Using DiscoveRx's ADP Quest™ for Academic Screening of Novel Kinases

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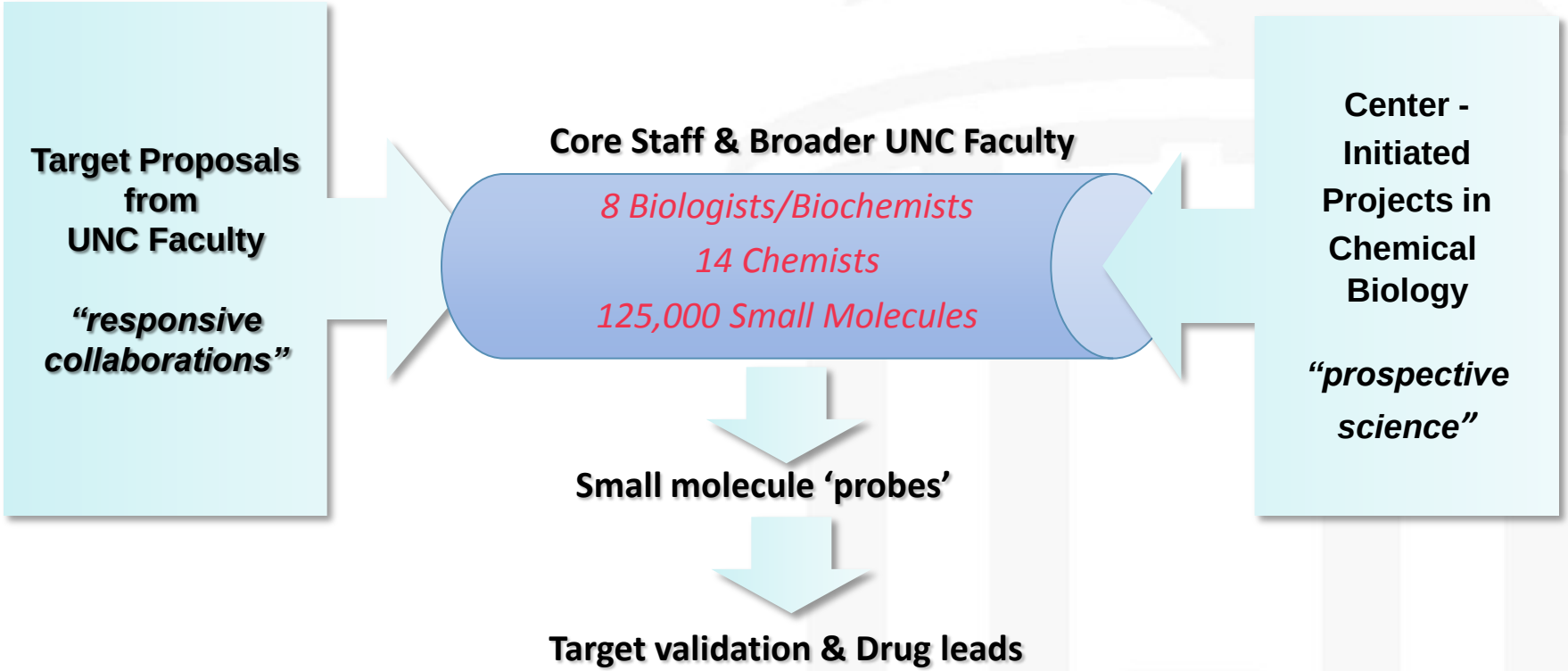
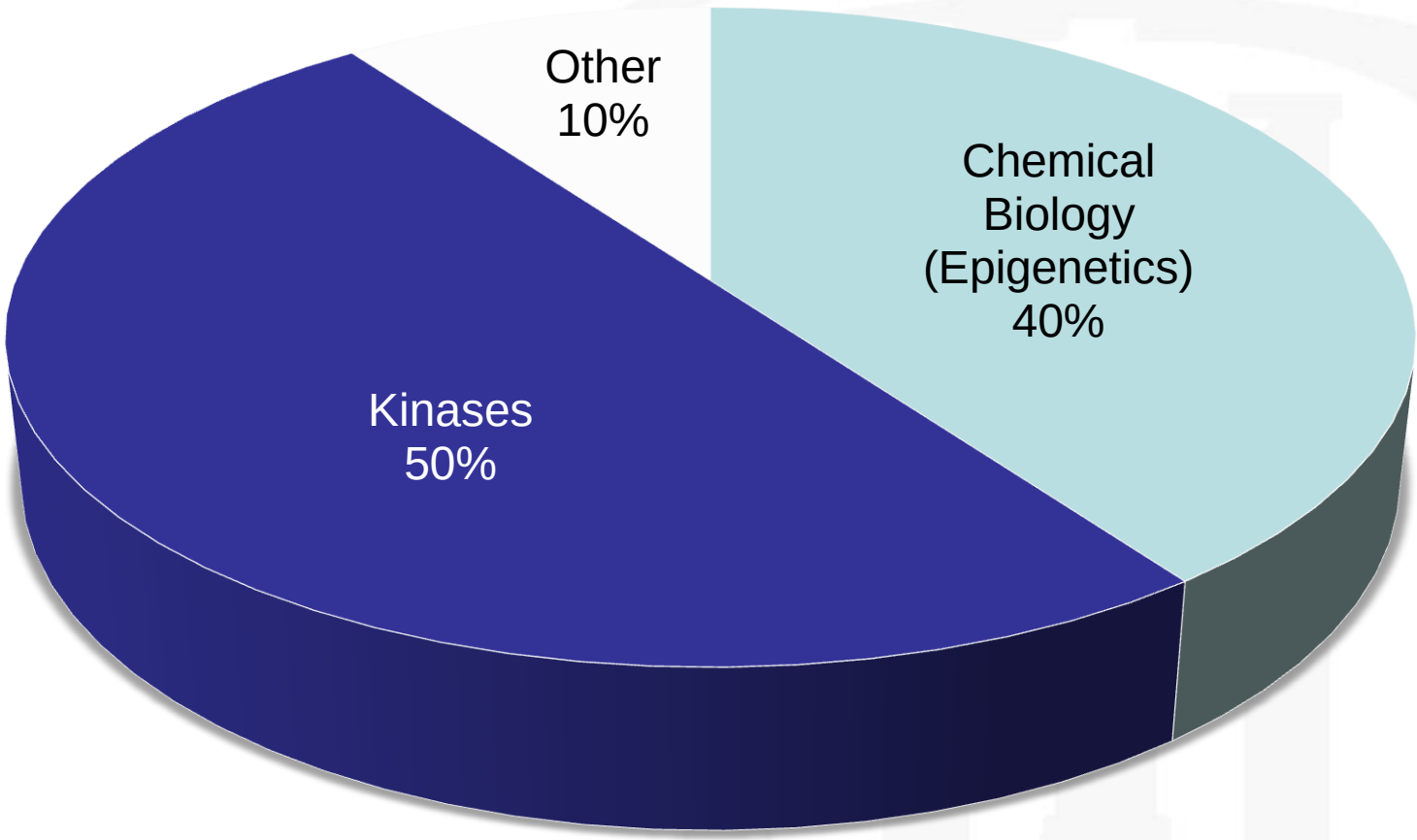
CICBDD Mission and Pipeline



