

PhosphoSens® Cell Lysate Activity Assay Format

ZAP70 Assay Validation Using the AQT1450 Selective Sensor Peptide Substrate

HGNC Name: ZAP70

Long Names: **Zeta-chain-Associated Protein kinase 70, ZAP-70**

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, GSK3 α/β , JNK1-3, ZAP70 $\alpha/\beta/\gamma/\delta$, and DNA-PK

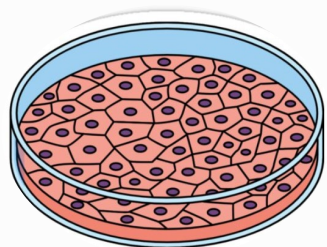
COMING SOON – From our active R&D pipeline of selective sensor peptide substrates for:
CDK1-3/5, CDK2, CDK5, CDK4/6, cKit D816V, cMet, JAK2, MEK1/2, PIM1/3, PKA α/β , SGK1/2/3, and SYK.

Robust process and pipeline of selective sensor peptides established

PhosphoSens-Lysate Assays

A Simple & Powerful Solution to a Complex Problem

1. Grow Cells
(+/- Pathway Activation)
or Access Tissues



2. Harvest

ADD **PhosphoPreserve**
Lysis Buffer + Protease &
Phosphatase Inhibitors



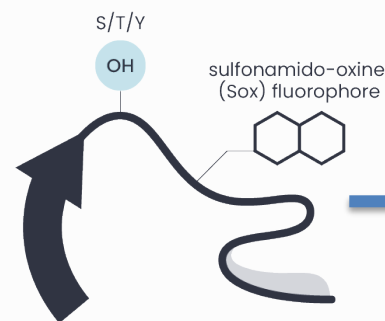
Study Kinase Enzyme Activity Where Biology Happens

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.

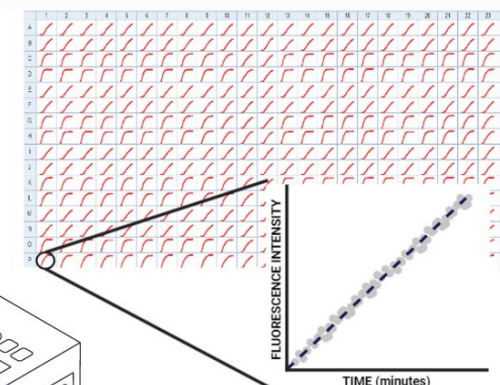
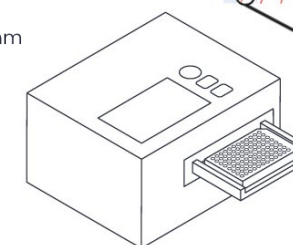
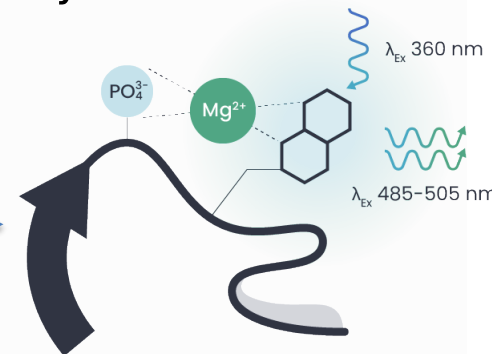


3. Prepare Reaction Mix
(PhosphoSens Sensor Peptide
Substrate, ATP, Mg^{2+})

Easy-to-use Kits &
Bulk Sensor Peptide
(Native substrates, stable,
scalable, excellent lot consistency)



ADD Lysate & Read

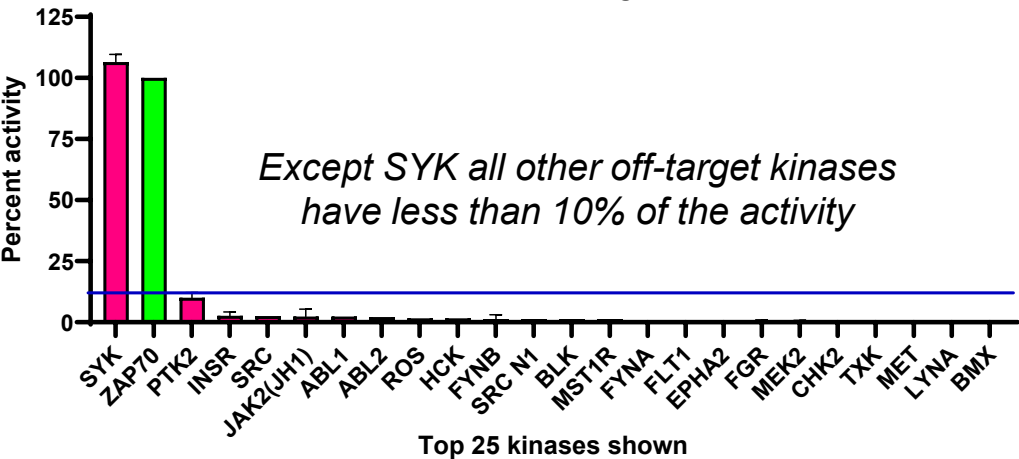


Immediate Progress Curve
Visualization in Every Well

Selectivity of AQT1450 for ZAP70 determined with AQT's Kinome Profiling Service

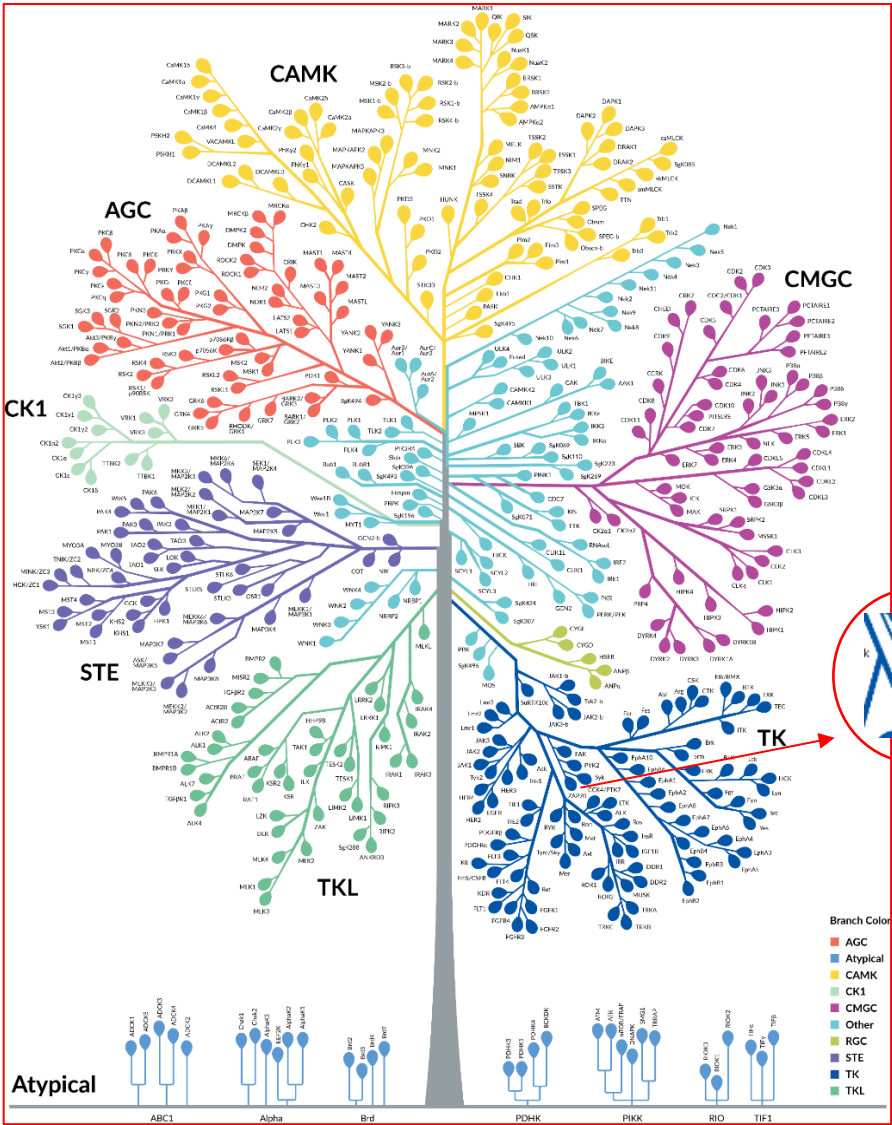
Top 25 Kinases from Kinome Profiling

AQT1450 Profiling data



AQT1450		
Kinase tested	Average % activity	Average rate (RFU/pmol/min)
SYK	106.6	2,000.0
ZAP70	100.0	1,876.8
PTK2	10.0	187.9
INSR	2.7	50.5
SRC	2.5	46.9
JAK2(JH1)	2.3	43.3
ABL1	2.3	42.9
ABL2	2.1	40.0
ROS	1.6	30.0
HCK	1.6	30.0
FYNB	1.3	25.0
SRC N1	1.3	25.0
BLK	1.3	25.0
MST1R	1.1	20.0
FYNA	0.8	15.0

FLT1	0.8	15.0
EPHA2	0.8	15.0
FGR	0.6	10.9
MEK2	0.4	7.3
CHK2	0.3	5.0
TXK	0.2	4.6
MET	0.1	2.5
LYNA	0.1	2.5
BMX	0.1	2.5



AssayQuant's Kinome Tree

AssayQuant's Kinome profiling service platform features 411 wild-type kinases. All assays are run kinetically to assess compound or sensor peptide selectivity. Compounds are run at ATP K_m and/or 1 mM ATP (physiological). Sensor peptides are run at 1 mM ATP.



Outline for this Study

PhosphoSens-Lysate Assay Validation Using the AQT1450 Sensor Peptide Substrate



Lysate Source:

JURKAT cells (passage 6) treated +/- 10 µg/mL anti-CD3 Monoclonal Antibody (OKT3, Invitrogen - Lot Number: 3419574), 10 µg/mL anti-CD28 Monoclonal Antibody (Invitrogen Lot Number: 3367568), and Goat Anti-Mouse Kappa Light Chain, F(ab')₂ (Cell Signaling #75861).

Reference Compound Information:

1. RDN2150 (ZAP70 inhibitor): MedChemExpress -Cat. No.: HY-155978
2. PRT062607 (SYK inhibitor): MedChemExpress - Cat. No.: HY-15322
3. RO9021 (SYK inhibitor): MedChemExpress - Cat. No.: HY-16902

Experimental Validation Performed:

1. Lysate from JURKAT cells (with +/- 10 µg/mL anti-CD3 Monoclonal Antibody and 10 µg/mL anti-CD28 Monoclonal Antibody crosslinked with Goat Anti-Mouse Kappa Light Chain)
2. Phosphopeptide Control (AQT1544) and comparison to phosphorylation of AQT1450 sensor peptide with recombinant ZAP70 (Carna, Cat: 08-177)
3. AQT1450 sensor peptide substrate K_m determination
4. DMSO Tolerance Test
5. Reference Compound IC_{50} Determinations with RDN2150, PRT062607, and RO9021 using recombinant ZAP70.
6. Reference Compound IC_{50} Determinations with RDN2150, PRT062607, and RO9021 using JURKAT lysates.

Preparation of JURKAT Cell lysates Treated +/- Anti-CD3 or CD3+CD28 Antibodies (Plus Anti-IgG Ab) to Activate ZAP70

- 1) JURKAT cells (passage 5) were grown in T175 flasks for 48 hours at 37 °C in RPMI medium supplemented with 10% FBS (Thermo Fisher, A56708-01) and 1% Penicillin-Streptomycin (Thermo Fisher, 15140122) in a 5% CO₂ atmosphere.
- 2) On the day before, the cells were serum-starved **overnight** in a culture medium containing 0% FBS and 1% Penicillin-Streptomycin.
- 3) Subsequently, buffer (negative control) or 10 µg/mL anti-CD3 Monoclonal Antibody (OKT3, Invitrogen - Lot Number: 3419574) and 10 µg/mL anti-CD28 Monoclonal Antibody (Invitrogen Lot Number: 3367568) were added directly to 2 mL of JURKAT cells (1 x 10⁷ cells/mL) and incubated on ice for 10 minutes. Then, 20 µg/mL of Goat Anti-Mouse Kappa Light Chain, F(ab')₂ (Cell Signaling #75861) was added and incubated on ice for 10 more minutes to initiate crosslinking of the antibodies bound to the cell surface. The samples were then incubated at 37°C for 5 minutes to stimulate ZAP70 signaling.
- 4) Cells were immediately chilled on ice for 5 minutes, and then centrifuged at 4°C, RPMI medium was removed, and the cells were lysed with 0.6 mL of Cell Extraction Buffer (CEB) (see recipe below) for 30 minutes on ice.

