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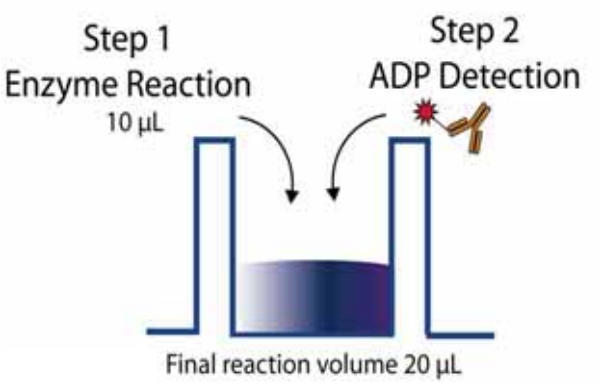
Biology at Work

Analyzing Kinase Inhibitor Residence Times using the Transcreener ADP Assay

Meera Kumar
BellBrook Labs

TRANSCREENER® HTS Assays



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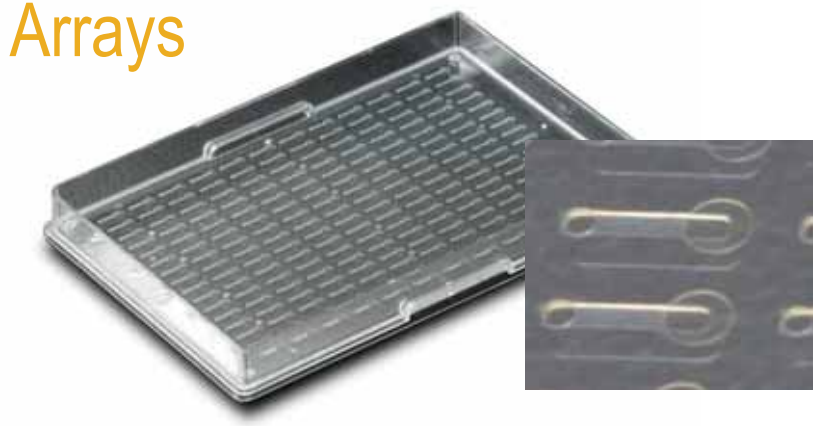
IUVO™ Microconduit Arrays



TRANSCREENER® Assays

Four assays, thousands of targets

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Our Transcreener® assays enable detection of thousands of different nucleotide dependent enzymes.

Transcreener Assay →	Target Families
AMP/GMP	Phosphodiesterases, Sialytransferases
UDP	Glycosyltransferases, Ligases (DNA, Amino Acid, Protein)
GDP	GTPases (Small G Proteins), GAPs, Fucosyltransferases
ADP	Kinases (Protein, Lipid, CHO), ATPases, Carboxylases, Helicases
EPIGEN	Methyltransferases, Acetyltransferases

Stuck on your screen? Bypass HTS assay development roadblocks with BellBrook's Transcreener® Assay Development Services.

Webinar Presenters

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Agenda

- Overview of Transcreener technology.
- Overview of Drug Residence Times.
- Jump dilution Method using Transcreener assays
- Data Analysis
- Conclusions

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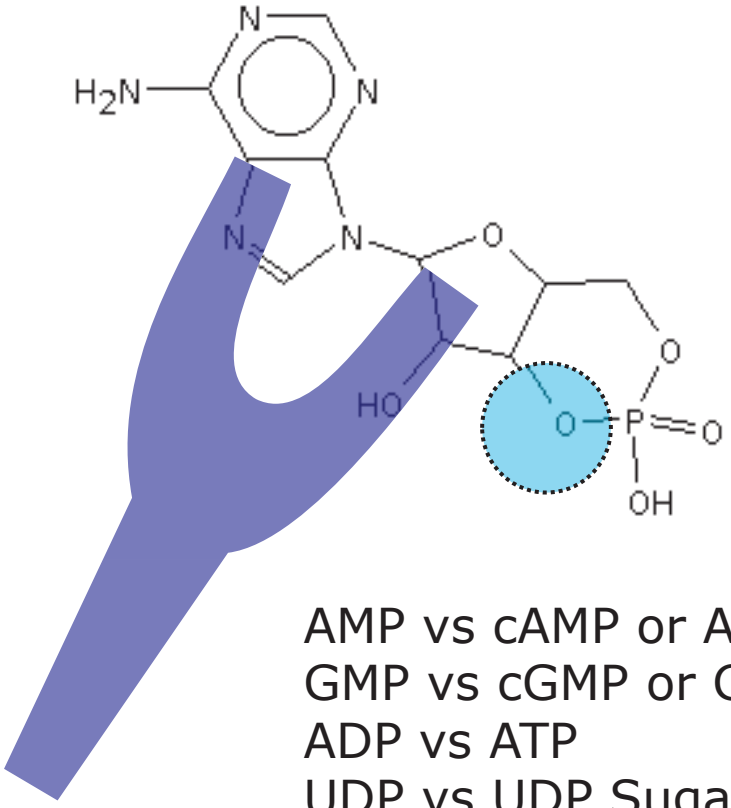
Transcreener Technology