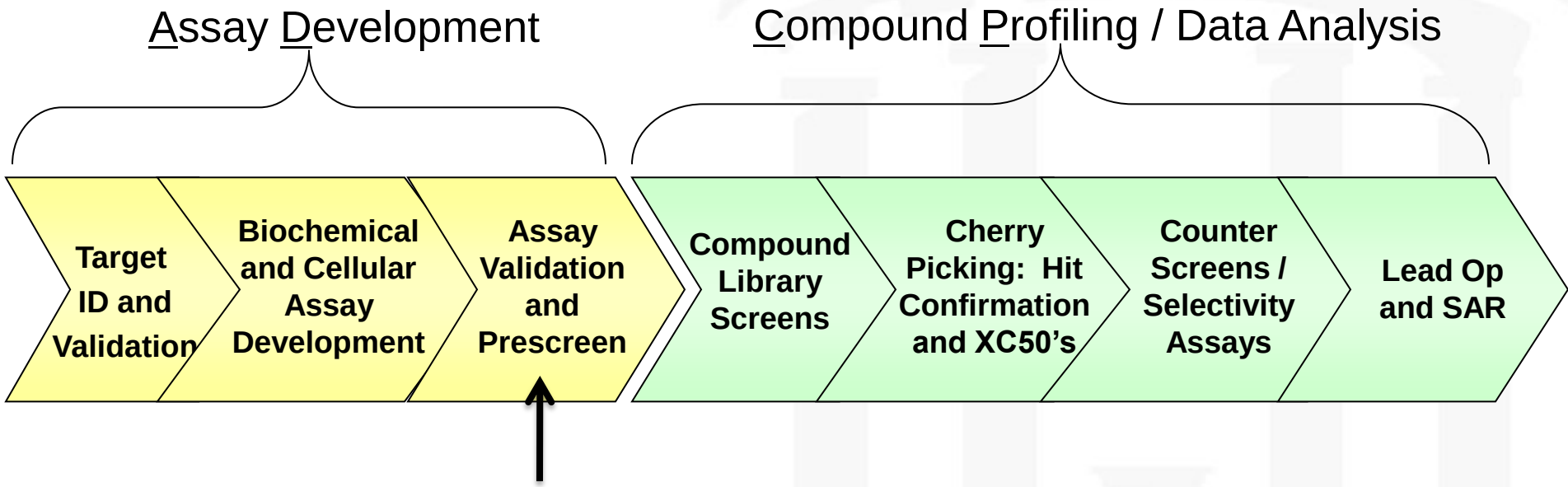
- New Center, research operations began in January 2009; mostly grant funded
- Portfolio of 18-24 pre-projects and projects running concurrently
- Extensive pharma experience among center leadership and staff, including contributions to several FDA and multiple clinical candidates
- Selected as NCI Comprehensive Chemical Biology Screening Center in 2009 (NCI CBC)

✓ **GOAL: Bring UNC to forefront of translational medicine**

CICBDD Research Areas of Interest

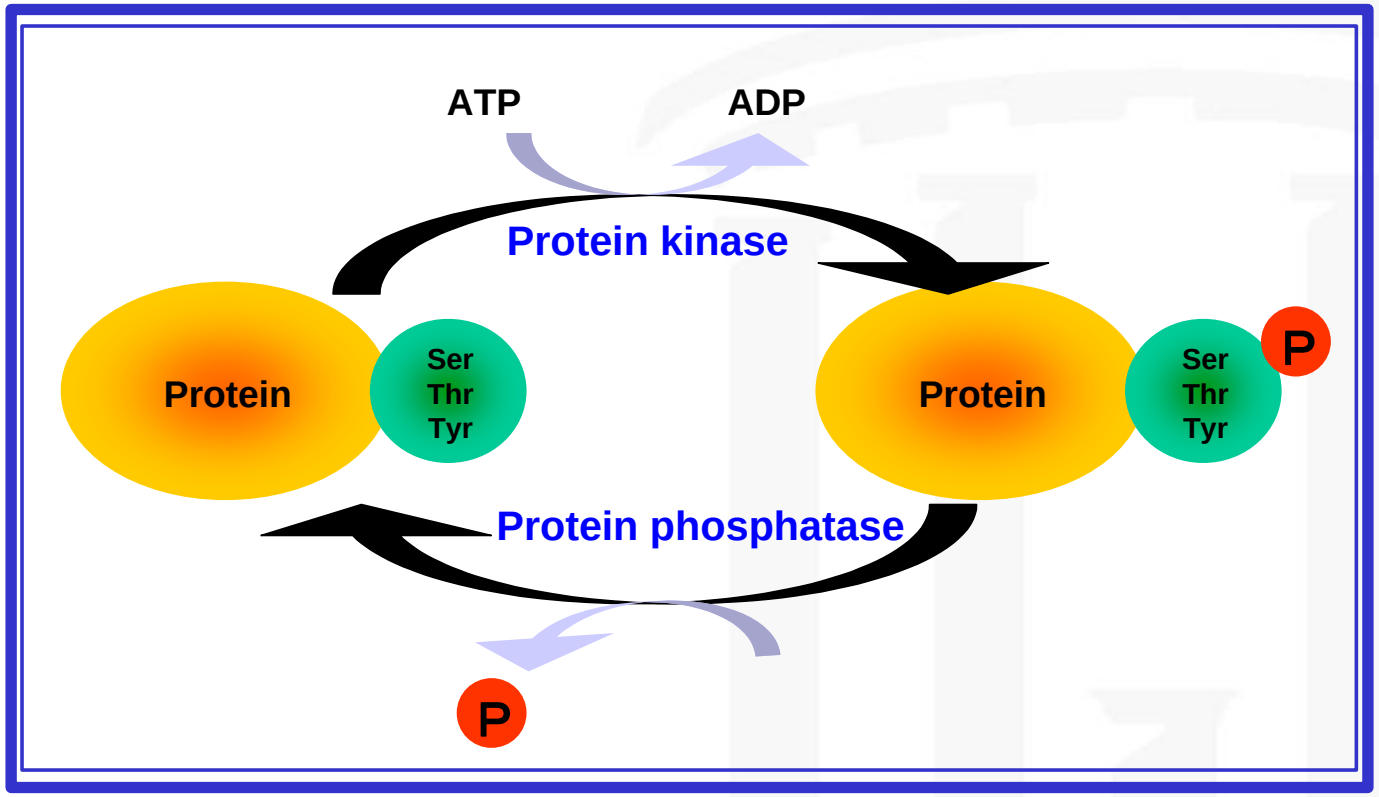


Drug Discovery Path for Kinases in CICBDD



LOPAC: Library of Pharmacologically Active Compounds
Source Sigma Aldrich
1280 Compounds