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
- PhosphoPreserve Phosphatase Inhibitor Cocktail - 1, 100X
- PhosphoPreserve Phosphatase Inhibitor Cocktail - 3, 60X
- Bisperoxovanadium(phen), Phosphatase Inhibitor, 10X (250 µM)
- PhosphoPreserve Protease Inhibitor Cocktail, 60X

Assay Conditions for a Basic Lysate Assay

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 MgCl₂

12 μM AQT1450 sensor peptide substrate

Tool Compounds – All tool compounds were tested at a top concentration of 10 μM, serially diluted 3-fold (11-points) in DMSO (Final DMSO concentration is 2%)

RDN2150 (ZAP70 inhibitor) in 2% DMSO

PRT062607 (SYK inhibitor)

RO9021 (SYK inhibitor)

JURKAT cell lysates – 5 μL of 0.25 mg/mL (5 μg final concentration) in 25 μL total assay volume

Reaction Set Up:

- Add **14.5 μL 1.72X Reaction Mix with ATP, & DTT**
- Add **5 μL of 5X AQT1450**
- Add **0.5 μL of 50X tool compounds**
- Add **5 μL JURKAT Lysate or Blank buffer (lysate buffer + EDB)**, to obtain **25 μL** Final reaction volume

Notes:

Reaction was run at 30 °C for 480 minutes in a Corning® 96-well Half Area White Flat Bottom Polystyrene NBS Microplate at 25 μL final well volume after sealing with 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.

Total Protein Determination: Total protein was determined by the Bradford method

Assay Conditions for a Recombinant Assay

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 MgCl₂

15 μ M AQT1450 or AQT1544 (phosphopeptide control)

Kinases – 1.5 nM ZAP70 (Carna, Cat: 08-177)

Reaction Set Up:

- Add **15 μ L 1.72X Reaction Mix with ATP, & DTT**
- Add **5 μ L of 5X AQT1450**
- Add **5 μ L kinase or Blank buffer (lysate buffer + EDB),**
to obtain **25 μ L** Final reaction volume

Notes:

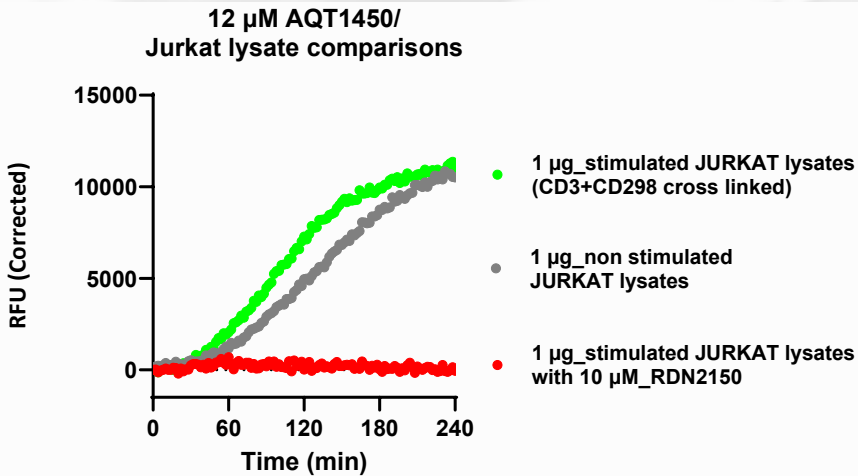
Reaction was run at 30 °C for 480 minutes in a Corning® 96-well Half Area White Flat Bottom Polystyrene NBS Microplate at 25 μ L final well volume after sealing with optically clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a BioTek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

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.

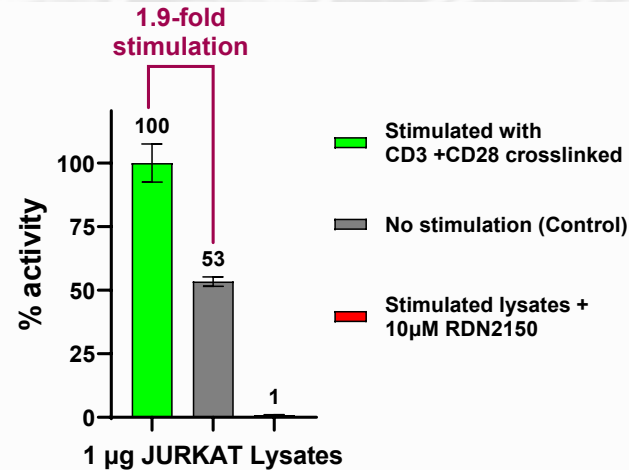
JURKAT Cell Stimulation to Activate ZAP70 activity

Detection of ZAP70 Activity in Lysates from JURKAT Cells Using AQT1450

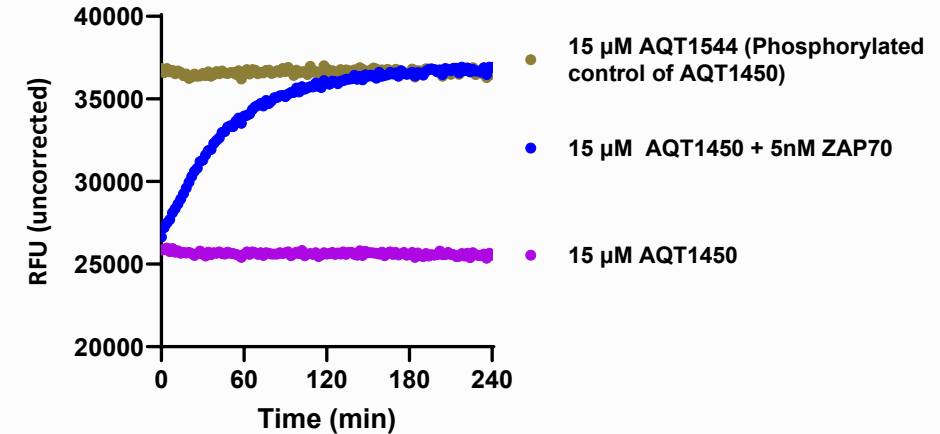
A. Complete Progress Curves



B. Reaction Rate Comparison



C. PhosphoPeptide Control (AQT1544)



- ❖ Serum-starved (overnight) Jurkat cells were stimulated with anti-CD3 and anti-CD28 antibodies, followed by crosslinking with an anti-IgG antibody.
- ❖ Stimulated cell lysates exhibited up to a 1.9-fold increase in phosphorylation rate relative to unstimulated controls (Fig. A and B). Phosphorylation of AQT1450 was inhibited completely by 10 μ M RDN2150.
- ❖ Recombinant ZAP70 (5 nM) completely phosphorylated AQT1450 (Fig. C), as evidenced by convergence of the signal with that of AQT1544, the phosphopeptide positive control.

Assay Conditions for Lysate Titration

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 MgCl₂
12 μM AQT1450 sensor peptide substrate

JURKAT cell lysates – 5 μL of lysates 1.5 –fold serially diluted in a mixture of enzyme dilution buffer and lysate buffer (11-point) with 5 μg top concentration in the wells

Reaction Set Up:

- Add **14.5 μL 1.72X Reaction Mix with ATP, & DTT**
- Add **5 μL of 5X AQT1450**
- Add **0.5 μL of DMSO**
- Add **5 μL JURKAT Lysate or Blank buffer (lysate buffer + EDB)**, to obtain **25 μL** Final reaction volume

Notes:

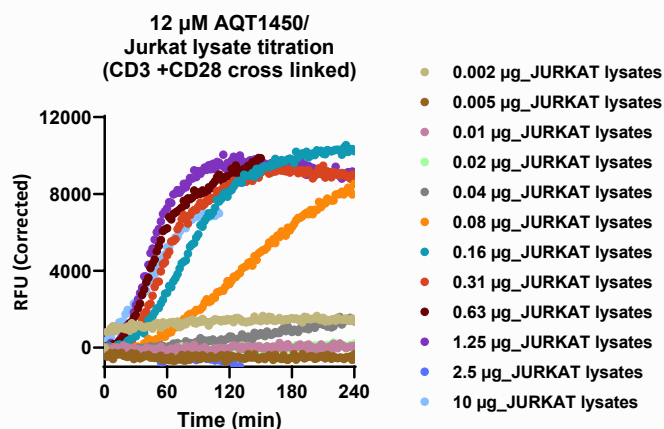
Reaction was run at 30 °C for 480 minutes in a Corning® 96-well Half Area White Flat Bottom Polystyrene NBS Microplate at 25 μL final well volume after sealing with optically clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a BioTek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

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.

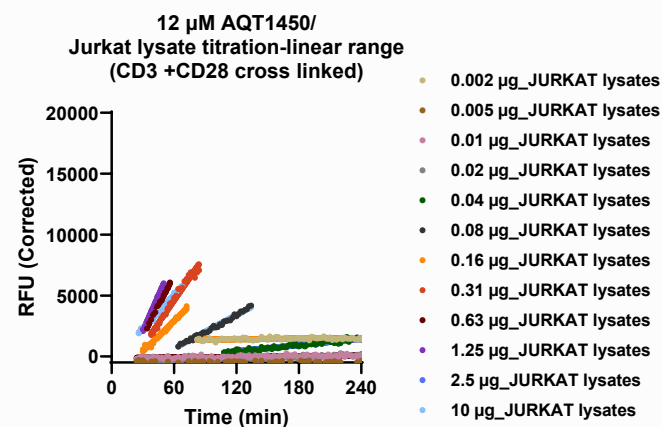
Activated JURKAT Cell Lysate Titration Data

Progress Curves, Reaction Rates & Linearity for ZAP70 Activity with AQT1450

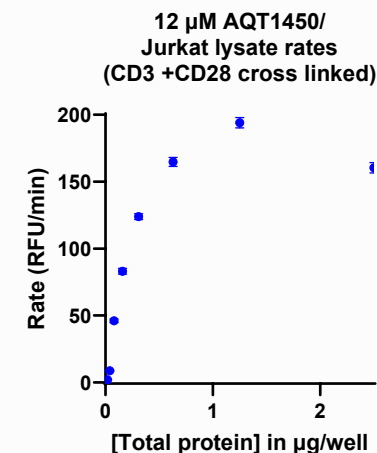
A. Lysate Titrations Full Progress Curve



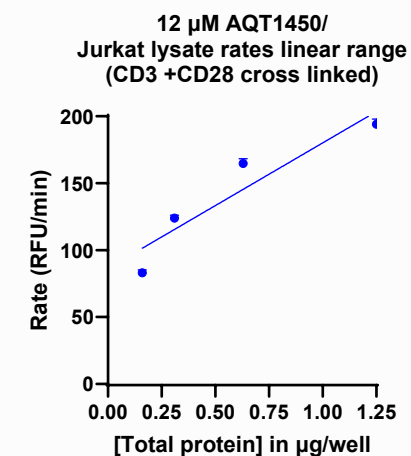
B. Lysate Titrations Linear Range



C. Lysate Titration Rates



D. Lysate Linear Range



- ❖ A plot of phosphorylation rate (RFU/min) vs lysate concentration shows that the reaction rate decreases at higher lysate concentrations above 1.25 μ g.
- ❖ The linear range was found to be between 0.16 and 1.25 μ g of lysate/well.
- ❖ A lysate concentration of 1.25 μ g was used for further experiments.