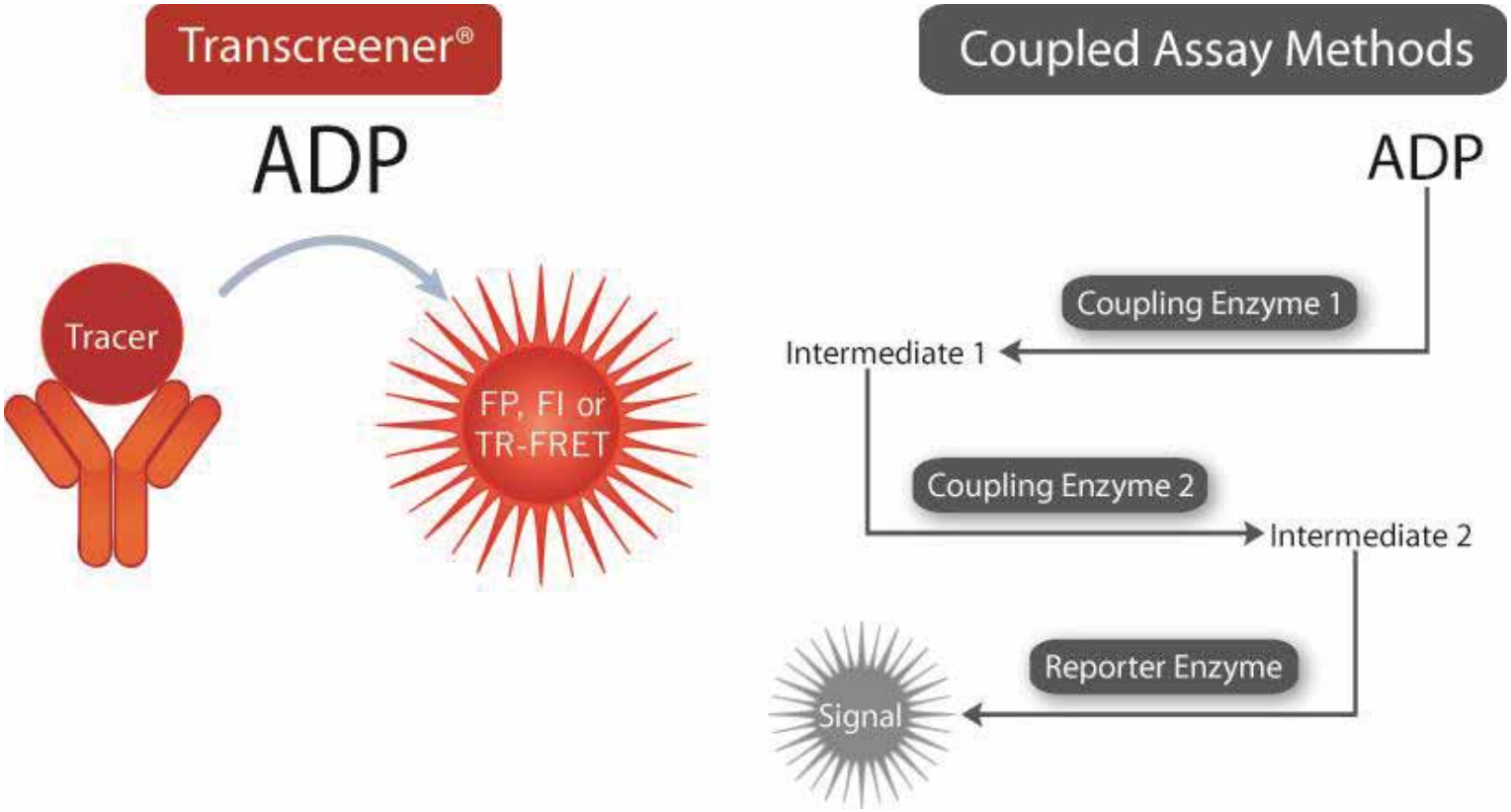
Selective Nucleotide Detection

The Transcreener® assays rely on antibodies that are able to differentiate between nucleotides on the basis of subtle structural differences. This makes detection of the product nucleotide possible even in the presence of excess substrate nucleotide.