Protein Kinases as Targets

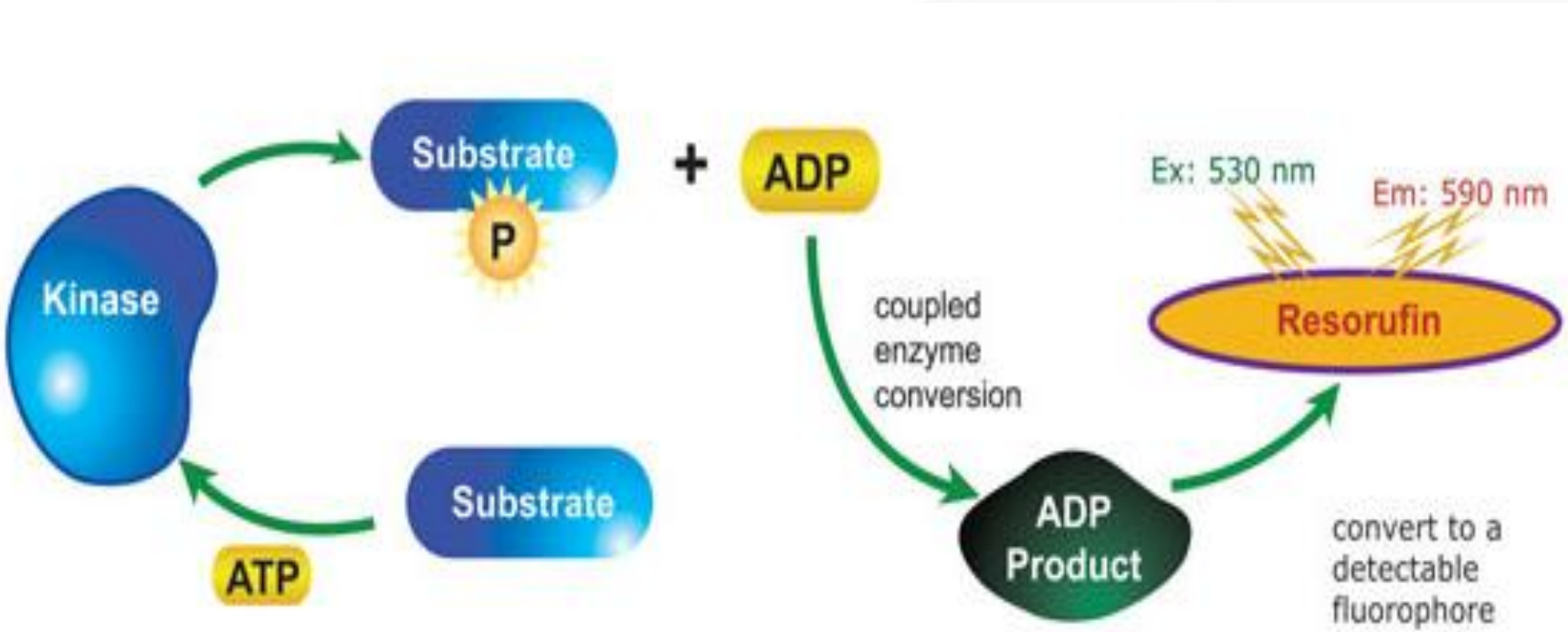


Phosphorylation is the most common post-translational modification of proteins

Regulates: activity, location, degradation, conformation…….

Many implicated in disease - *especially cancer & inflammation…..*

ADP Quest™ DiscoveRx Technology



DiscoveRx

Simple Solutions for Complex Biology

Novel ATP Utilizing Kinases

- **Receptor Tyrosine Kinase-like Orphan Receptor 2 (Ror2)** - Collaboration with Dr. Kim Rathmell, UNC Lineberger Comprehensive Cancer Center

- **LKB1** -Collaboration with Dr. Ned Sharpless UNC Lineberger Comprehensive Cancer Center

- **ATPase** -Collaboration with Dr.Matthew Redinbo UNC Department of Chemistry

ROR2 Background

- Ror2 is part of a family of orphan Receptor Tyrosine Kinase that display extracellular Carbohydrate recognition domain that bind Wnt ligands

- The Rathmell lab identified Ror2 as an abnormally expressed Tyrosine kinase in Renal Cell Carcinoma cell lines and human tumors

- Ror2 shows intrinsic tyrosine kinase activity and has since been identified in other cancers Expression associated with invasive phenotype

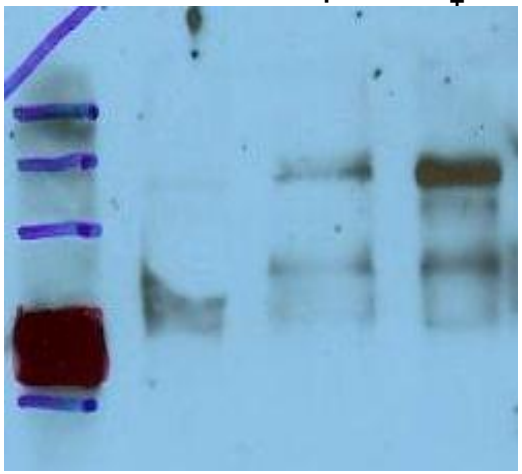
- Ror2 is a kinase primarily expressed in early development.

- Ror2 expression promotes cell migration and 3D growth *in vivo and in vitro.*

The Ror receptor tyrosine kinase family, W. C. Forrester, CMLS, Cell. Mol. Life Sci. **2002,** *59***,** 83.

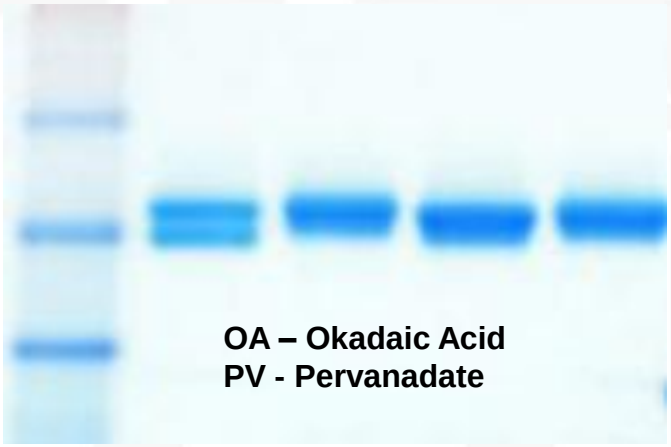
In-house Production of Active Ror2

Commercial Prep



pTyr Blot

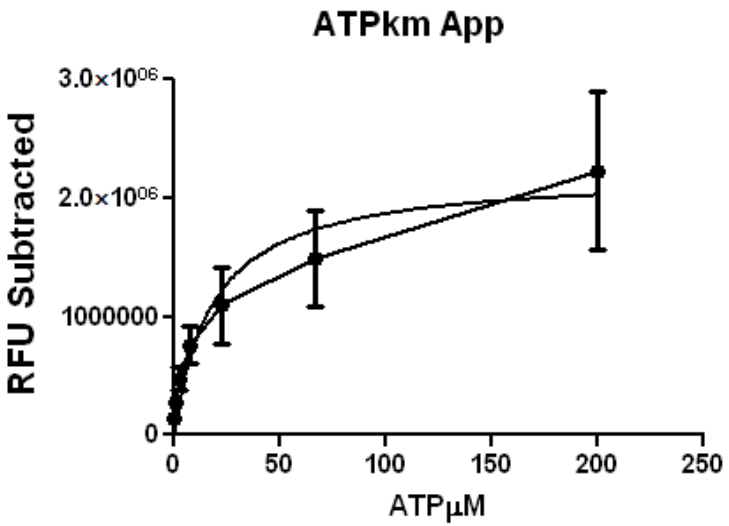
In-house Prep



	Lane													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ROR		+	+	+	+	+	+	+	+	+	+	+	+	+
MBP	+	+		+			+			+			+	
OA		+	+	+	+				+	+	+			
PV		+	+	+	+	+	+	+						
ATP		+		+	+		+	+		+	+		+	+