Assay Conditions For Sensor Peptide Titration

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 MgCl₂

Sensor Peptide - 1.5 –fold serially diluted in water
(11-points) with 50 µM top concentration in the wells

JURKAT cell lysates – 5 µL of 0.25 mg/mL (1.25 µg
final concentration) in 25 µL total assay volume

Reaction Set Up:

- Add **14.5 µL 1.72X Reaction Mix with ATP, & DTT**
- Add **5 µL of 5X serially diluted AQT1450**
- Add **0.5 µL of DMSO**
- Add **5 µL JURKAT Lysate or Blank buffer (lysate buffer + EDB)**, to obtain **25 µL** Final reaction volume

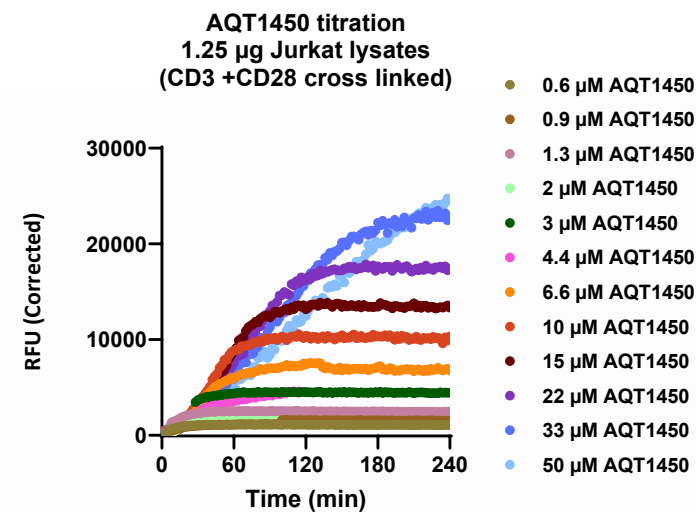
Notes:

Reaction was run at 30 °C for 480 minutes in Corning® 96-well Half Area White Flat Bottom Polystyrene NBS Microplate at 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.

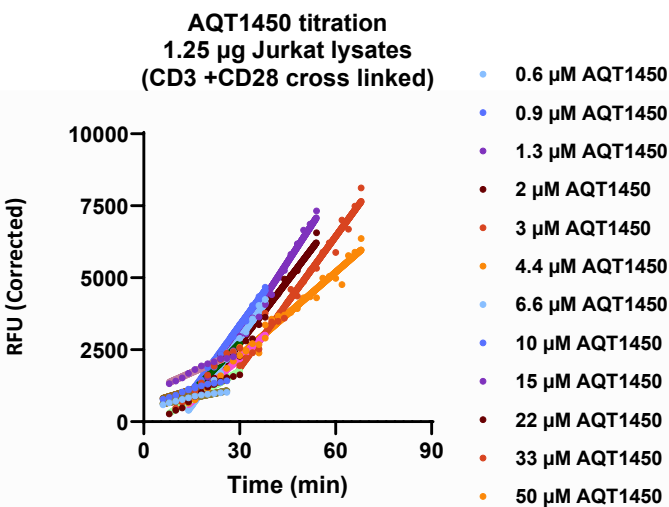
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

Sensor Peptide Substrate K_m In JURKAT Lysates

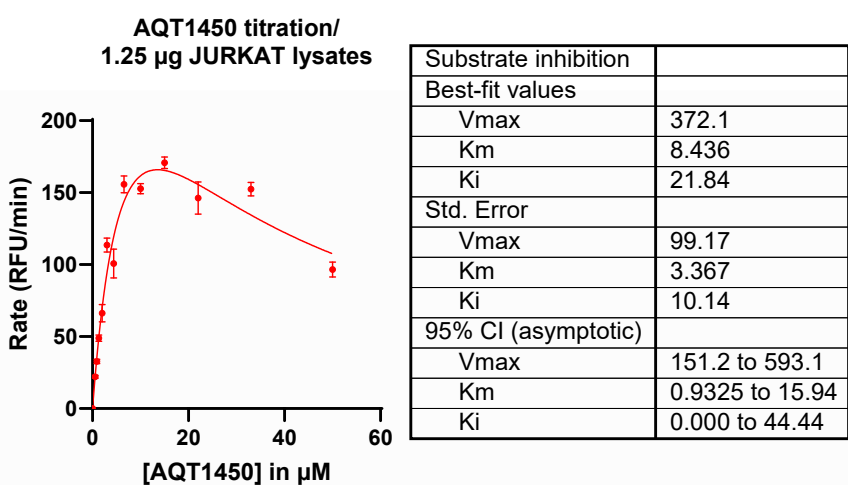
Sensor peptide titrations full progress curve



Sensor peptide Titrations linear range

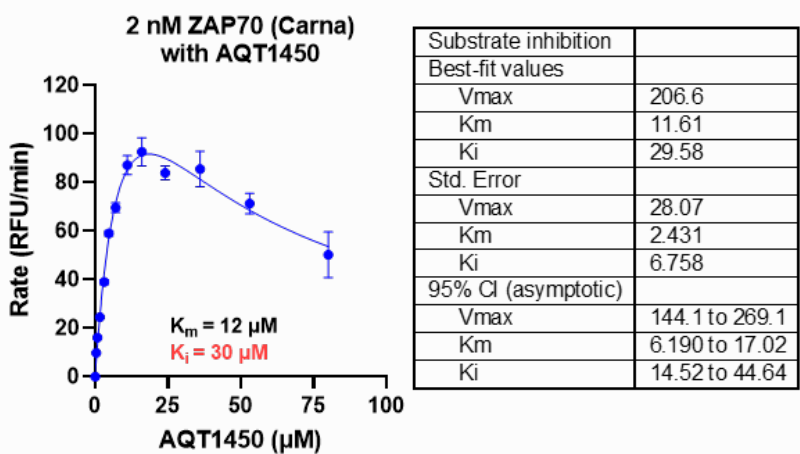


Sensor peptide K_m in Lysates



- ❖ AQT1450 concentrations above 15 μ M resulted in reduced reaction rates, consistent with the substrate inhibition trend observed (right hand side).
- ❖ The rate versus substrate concentration data did not fit a standard Michaelis-Menten model. However, fitting the data to a substrate inhibition model yielded a K_m of 8.4 μ M and a K_i of 22 μ M.
- ❖ The K_m value determined using Jurkat lysates was comparable to that obtained in the recombinant ZAP70 assay (K_m = 12 μ M; K_i = 30 μ M), supporting consistent substrate behavior across both assay formats.

Sensor peptide K_m for recombinant full length ZAP0 (Carna)



Assay Conditions for DMSO Titration

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 MgCl₂

12 μM AQT1450 sensor peptide substrate

DMSO titration - 1.5 –fold serially diluted in water (11-points) with 10 % top concentration in the wells

JURKAT cell lysates – 5 μL of 0.25 mg/mL (1.25 μg final concentration) in 25 μL total assay volume

Reaction Set Up:

- Add **12 μL 2.08 X Reaction Mix with ATP, & DTT**
- Add **5 μL of 5X AQT1450**
- Add **2.5 μL of 10X DMSO**
- Add **5 μL JURKAT Lysate or Blank buffer (lysate buffer + EDB)**, to obtain **25 μL** Final reaction volume

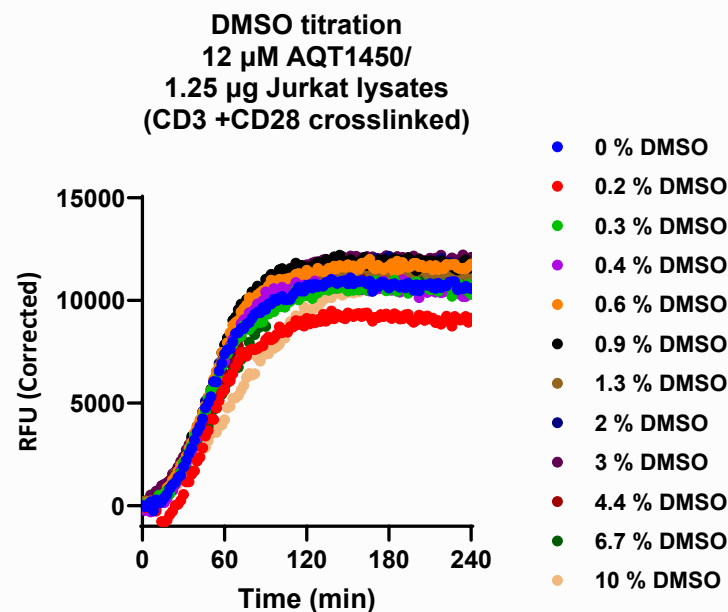
Notes:

Reaction was run at 30 °C for 480 minutes in a Corning® 96-well Half Area White Flat Bottom Polystyrene NBS Microplate at 25 μL final well volume after sealing with 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.

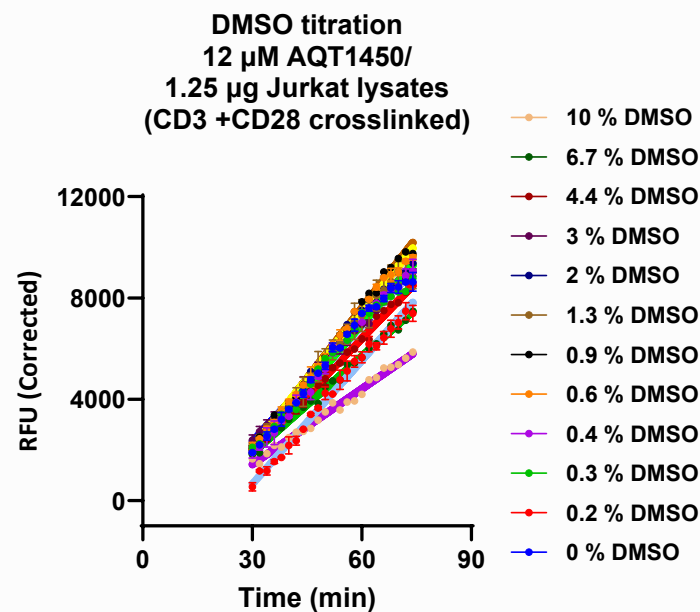
Enzyme Dilution Buffer (EDB): 20 mM HEPES, pH 7.5, 0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

DMSO Titration in JURKAT Cell Lysates

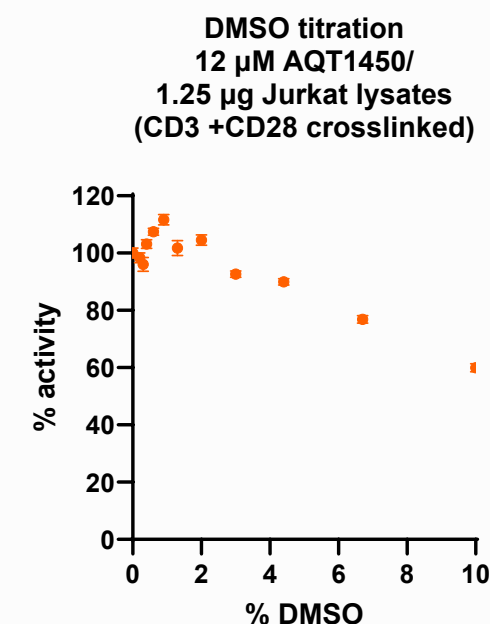
A. DMSO titrations full progress curve



B. DMSO titrations linear range



C. DMSO Titration plot



- ❖ DMSO titration curves with AQT1450 yielded progress curves at various DMSO concentrations.
- ❖ No significant loss in AQT1450 phosphorylation rates was observed up to 2 % DMSO.
- ❖ About 50% of AQT1450 phosphorylation activity was retained at 10% DMSO.
- ❖ A final DMSO concentration of 2% was used for inhibitor testing.

