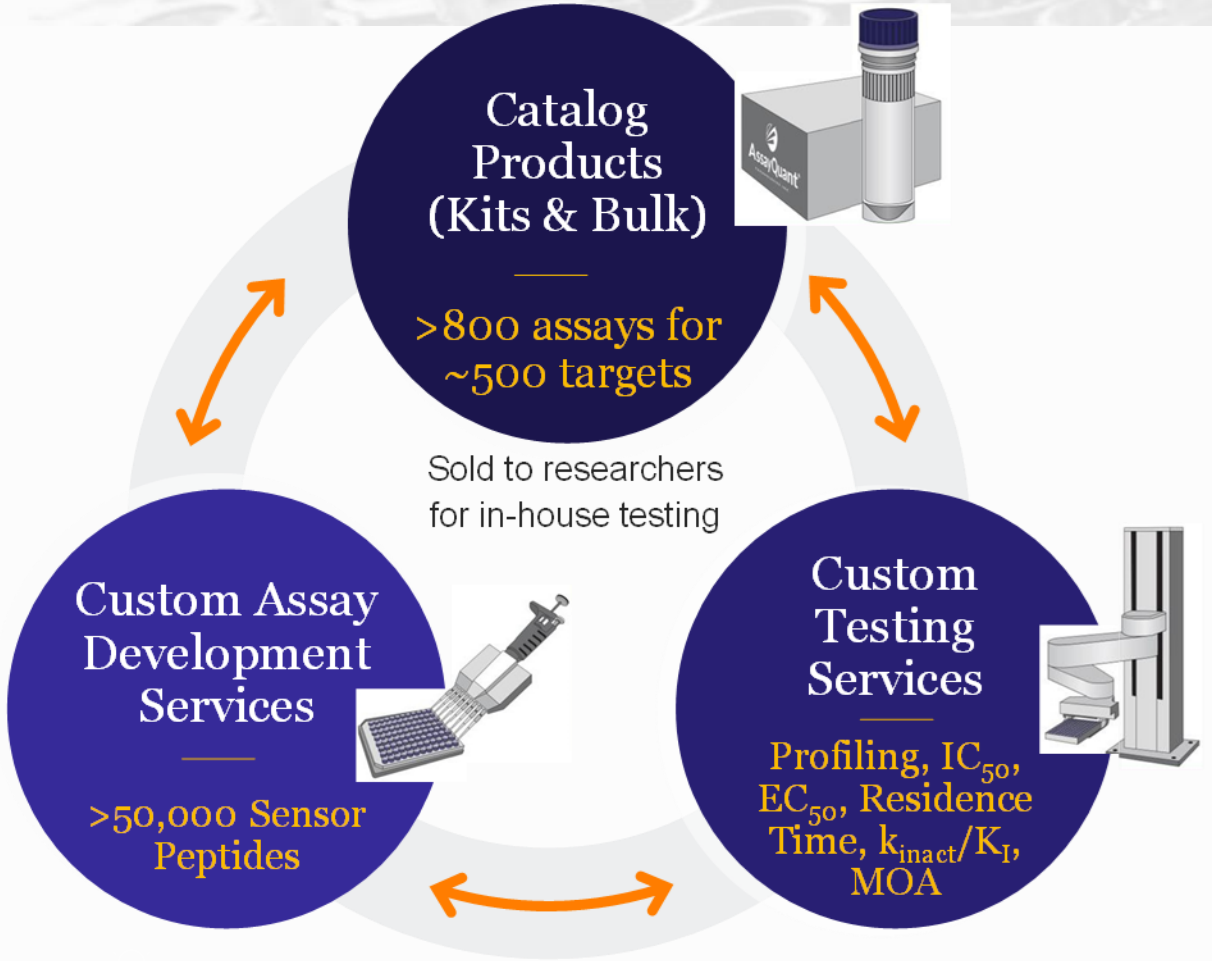
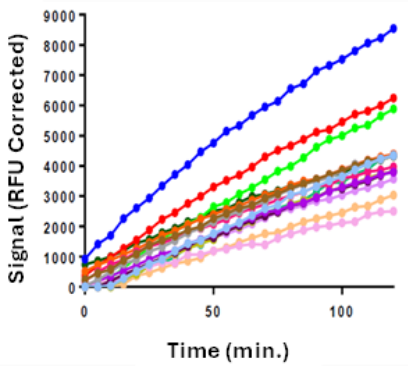