ROR2 Assay Development

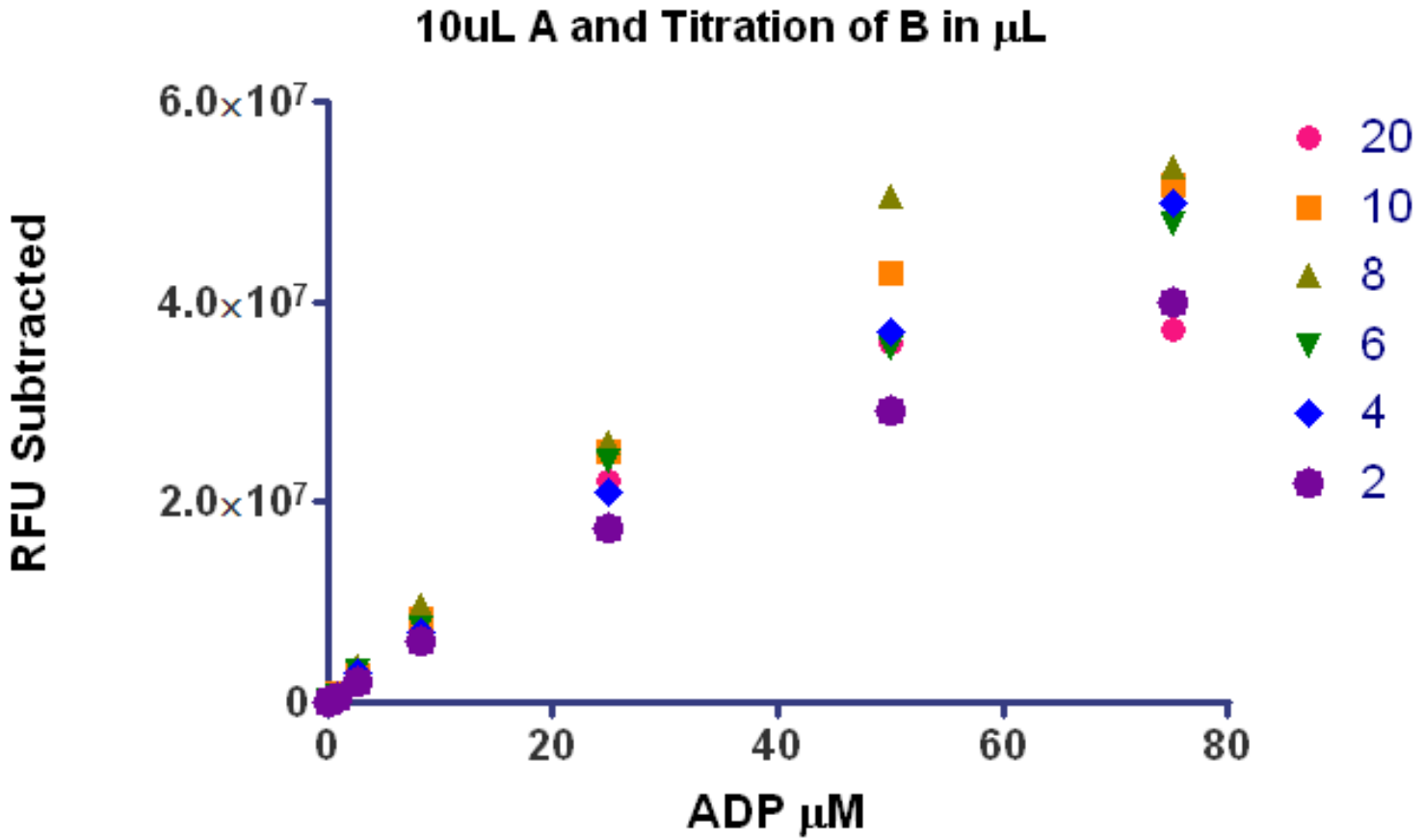
- Titrated ROR2 Kinase to determine activity range
- Titrated ATP to determine K_m Apparent
- Titrated MBP substrate
- DMSO titration to test Kinase tolerance
- Final reaction conditions in 10µL Kinase Rxn:
  - 1% DMSO
  - 200nM ROR2
  - 25µM ATP K_m Apparent
  - 5µM MBP



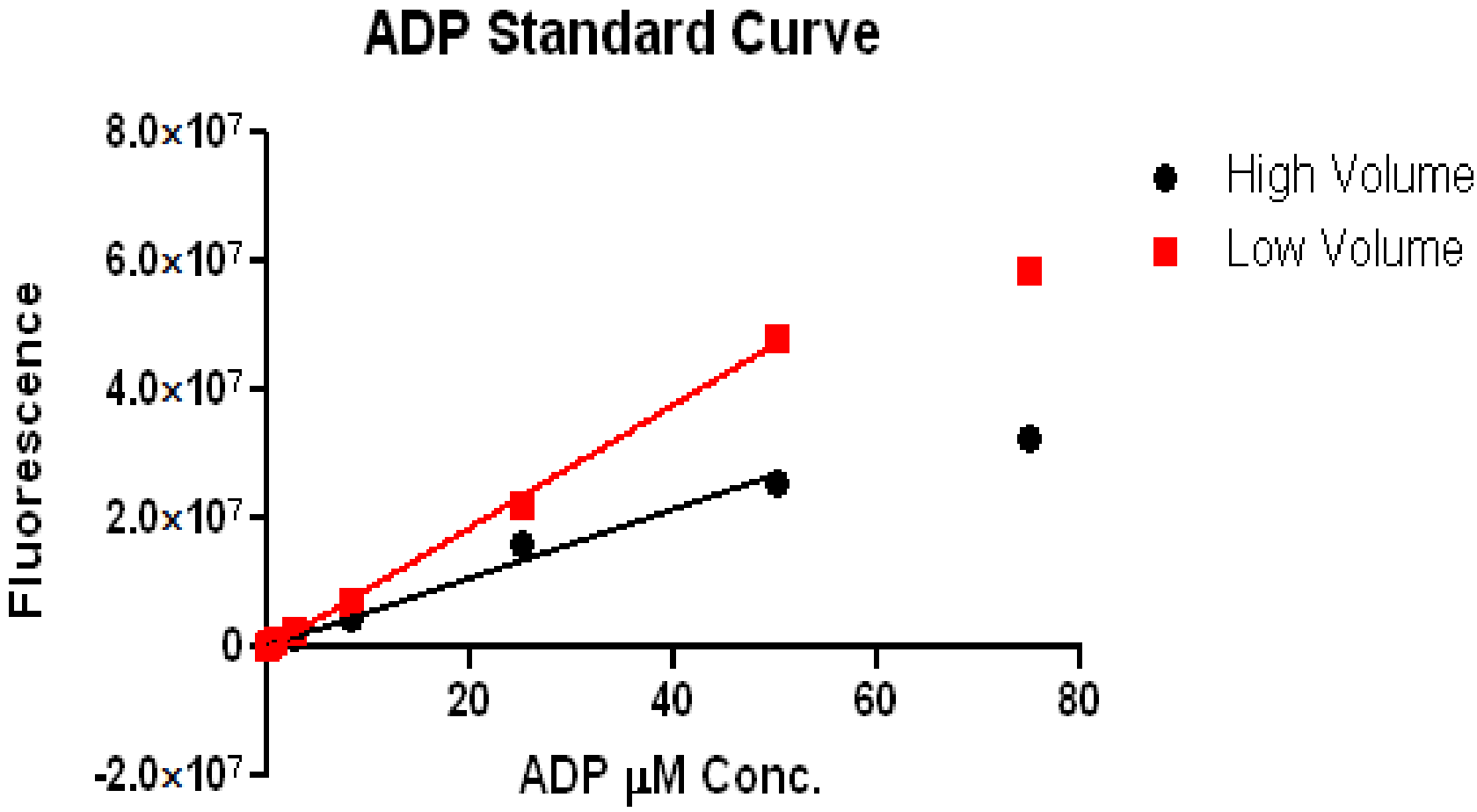
Michaelis-Menten	
Best-fit values	
Vmax	2.208e+006
Km	18.22