Assay Conditions for Recombinant IC₅₀ Assay

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 MgCl₂
12 μM AQT1450

Tool Compounds – All tool compounds were tested at a top concentration of 10 μM, serially diluted 3-fold (11-points) in DMSO (Final DMSO concentration is 2%)

RDN2150 (ZAP70 inhibitor)

PRT062607 (SYK inhibitor)

RO9021 (SYK inhibitor)

Kinases – 1.5 nM ZAP70 (Carna, Cat: 08-177) and 1 nM SYK (Sino Bio, Cat: S52-10G)

Reaction Set Up:

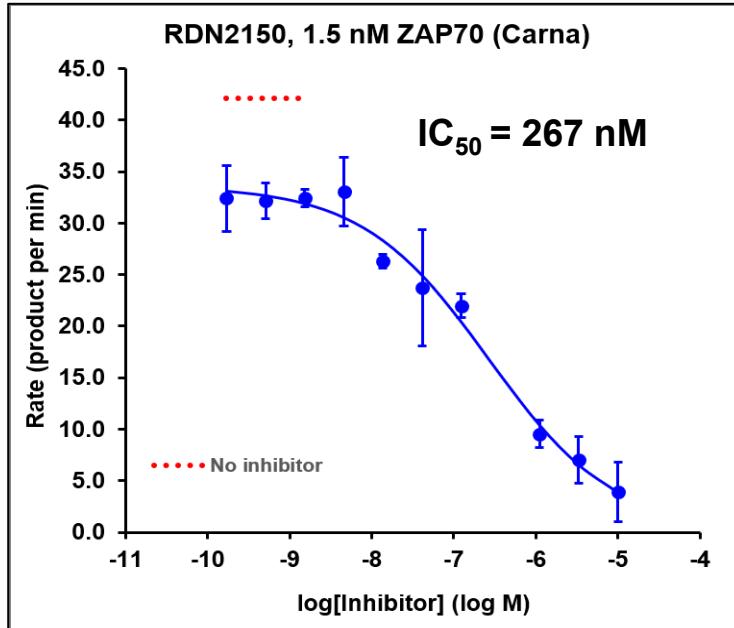
- Add **14.5 μL 1.72X Reaction Mix with ATP, & DTT**
- Add **5 μL of 5X AQT1450**
- Add **0.5 μL of 50X tool compounds**
- Add **5 μL kinase or Blank buffer (lysate buffer + EDB),** to obtain **25 μL** Final reaction volume

Notes:

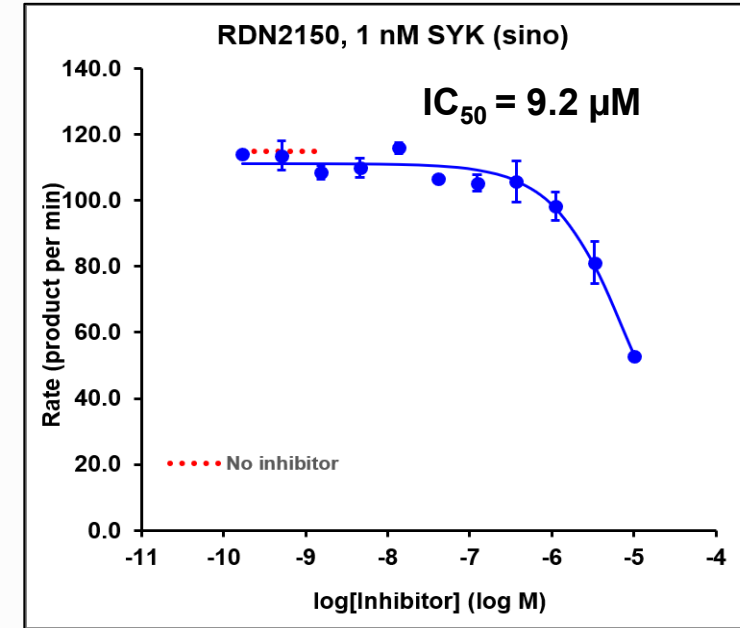
Reaction was run at 30 °C for 480 minutes in Corning® 96-well Half Area White Flat Bottom Polystyrene NBS Microplate at 25 μL final well volume after sealing using optically- clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

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

IC₅₀ Data for RDN2150 Against Recombinant ZAP70 and SYK Using the AQT1450 Sensor Peptide Substrate



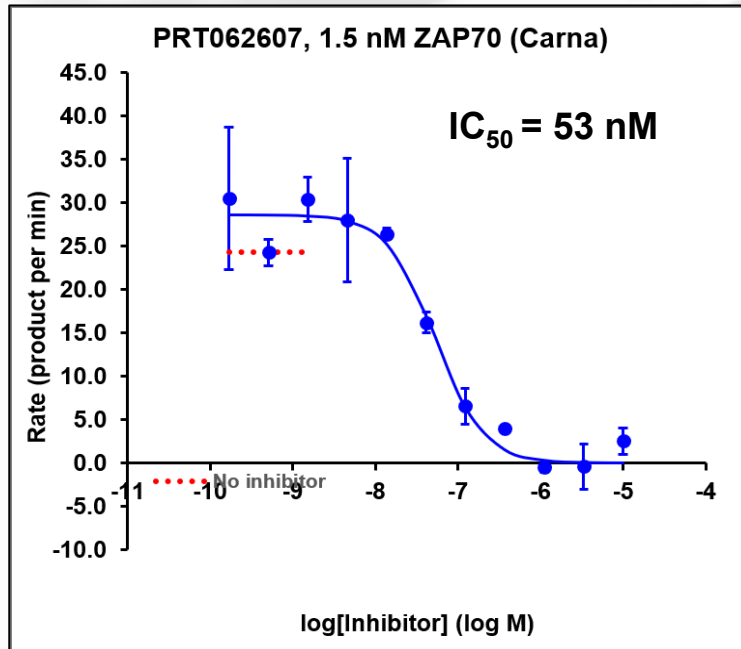
Parameter	Value
Bottom	0.0
Top	33.6
log IC ₅₀	-6.57
IC ₅₀ (nM)	266.58
K _i (nM)	14.85
Slope	-0.563
R squared	0.986
IC ₅₀ approx SE (nM)	14.93
50% inhibition (nM)	266.58



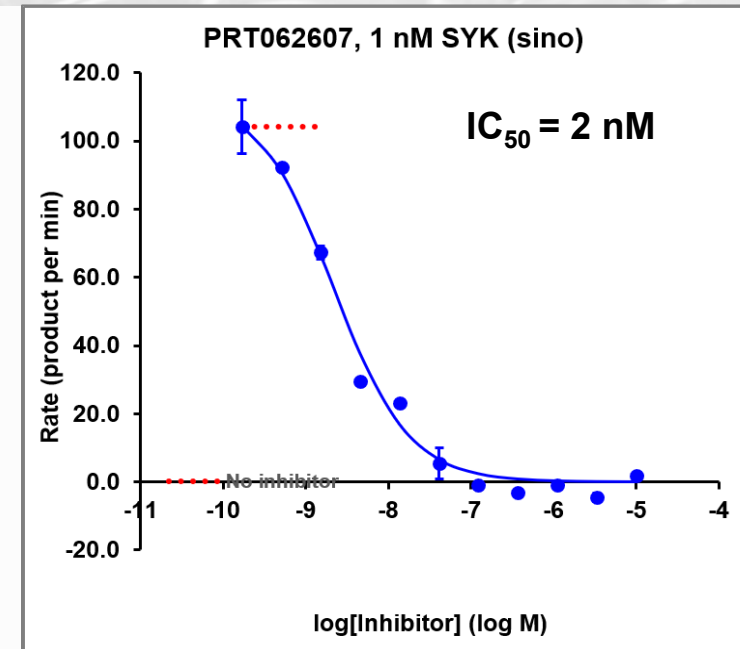
Parameter	Value
Bottom	0.0
Top	111.1
log IC ₅₀	-5.04
IC ₅₀ (nM)	9206.87
K _i (nM)	512.94
Slope	-0.934
R squared	0.978
IC ₅₀ approx SE (nM)	257.64
50% inhibition (nM)	9206.87

- ❖ RDN2150 inhibits ZAP70 with an IC₅₀ of 267 nM. However, the compound is a weak SYK inhibitor with an IC₅₀ value of 9200 nM.
- ❖ RDN2150 exhibits 34-fold selectivity over SYK.

IC₅₀ data for PRT062607 against recombinant ZAP70 and SYK Using the AQT1450 Sensor Peptide Substrate



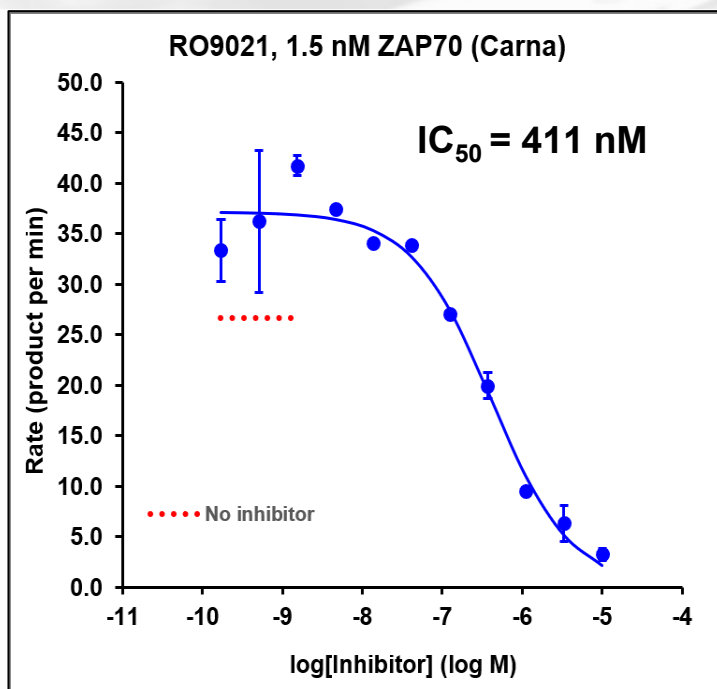
Parameter	Value
Bottom	0.0
Top	28.7
log IC ₅₀	-7.27
IC ₅₀ (nM)	53.35
K _i (nM)	2.97
Slope	-1.486
R squared	0.972
IC ₅₀ approx SE (nM)	3.24
50% inhibition (nM)	53.35



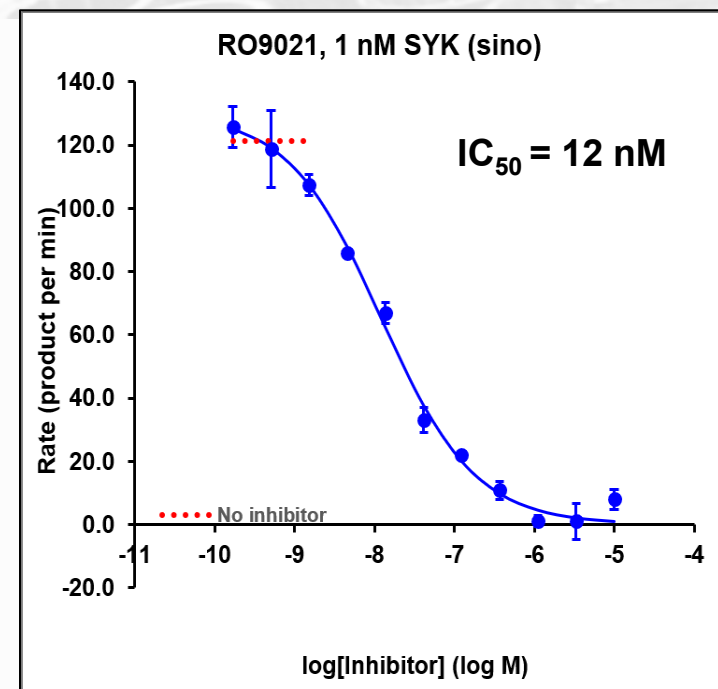
Parameter	Value
Bottom	0.0
Top	113.9
log IC ₅₀	-8.66
IC ₅₀ (nM)	2.17
K _i (nM)	0.12
Slope	-0.949
R squared	0.985
IC ₅₀ approx SE (nM)	0.10
50% inhibition (nM)	2.17

- ❖ PRT062607 inhibits ZAP70 with an IC₅₀ of 53 nM. However, the compound is an even more *potent* SYK inhibitor with an IC₅₀ value of 2 nM.
- ❖ Although PRT062607 shows some selectivity over ZAP70 (25-fold), it would be difficult to use this tool compound to differentiate ZAP70 and SYK activity in lysates. By comparison, RDN2150 exhibits 34-fold selectivity over SYK.

IC₅₀ Data for RO9021 Against Recombinant ZAP70 and SYK Using the AQT1450 Sensor Peptide Substrate



Parameter	Value
Bottom	0.0
Top	37.2
log IC50	-6.39
IC50 (nM)	411.05
Ki (nM)	22.90
Slope	-0.869
R square	0.969
IC50 approx SE (nM)	20.07
50% inhibition (nM)	411.05



Parameter	Value
Bottom	0.0
Top	130.5
log IC50	-7.92
IC50 (nM)	12.13
Ki (nM)	0.68
Slope	-0.738
R square	0.996
IC50 approx SE (nM)	0.41
50% inhibition (nM)	12.13

- ❖ RO9021 is a *weak* ZAP70 inhibitor with an IC₅₀ of 411 nM. However, the compound is a *potent* SYK inhibitor with an IC₅₀ value of 12 nM.
- ❖ RO9021 exhibits 34-fold selectivity over ZAP70.