PhosphoSens® Platform Continuous Workflow Applications

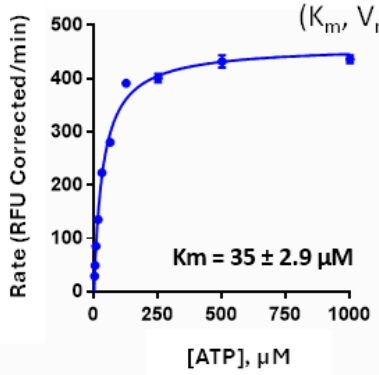
AssayQuant TECHNOLOGIES INC.

Assay Development



Enzyme Kinetics with Recombinant Proteins

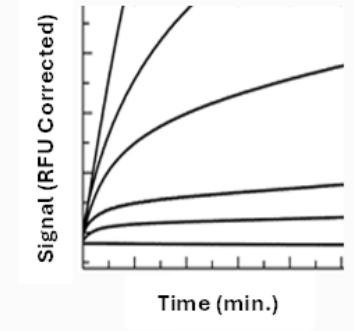
(K_m, V_{max}, k_{cat}, k_{cat}/K_m)



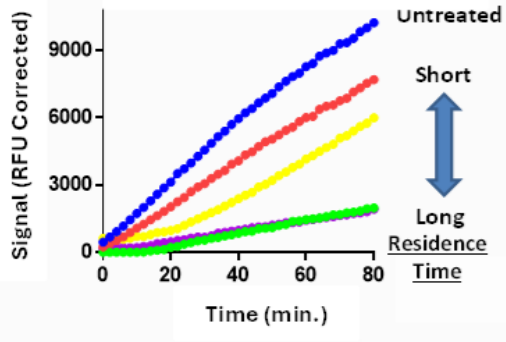
Fluorescence increase	[Km (µM)]	V_{max} (µmol.mg^{-1}.min^{-1})
3.5 - fold	0.01	1.8
3.9 - fold	0.69	2.5
4.4 - fold	1.2	1.3

Drug Potency (IC_{50}, K_i, Residence Time, k_{inact}/K_I, MOA, EC_{50})

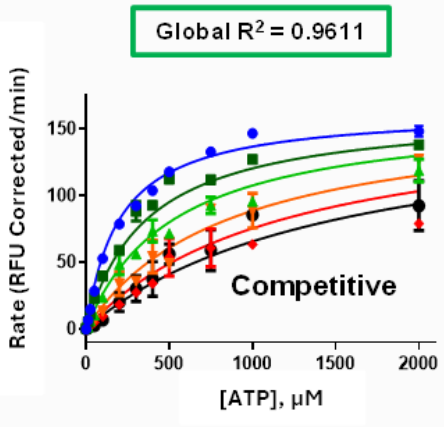
Time-Dependent Inhibition (TDI)



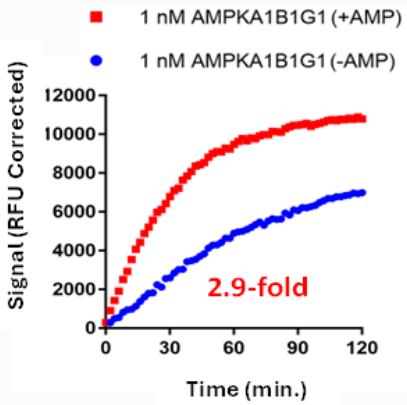
EGFR (T790M/C797S/L858R)