Assay development of ADP Quest™

- Recommend Titrating Kit reagents A and B (Coupled Enzyme reaction)

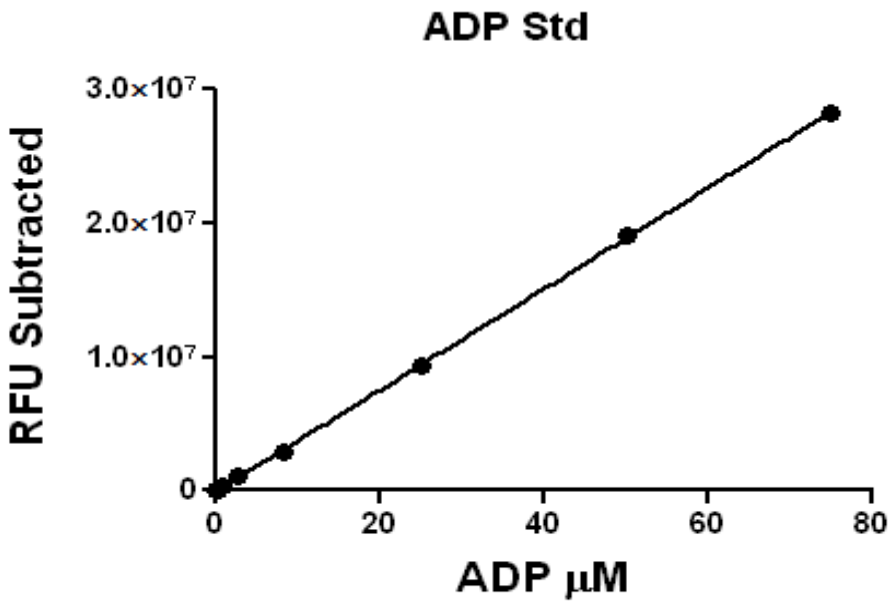


Assay Validation ADP Standard Curves



Assay development of ADP Quest™

- Final Detection Reagent Conditions:
  - 4µL Reagent A
  - 2.5µL Reagent B



Best-fit values	
Slope	378657 ± 1817
Y-intercept when X=0.0	-70107 ± 60343
X-intercept when Y=0.0	0.1851
1/slope	2.641e-006
95% Confidence Intervals	
Slope	374212 to 383102
Y-intercept when X=0.0	-217767 to 77553
X-intercept when Y=0.0	-0.2063 to 0.5710
Goodness of Fit	
R square	0.9999

Assay and High Throughput Screening (HTS) Validation

- Assay Validation:
  - An automated run of three consecutive days of full plates of Max and Min signals to test uniformity and separation of signals, using DMSO at the concentration to be used in screening.
  - "Max" signal: measures the highest signal. This would be the final kinase reaction condition in the presence of DMSO
  - "Min" signal: This measures the lowest signal. This would include all components in the assay and a known inhibitor that fully inhibits the kinase and done in the presence of DMSO
- HTS Validation:
  - A run of 2 plates over three consecutive days with use of automation and final plate layout for the screen.

Plate Layout for Assay and HTS Validation

Assay Validation

TOP	TOP	TOP	TOP	TOP	TOP	TOP
TOP	TOP	TOP	TOP	TOP	TOP	TOP
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Bottom	Bottom	Bottom	Bottom	Bottom	Bottom	Bottom

HTS Validation

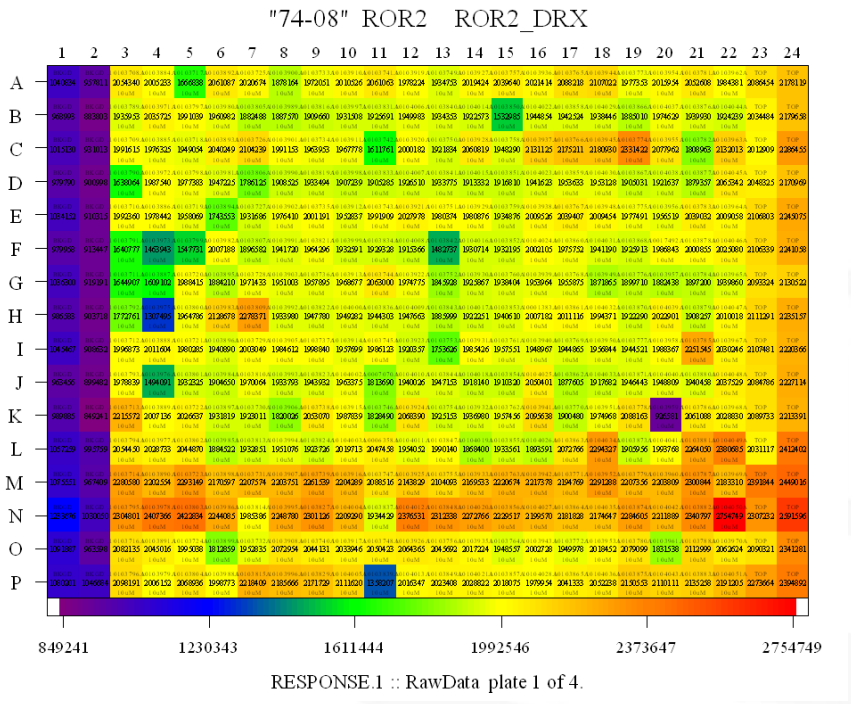
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	B...	B...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	A...	T...	T...
B	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	B...	T...	T...
C	B...	B...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	C...	T...	T...
D	B...	B...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	D...	T...	T...
E	B...	B...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	E...	T...	T...
F	B...	B...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	F...	T...	T...
G	B...	B...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	G...	T...	T...
H	B...	B...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	H...	T...	T...
I	B...	B...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	I...	T...	T...
J	B...	B...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	J...	T...	T...
K	B...	B...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	K...	T...	T...
L	B...	B...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	L...	T...	T...
M	B...	B...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	M...	T...	T...
N	B...	B...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	N...	T...	T...
O	B...	B...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	O...	T...	T...
P	B...	B...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	P...	T...	T...