Assay Conditions for Tool Compound IC₅₀ in JURKAT Cell Lysates



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 MgCl₂
12 μM AQT1450 sensor peptide substrate

Tool Compounds – All tool compounds were tested at a top concentration of 10 μM serially diluted 3-fold (11-points) in DMSO (Final DMSO concentration is 2%)

RDN2150 (ZAP70 inhibitor)

PRT062607 (SYK inhibitor).

RO9021 (SYK inhibitor)

JURKAT cell lysates – 5 μL of 0.25 mg/mL (5 μg final concentration) in 25 μL total assay volume

Reaction Set Up:

- Add **14.5 μL 1.72X Reaction Mix with ATP, & DTT**
- Add **5 μL of 5X AQT1450**
- Add **0.5 μL of 50X tool compounds**
- Add **5 μL JURKAT Lysate or Blank buffer (lysate buffer + EDB), to obtain 25 μL Final reaction volume**

Notes:

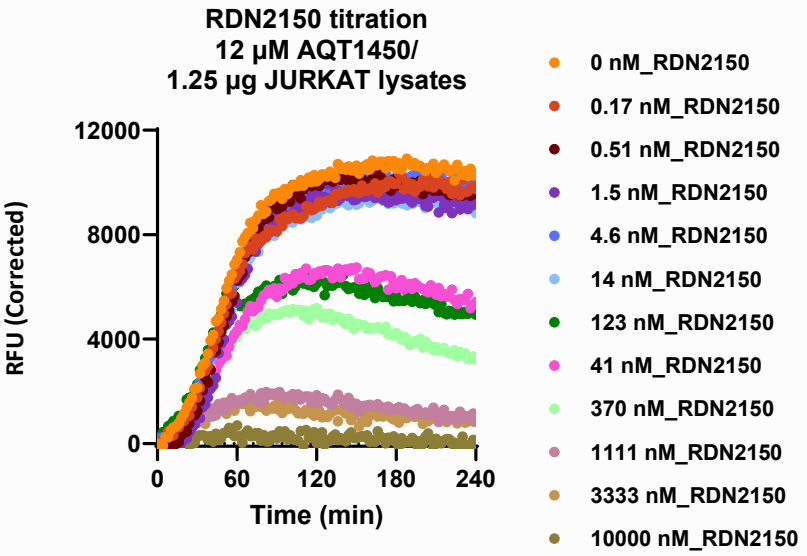
Reaction was run at 30 °C for 480 minutes in a Corning® 96-well Half Area White Flat Bottom Polystyrene NBS Microplate at 25 μL final well volume after sealing with optically clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a BioTek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

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.

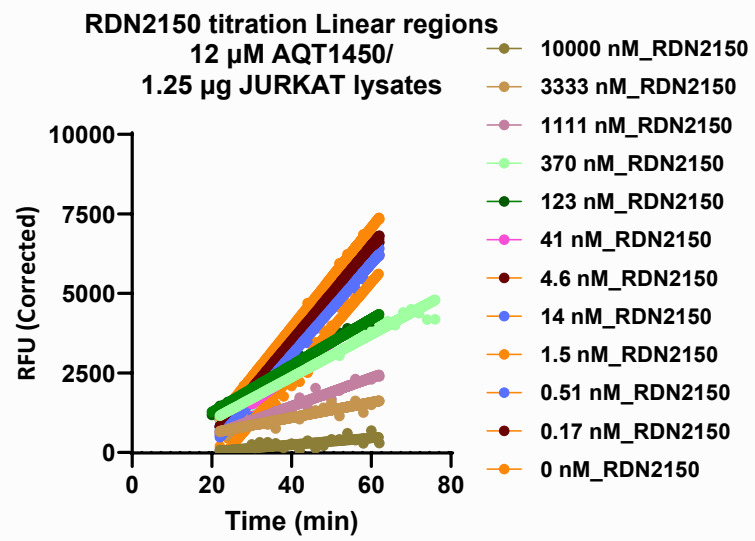
RDN2150 Titration to Determine the IC₅₀ in JURKAT Cell Lysates Using the AQT1450 Sensor Peptide Substrate



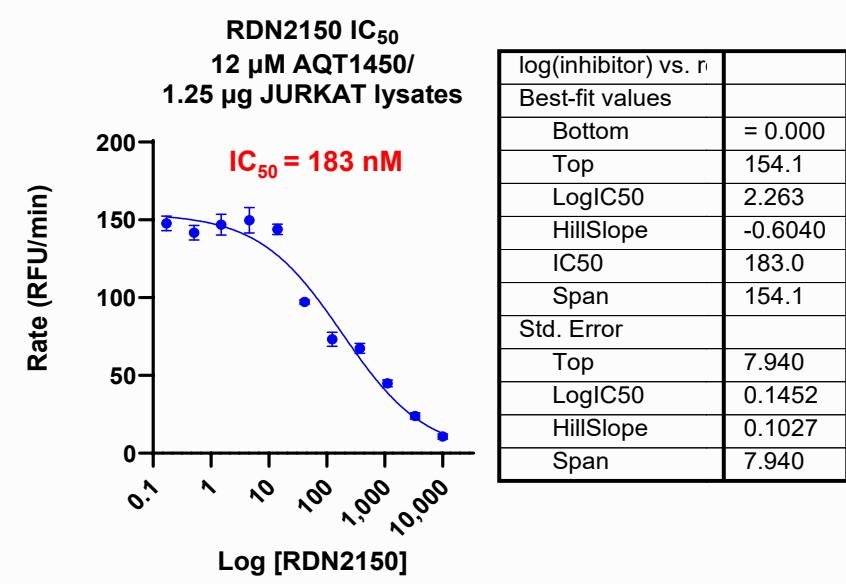
A. RDN2150 Titrations Full Progress Curve



B. RDN2150 Titrations Linear Range



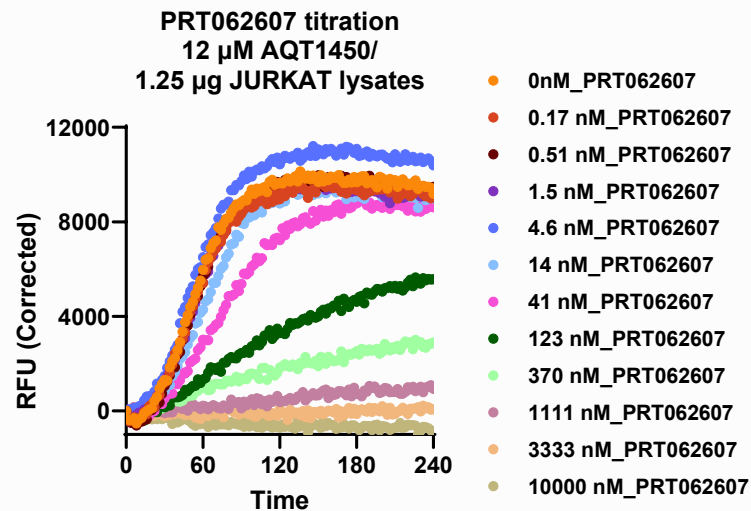
C. RDN2150 Titration Plot



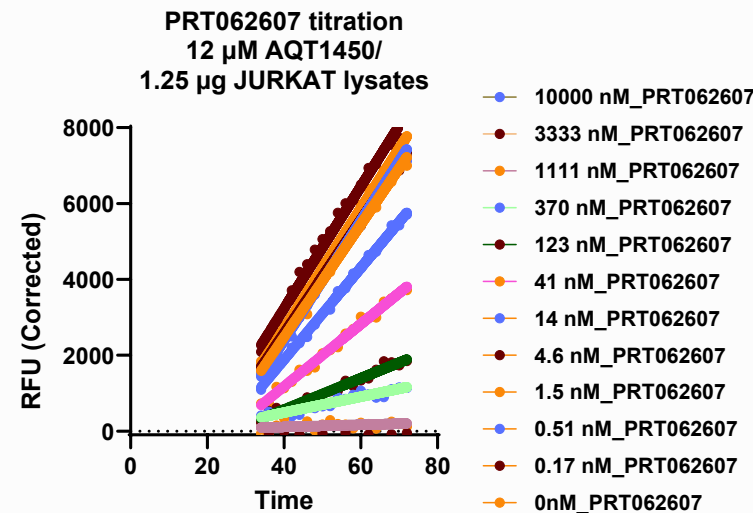
- ❖ RDN2150 titration using JURKAT lysates resulted in an IC₅₀ value of 183 nM.
- ❖ The lysate IC₅₀ value for RDN2150 is comparable to its IC₅₀ of 267 nM against recombinant ZAP70 (Carna-FL), and not that for SYK (9,200 nM). Compare to the recombinant data shown in slide 17.

PRT062607 Titration to Determine the IC₅₀ in JURKAT Cell Lysates Using the AQT1450 Sensor Peptide Substrate

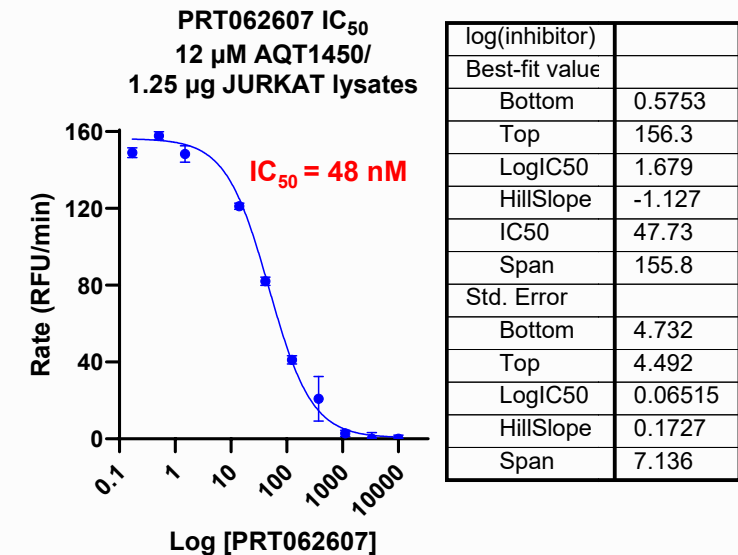
A. PRT062607 titrations full progress curve



B. PRT062607 titrations linear range



C. PRT062607 Titration plot

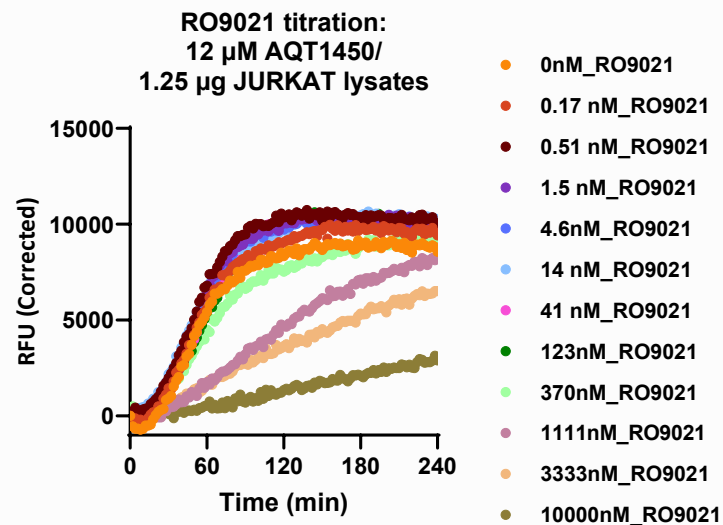


- ❖ The IC₅₀ value for PRT062607 in JURKAT lysates is 48 nM.
- ❖ The lysate IC₅₀ value for PRT062607 is closer to the recombinant ZAP70 (Carna) value (53 nM), and not that for SYK (2 nM). Recombinant data shown in slide 18.