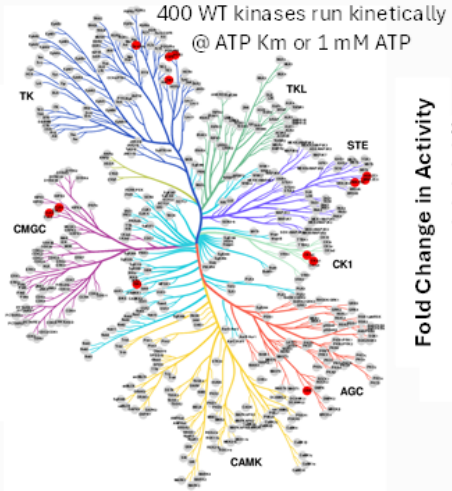
Mechanism of Inhibition (MOI)



Target Activation

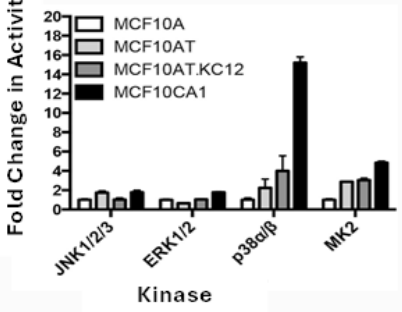


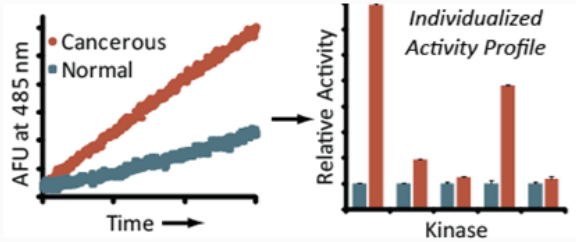
Kinome Profiling



Quantitative Enzyme Activity Measurements in Unfractionated Cell or Tissue Lysates

Direct-to-Phase II SBIR from NIH/NCI funded in August 2024





Crude lysate figures from Imperiali lab (MIT): Peterson *et al.* Biochemistry 2014, 53: 5771-5778; Stains *et al.* Chem Biol. 2012, 19(2): 210–217.

PhosphoSens® Platform Advantages:

- **Sensitive** and **continuous** (**kinetic**) format based on Chelation-enhanced fluorescence.
- **Homogeneous (add & read) and direct** measure of enzyme activity resulting in a complete **progress curve in every well** for each condition.
- Sensor peptides or lipids based on **physiologically-relevant** substrates.
- Run under **optimal enzyme conditions**, with physiological **Mg^{2+}, Mn^{2+} & Ca^{2+}** ions and **any ATP** concentration (1-2 mM = physiological; or at ATP K_m) or sample type (lysates via selective sensor peptides or IP-kinase assays).
- Determination of **initial reaction rate** from **linear** portion of curve provides **high accuracy & precision** (Z'>0.7) and enables assessment of time-dependent inhibitor or activator potency (IC_{50}, EC_{50}, k_{inact}/K_I, residence time).
- **Corrects for any compound autofluorescence** – **Kinetic:** Background signal doesn't change over time; **Red:** Uses Europium (Eu^{3+}) for time-resolved fluorescence (TRF) with a 100 µsec delay & 620 nm emission.
- **Rapid & predictable development** of new assays is achieved even with difficult targets using >30,000 sensor peptide panel for protein kinases or phosphatases and any DAG species for DGK assays.
- **Rigorous manufacturing** process for PhosphoSens® sensors provides excellent lot to lot consistency.
- **Works across entire drug development workflow** – Improving efficiency and performance

We apply our >200 years of collective experience in kinase biology, drug or assay development, and automation to address complex biology and evaluate drugs in diverse ways, delivering high-quality data to accelerate your programs.