ROR2 HTS Screen

- Trimmed Z' scores were >0.8

	Z_FACTOR	ASSAY	STATUS	TOP	BKGD
1.1	0.823	ROR2_DRX	PASS	2,358,3...	1,010,734...
1.2	0.806	ROR2_DRX	PASS	2,284,3...	991,907.0...
1.3	0.780	ROR2_DRX	PASS	2,228,4...	989,145.7...
1.4	0.816	ROR2_DRX	PASS	2,342,9...	1,162,688...

PASS (SB00000039



ROR2 HTS Screen

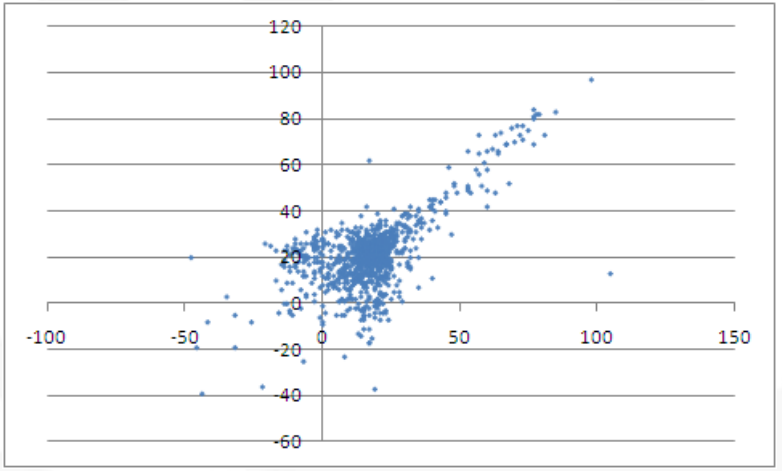
Focused Screening
DiscoveRx: MBP substrate
Single Point 10uM compound conc
Kinase Focused Library

Hit Confirmation
Retest/Test from DMSO Stock
IC 50 Curves

Secondary Assays
Retest/Test from DMSO stock
Caliper: peptide substrate
DiscoveRx: Selectivity assay
Confirm structure w/ LC/MS

Primary Assay	
Actives	74 (1.6%)
Tested/Confirmed in Potency	68/68 (100%)
Selective over DRx Counter Screen	8

Reproducibility: all duplicate measurements

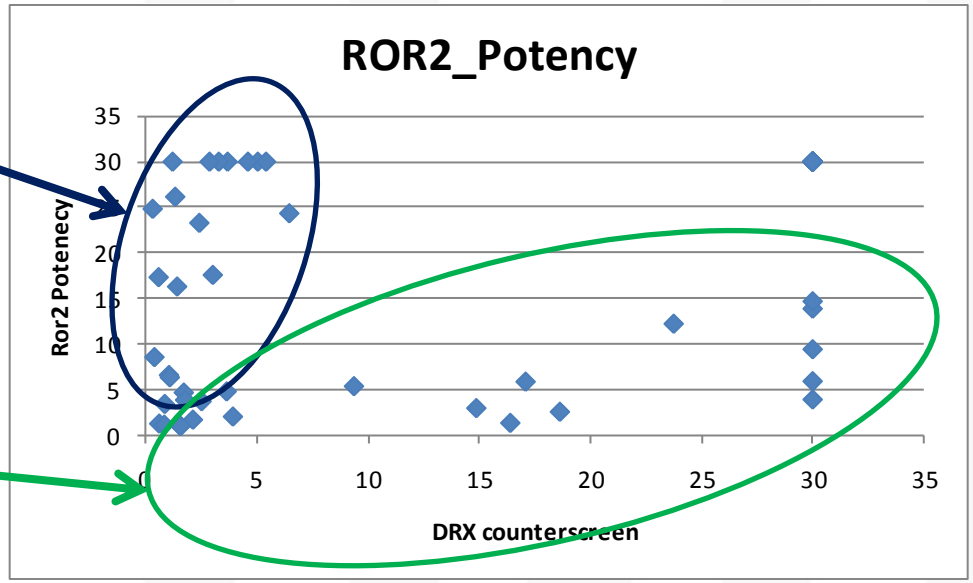


ADP Quest™ Counterscreen for False Positives

- Because the ADP Quest™ is a linked enzymatic assay we were able to run the assay in the presence of a fixed ADP concentration and compound to determine compound/assay interference.

Non-Selective

Ror2 Specific

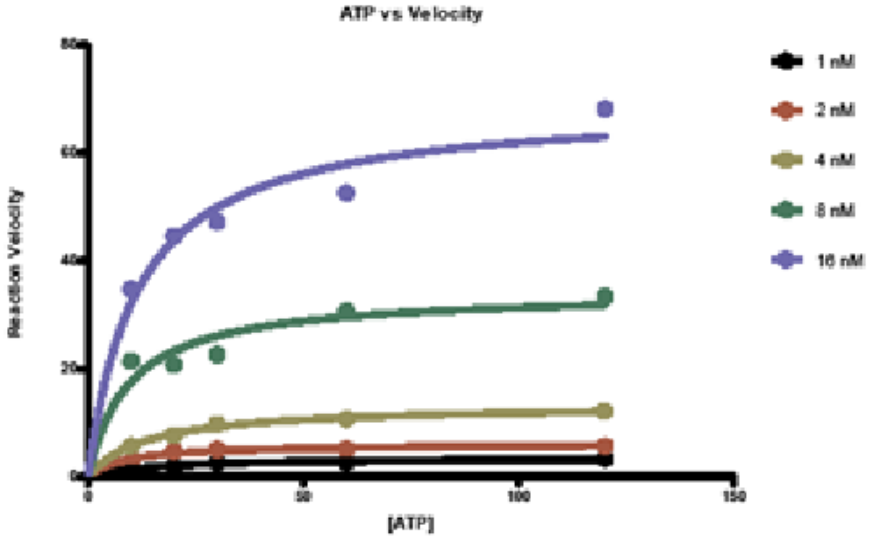


ATPase Background

- Catalyze ATP into ADP and a free phosphate ion
- Enzyme Class ATPase:
  - Kinase
  - Helicase
  - Ligase
- Screening **this** specific ATPase to find a chemical probe that will allow for further information into how these enzymes behave