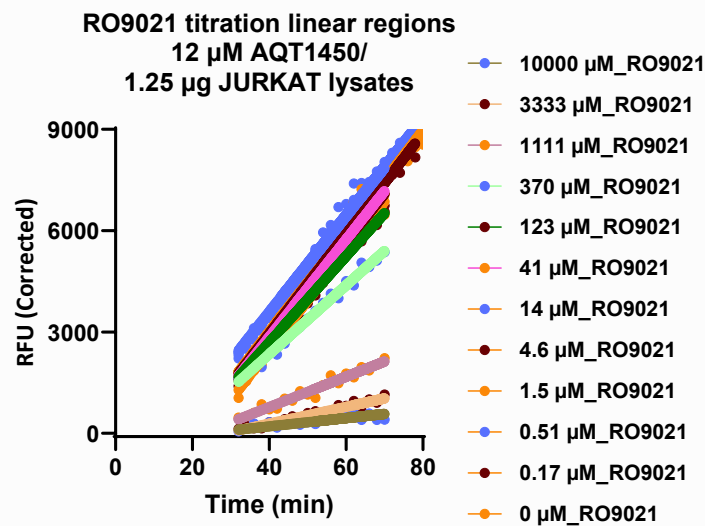
RO9021 Titration to Determine the IC₅₀ in JURKAT Cell Lysates Using the AQT1450 Sensor Peptide Substrate



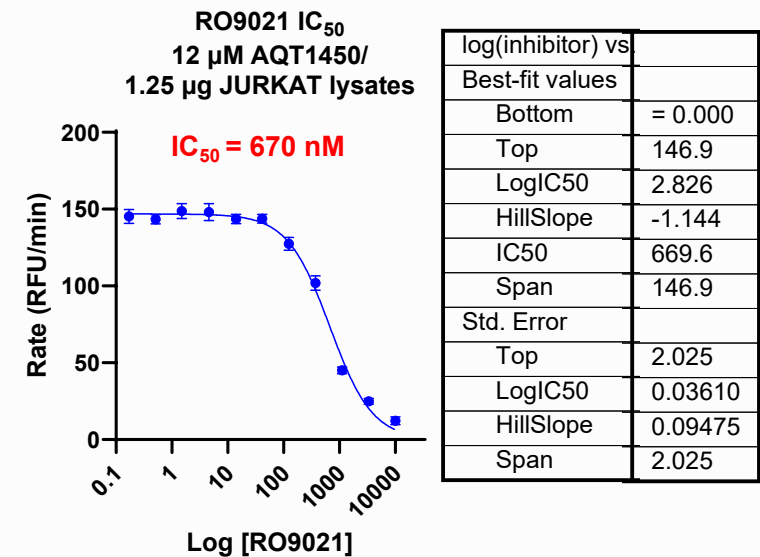
A. RO9021 titrations full progress curve



B. RO9021 titrations linear range



C. RO9021 Titration plot



- ❖ RO9021 titration using JURKAT lysates gave an IC₅₀ value of 670 nM.
- ❖ The lysate IC₅₀ value for RO9021 is comparable to the recombinant ZAP70 (Carna) value (411 nM), and not that for SYK (2 nM). Recombinant data shown in slide 19.

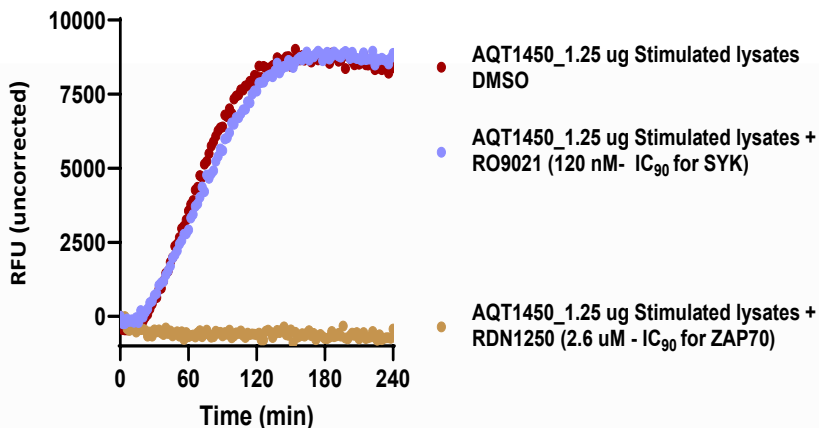
Comparison of IC₅₀ Values Between Recombinant and Lysate Assay Formats Using the AQT1450 Sensor Peptide Substrate

Compound	Recombinant IC ₅₀ (nM)		Lysate IC ₅₀ (nM)	
	ZAP70 (Carna)	SYK (Sino)	JURKAT Cell (p. 5) Lysates	
RDN2150	267	9,200	183	Used as a tool compound in lysate assays to support ZAP70 activity measurements.
PRT062607	53	2	48	
RO9021	411	12	670	Used as a tool compound in lysate assays to support SYK activity measurements.

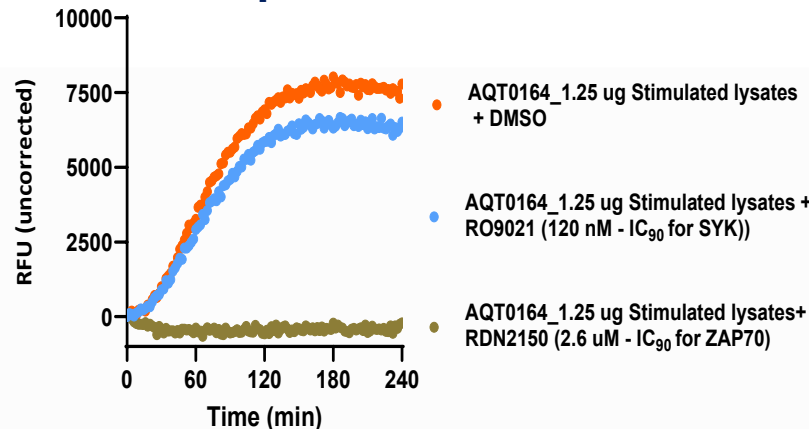
- ❖ The table shows IC₅₀ values determined in lysate and recombinant assays.
- ❖ The IC₅₀ values of the 3 tool compounds in crude JURKAT cell lysates compared well with the IC₅₀ values determined with recombinant full-length ZAP70 (Carna).
- ❖ The similarity in the potency values suggests that the likely target of these compounds in lysates is ZAP70.

Lysate testing using AQT0164 and AQT0166, which are SYK Sensor Peptide Substrates

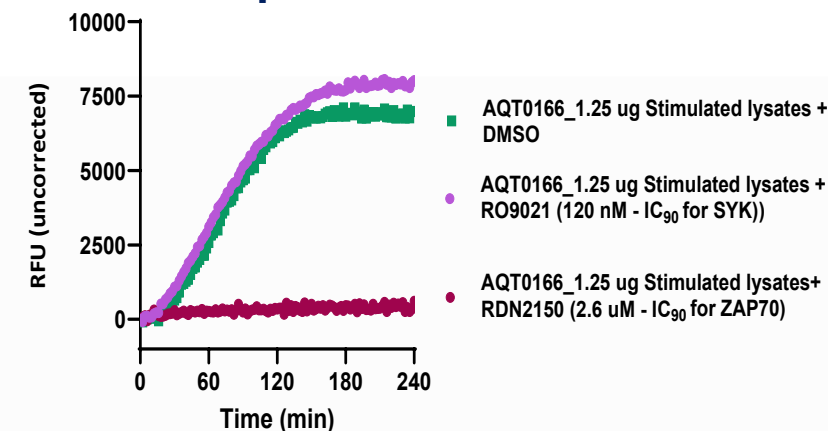
A. AQT1450 with Selective Tool Compounds for ZAP70 and SYK



B. AQT0164 with Selective Tool Compounds for ZAP70 and SYK



C. AQT0166 with Selective Tool Compounds for ZAP70 and SYK



- ❖ AQT0164 and AQT0166 are SYK substrates that also exhibit off-target ZAP70 activity. Both substrates were phosphorylated following treatment with 1.25 μ g of stimulated Jurkat cell lysate.
- ❖ Phosphorylation of AQT0164 and AQT0166 was inhibited by 2.6 μ M RDN2150 (ZAP70 IC₉₀), whereas no inhibition was observed with 0.12 μ M RO9021 (SYK IC₉₀).
- ❖ These findings further support that the phosphorylation of AQT1450 in CD3/CD28-stimulated Jurkat lysates is primarily driven by ZAP70 activity rather than SYK activity.

Table: Phosphorylation rates (RFU/min) and % activity in parentheses for each sensor peptide – tool compound pair

Sensor peptide	Rates in RFU/min (percent activity remaining)		
	DMSO alone or with Tool compounds		
	DMSO	RDN2150	RO9021
AQT1450	71 (100)	1.5 (2.1)	62 (87)
AQT0164	58 (100)	1.6 (2.7)	50 (86)
AQT0166	49 (100)	1.7 (3.5)	52 (100)

Summary

- ❖ The PhosphoSens-Lysate Activity Assay for ZAP70 using the sensor peptide AQT1450 demonstrates a robust, selective, and physiologically relevant assay that provides a functional assessment of endogenous ZAP70 activity with all the cellular components and signaling complexes. This ZAP70 activity assay is direct, highly quantitative, and easy to use.
- ❖ **Supporting results include:**
 - Treatment of serum-starved JURKAT cells with 10 µg/mL Anti-CD3 + 10 µg/mL Anti-CD28 (and 20 µg/mL Goat Anti-Mouse Kappa Light Chain, F(ab')₂) increases ZAP70 activation by 1.9-fold, as evidenced by the phosphorylation of AQT1450 (slide 9). This highly reproducible, but relatively low, level of activation is consistent with reports showing high basal activity in this cell line, in contrast to the low basal and high activation seen with primary T-cells or PBMC preparations.
 - ZAP70 activity with lysates from activated JURKAT cells was linear from 0.3 – 1.25 µg of lysates (total protein)
 - The K_M value of 9 µM was determined for AQT1450, which is comparable to the K_M from the recombinant assay (K_M = 12 µM).
 - DMSO titration experiments indicated that the AQT1450 phosphorylation activity was unaffected up to 2% DMSO, which was used for inhibitor testing.
 - IC₅₀ experiments in lysates with the 3 tool compounds, RDN2150, RO9021 and PRT062607 resulted in IC50 values that are comparable to the recombinant full-length ZAP70 IC50 values.
 - Including 2.6 µM of the selective ZAP70 inhibitor, RDN2150, resulted in complete inhibition of activity in the activated Jurkat cell lysates, while 0.12 µM of the selective SYK inhibitor, RO9021, resulted in minimal inhibition. Thus, the phosphorylation of AQT1450 in CD3/CD28-stimulated Jurkat lysates is primarily driven by ZAP70 activity rather than SYK activity.

AQT1450 enables selective and precise quantitation of ZAP70 isoform activity across cell types, providing a powerful tool for evaluating pathway activation or inhibition in complex samples from normal or disease states

Company & Technology Supporting Slides

AQT Senior Scientific Team

Experienced & Diverse Team from Leading Institutions



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



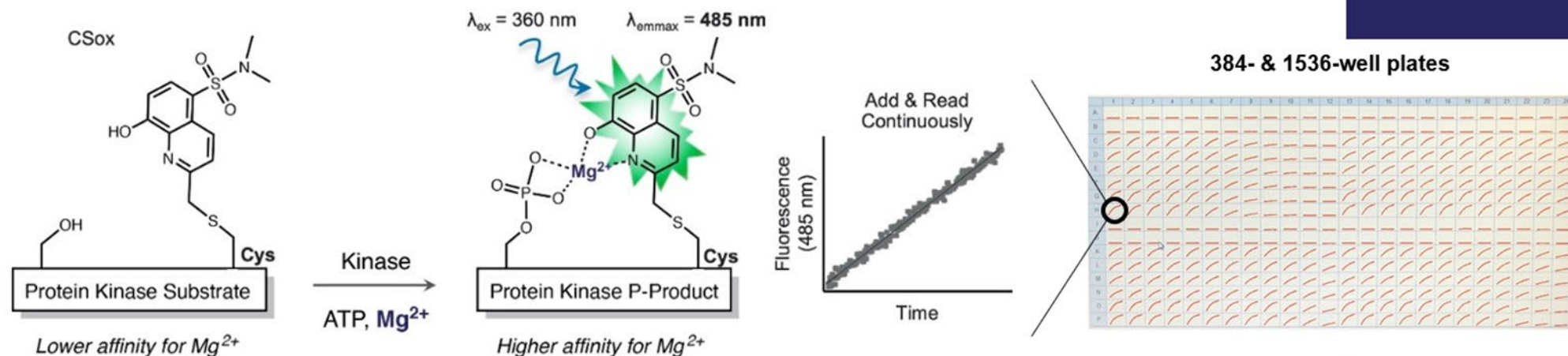
Susan Cornell-Kennon
Senior Scientist, DT



Dr. Satish Pimrale
Business
Development Leader



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)

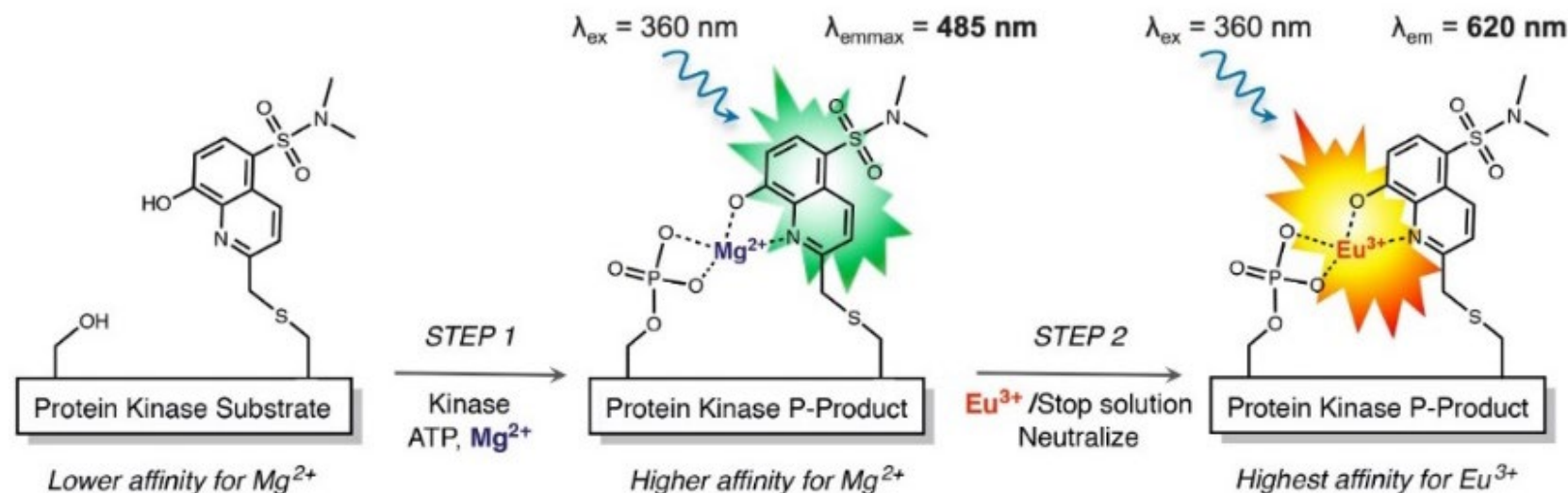
Simple, Powerful & Flexible!

*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**



Ideal for:

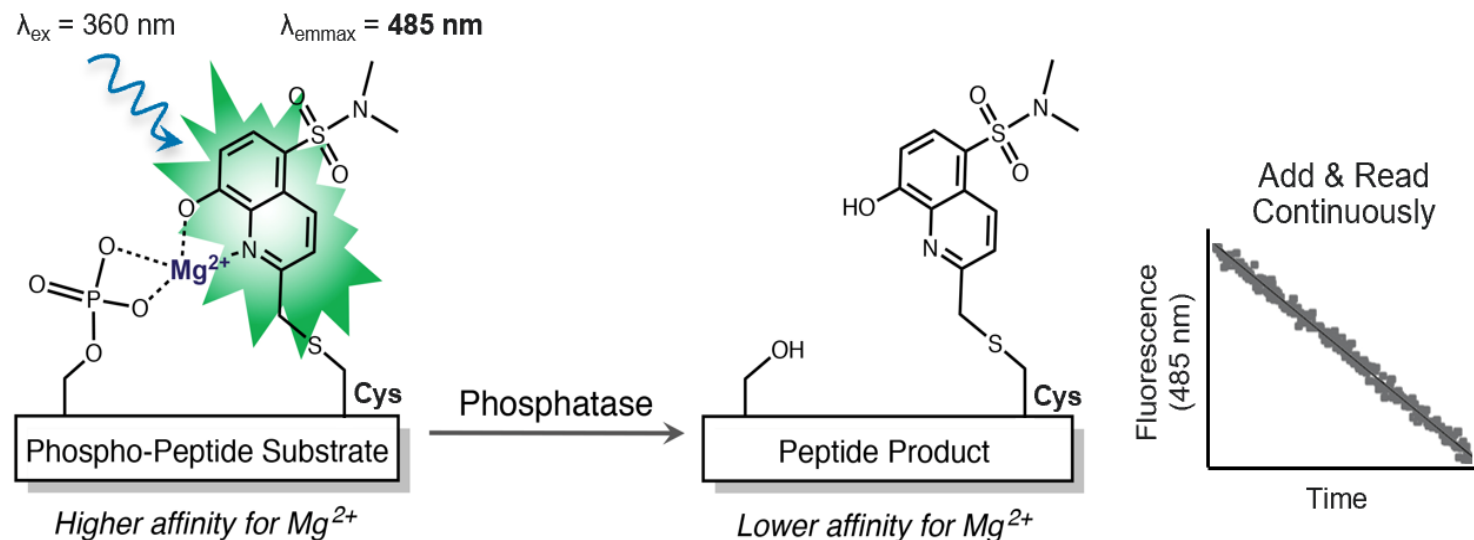
- HTS
- SAR

- ❖ 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

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

Kinetic and Endpoint

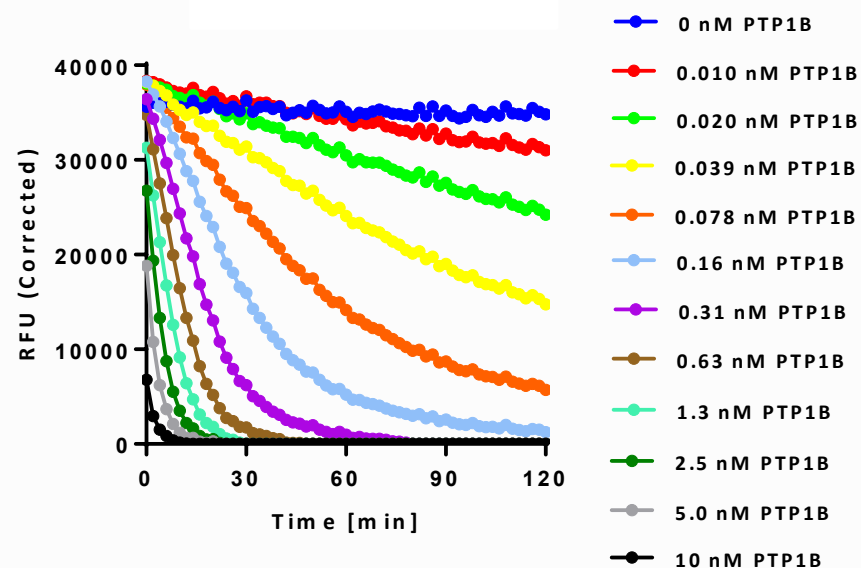
*One Technology,
Two Fit-for-Purpose
Formats*

Expanding menu
(currently 34 of
the
190 Protein
Phosphatases)

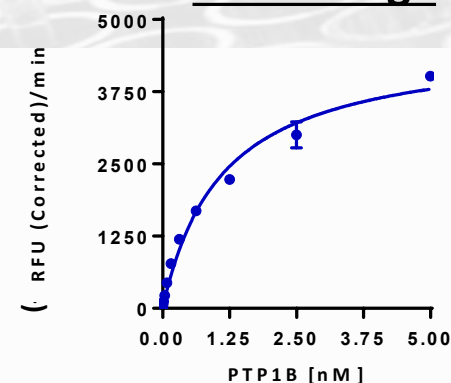
Analysis of PTP1B & PTPN2 Tyrosine Phosphatases

Optimized *PhosphoSens* Phosphopeptide Substrates

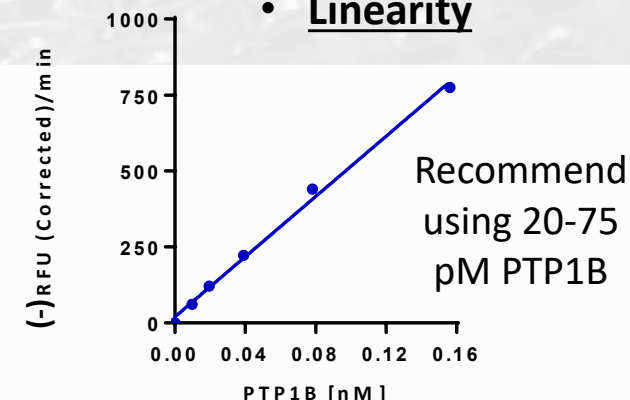
Progress Curves & Linearity for PTP1B with AQT0266 Sensor Peptide, Net Signal



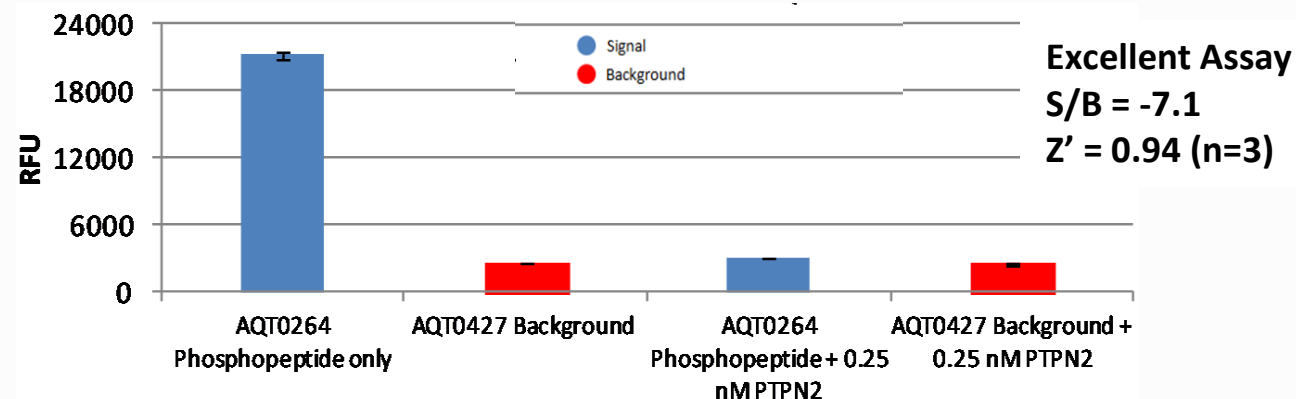
Total Range



Linearity



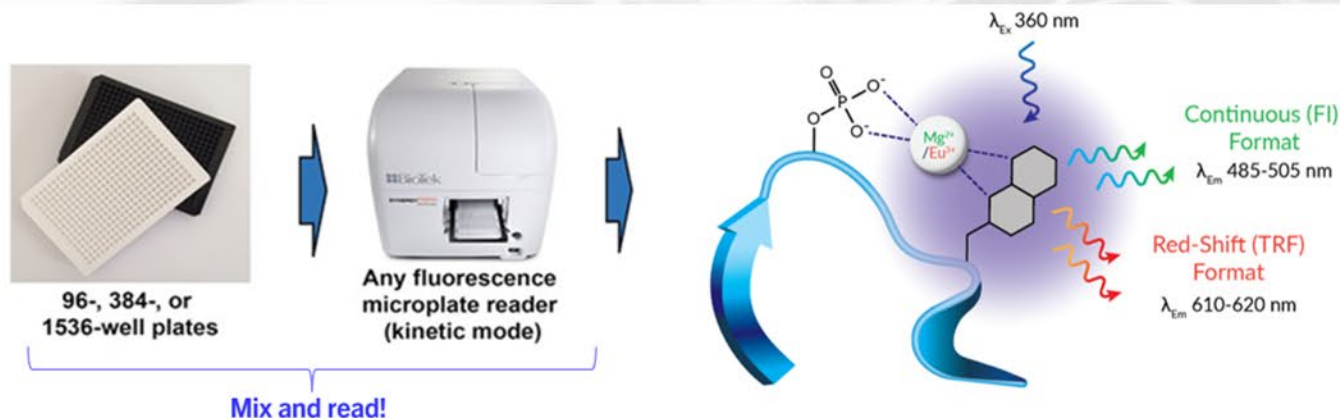
Total & Background for PTPN2 with AQT0264 in PhosphoSens®-Red