ATPase Assay Development

- Titration of ATPase to determine activity range
- Titration of ATP to determine K_m Apparent
- Titration of DNA substrate
- DMSO titration to test tolerance
- Final reaction conditions in 22.5µL Kinase Rxn:
  - 1.0% DMSO
  - 4nM ATPase
  - 11.5µM ATP K_m Apparent
  - 25nM DNA



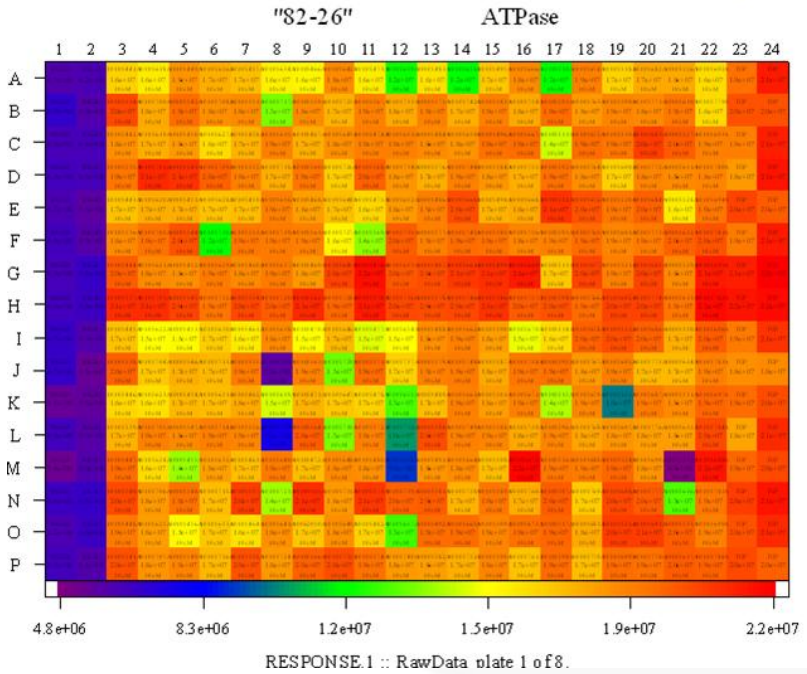
4nM ATPase
ATP K_m App 11.5uM

•ATPase HTS Screen

- Trimmed Z' scores were >0.8

	Z_FACTOR	ASSAY	STATUS	TOP	BKGD
1.1	0.831	ATPa...	PASS	20,193,...	6,287,301...
1.2	0.890	ATPa...	PASS	19,544,...	6,333,898...
1.3	0.830	ATPa...	PASS	20,476,...	6,183,702...
1.4	0.889	ATPa...	PASS	19,628,...	6,548,015...
1.5	0.871	ATPa...	PASS	19,436,...	6,344,723...
1.6	0.873	ATPa...	PASS	20,319,...	6,119,344...
1.7	0.890	ATPa...	PASS	20,332,...	6,056,757...
1.8	0.893	ATPa...	PASS	21,269,...	6,264,301...

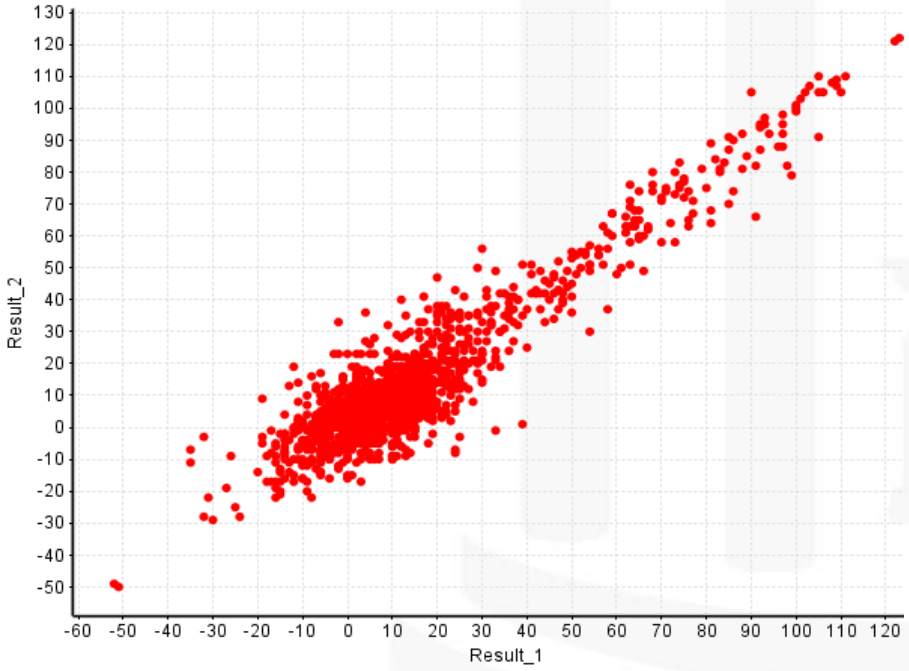
PASS (SB0000003607)



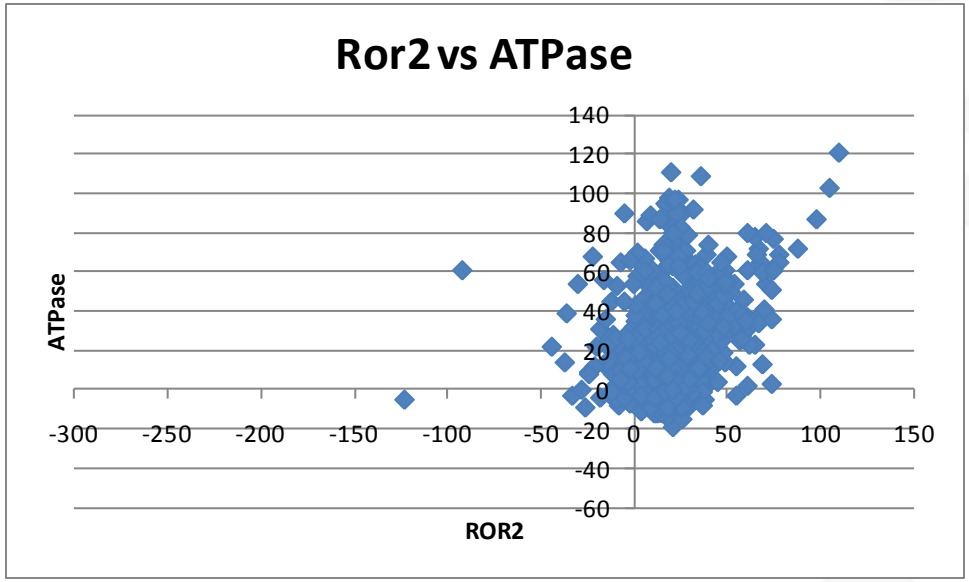
ATPase HTS Screen

$N_{unfiltered\ hits}$ = 249 (@ inh.>50%) 5% hit
$N_{filtered\ .counterscreen}$ = 163 (@ Counter Screen<40%) 3.3%
$N_{filtered\ .ROR2}$ = 148 3.0%

Reproducibility: all duplicate measurements



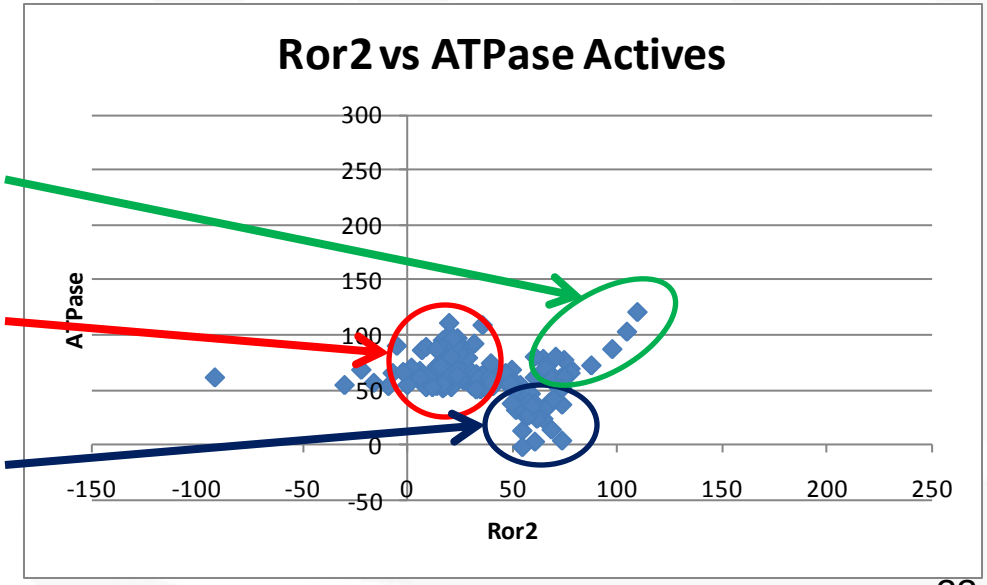
Hits from Ror2 and ATPase Screen



Non selective

ATPase specific

Ror2 specific



LKB1 Background

- A tumor suppressor that inhibits proliferation
- Has effects on cell polarity and on the ability of a cell to detect and respond to low cellular energy levels.
- Screening small molecules that inhibit LKB1 could be used to further explore it's signaling pathways
- Screening of small molecules that are activators for LKB1 could potentially be drug candidates to treat cancer

LKB1-Dependent Signaling Pathways, D. Alessi *et al*., *Annu. Rev. Biochem*. **2006**, *75*, 137.

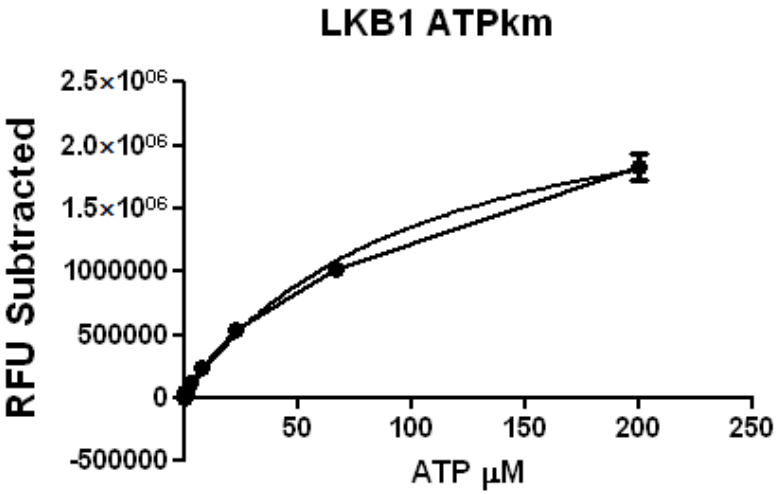
LKB1 Active Protein

- Purchased from Carna Biosciences LKB1／MO25α／STRADα

- Protein–protein interactions between LKB1 and its regulatory subunits STRADα and MO25α seem to be necessary for its kinase activity

LKB1 Assay Development

- Titrated LKB1 Kinase to determine activity range
- Titrated ATP to determine K_m Apparent
- Titrated LKBtide substrate
- DMSO titration to test Kinase tolerance
- Final reaction conditions in 8µL Kinase Rxn:
  - 1% DMSO
  - 30nM LKB1
  - 100µM ATP K_m Apparent
  - 50µM LKBtide



Michaelis-Menten	
Best-fit values	
Vmax	2.696e+006
Km	98.81

Summary

Successes

- ADP Quest™ was used successfully with trimmed Z' scores >0.8 in HTS screening
- False Positive Counter Screeen allowed us to rule out of compounds that interfered with the coupled kinase reaction of the kit reagents
- Screening with ADP Quest™ against two very different and novel ATPase was effective in both the detection of hits and determining selectivity between them

Learnings

- Kit reagents may need to be titrated to achieve linearity of the Standard Curve
- Counterscreen is needed to test for compound interference

Acknowledgements

Assay Development & Compound Profiling

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Tim Wigle, Post Doc (Now at Epizyme)

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Xiaodong Wang, Assistant Director
Jian Jin, Associate Director

CICBDD Administration

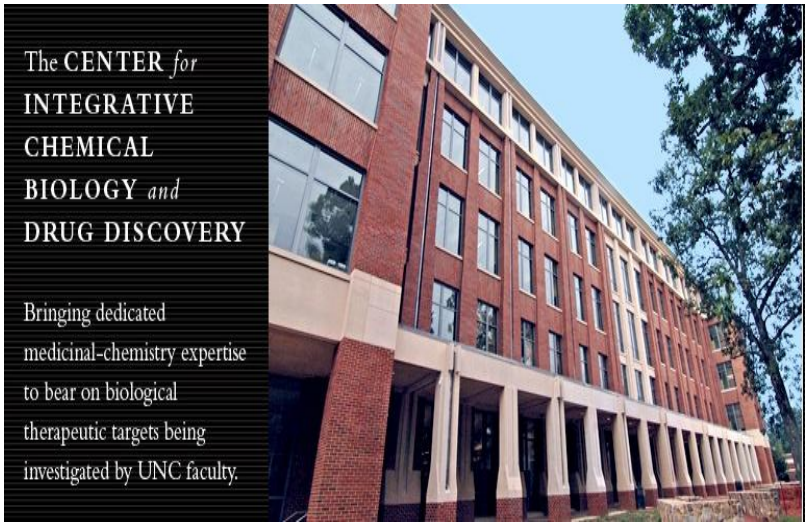
Stephen V. Frye, Director
Barbara Dearry, Business Services

Computational Drug Discovery

Dmitri Kireev, Director

Collaborators

Dr. Kim Rathmall, Lineberger Comprehensive Cancer Center
Neal Rasmussen, , Lineberger Comprehensive Cancer Center
Dr. Matthew Redinbo, Professor & Chair/Dir Structural Biology
Rebekah Nash, Graduate Student MD/Ph D (Just finished her defense)



http://www.pharmacy.unc.edu/labs/center-for-integrative-chemical-biology-and-drug-discovery

Wnt Signaling Pathway