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

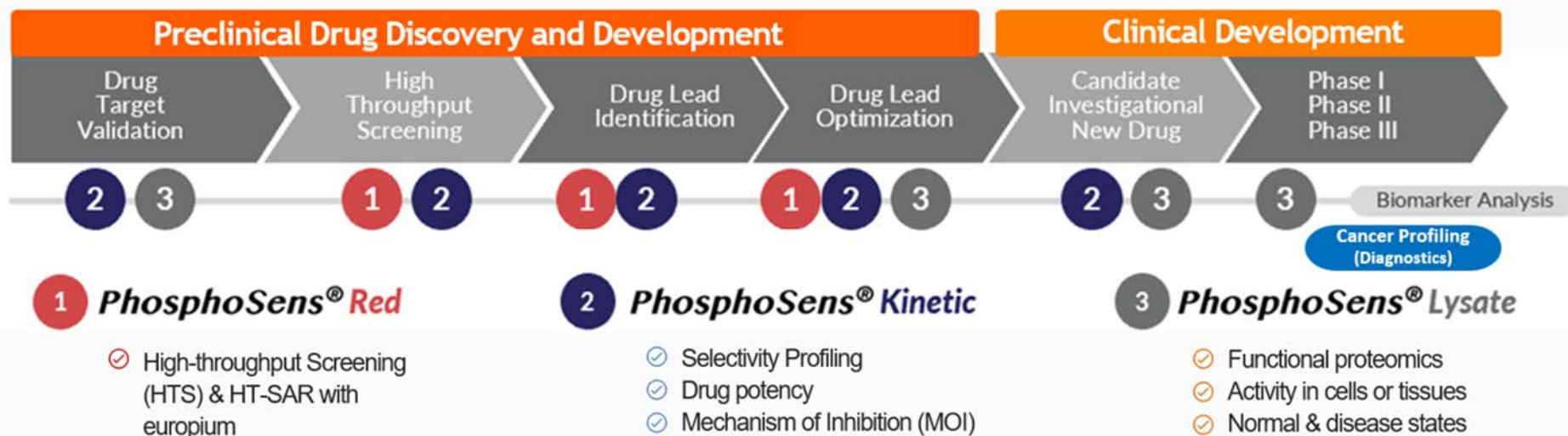
PhosphoSens® Platform

Enabling Analysis Across the Drug Development Workflow



**One Technology,
Two Fit-for-
Purpose Formats**

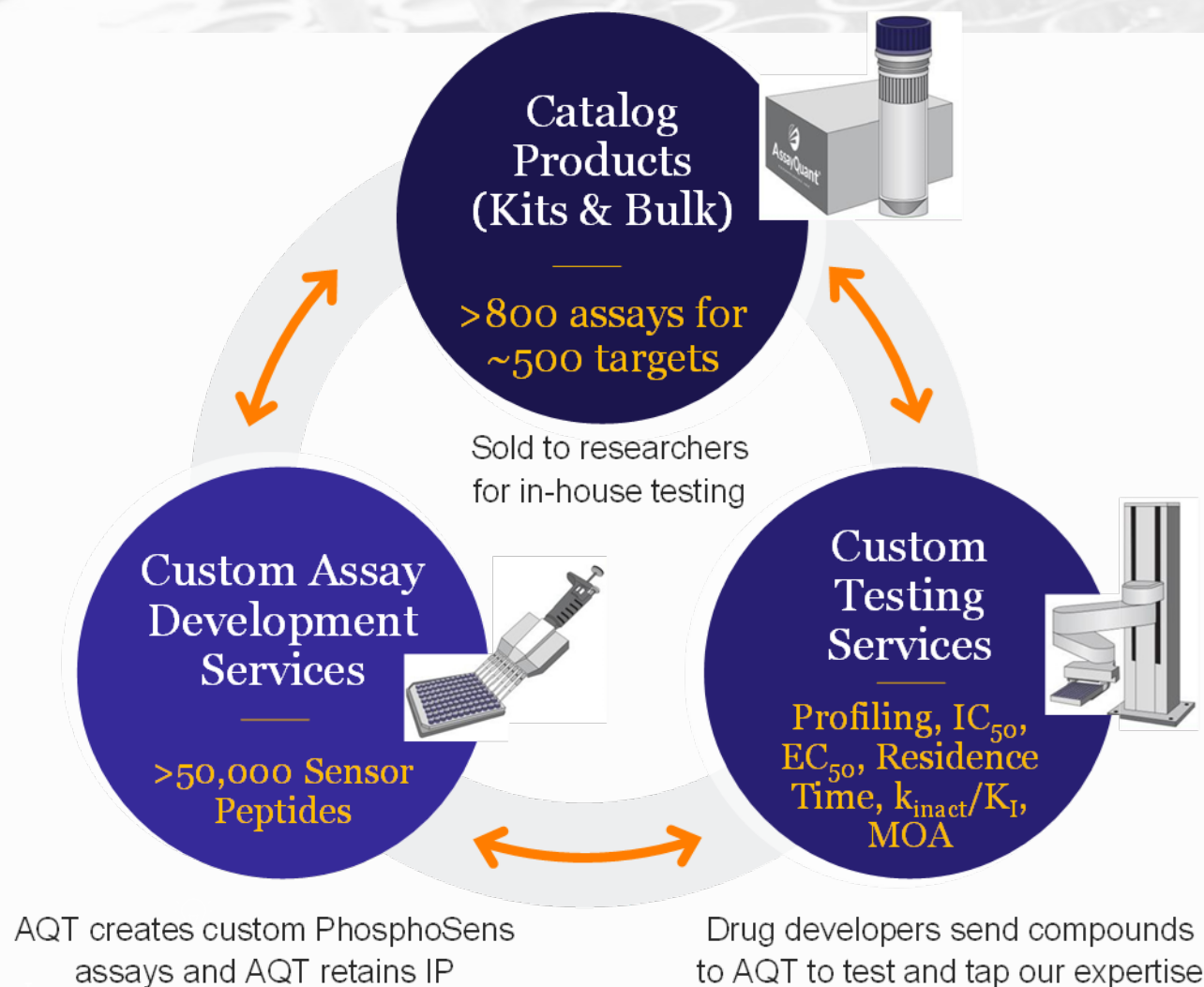
Kinetic and Endpoint



Accelerates progress and improves outcomes

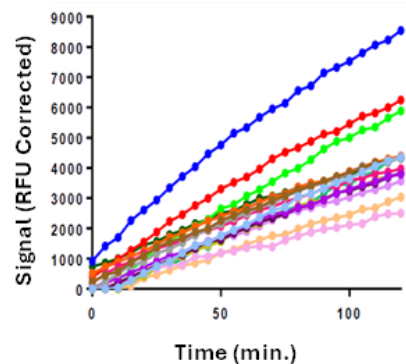
Integration of Catalog Products & Services

Multiple entry & transition points to address your needs

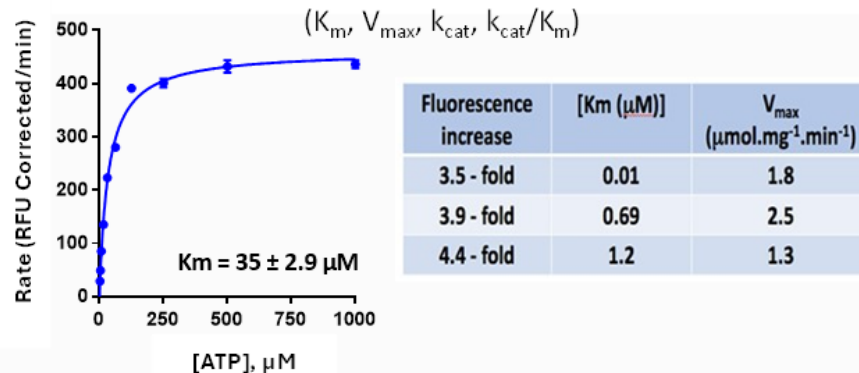


PhosphoSens® Platform Continuous Workflow Applications

Assay Development

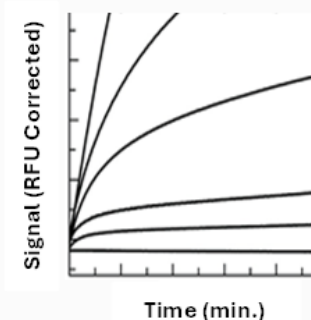


Enzyme Kinetics with Recombinant Proteins

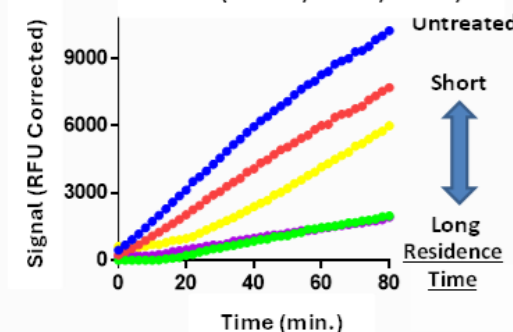


Drug Potency (IC_{50} , K_i , Residence Time, k_{inact}/K_i , MOA, EC_{50})

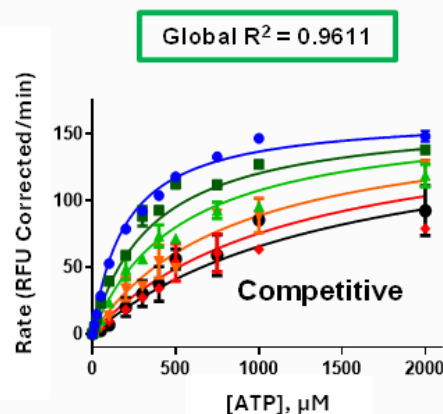
Time-Dependent Inhibition (TDI)



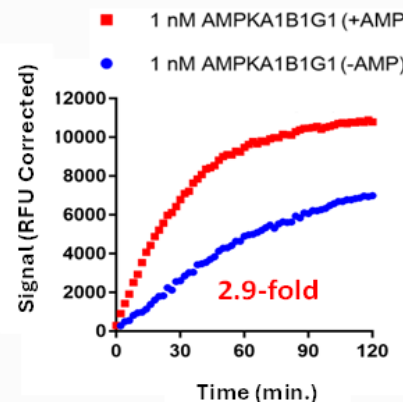
EGFR (T790M/C797S/L858R)



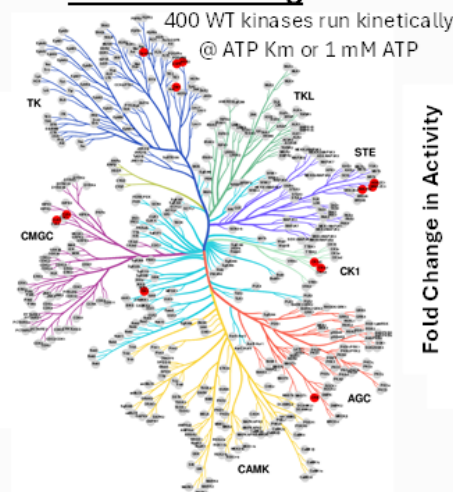
Mechanism of Inhibition (MOI)



Target Activation

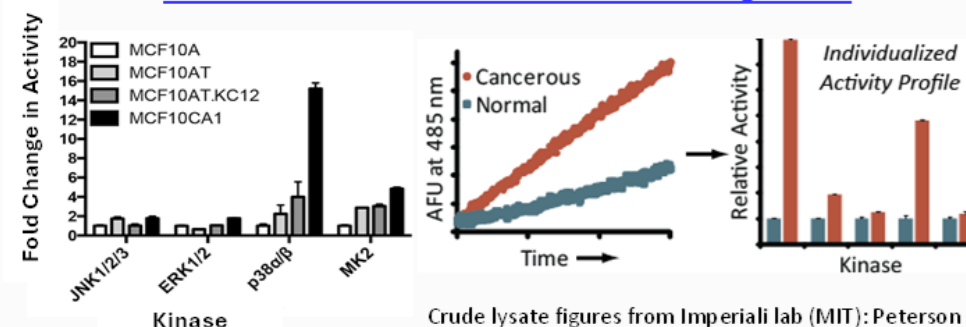


Kinome Profiling



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 **Mg²⁺, Mn²⁺ & Ca²⁺** 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 (IC₅₀, EC₅₀, k_{inact}/K_i, residence time).
- ❖ **Corrects for any compound autofluorescence** – **Kinetic**: Background signal doesn't change over time; **Red**: Uses Europium (Eu³⁺) 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.