AMP vs cAMP or ATP
GMP vs cGMP or GTP
ADP vs ATP
UDP vs UDP Sugar
GDP vs GTP

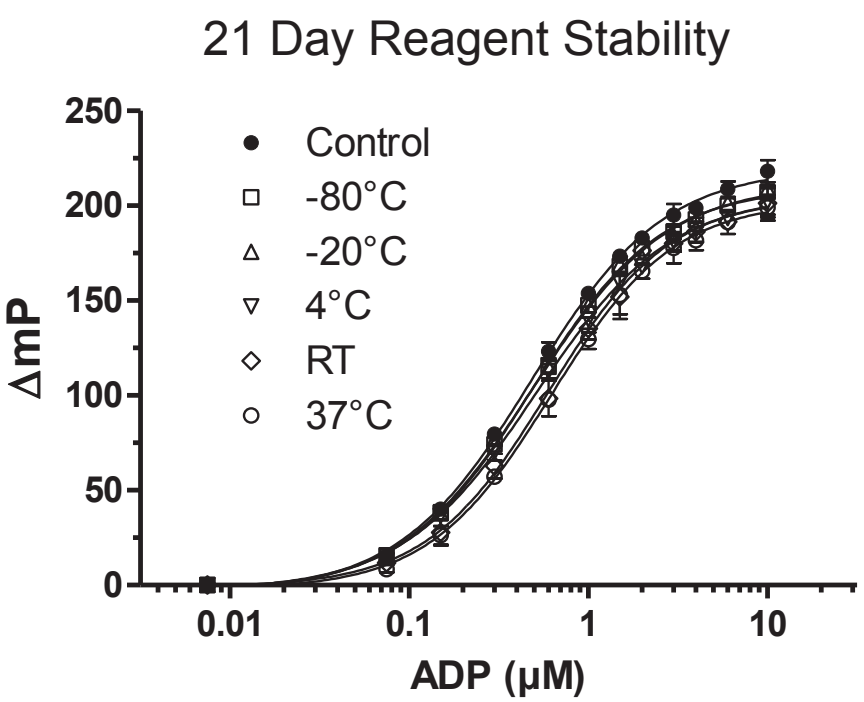
Direct Detection of Nucleotides decreases potential for interference

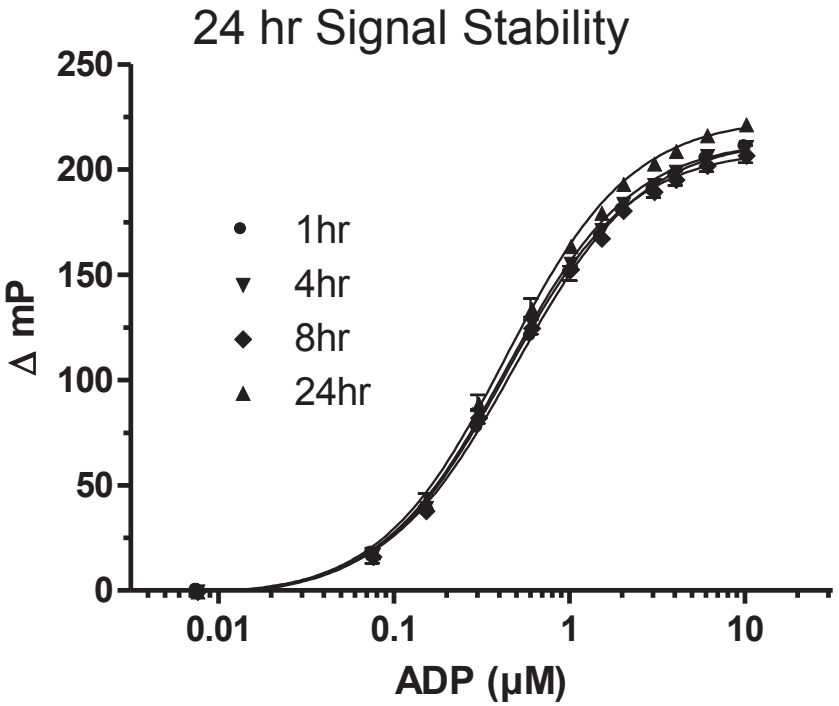


The Transcreener® assay relies on **direct detection** of ADP. Binding of tracer to antibody causes a change in fluorescence. There are just two components, and no intermediate steps.

All other ADP assays use **indirect detection** and are more complex. In a series of enzymatic steps, UDP is converted to a detectable product. Each step is subject to inhibition by library compounds.

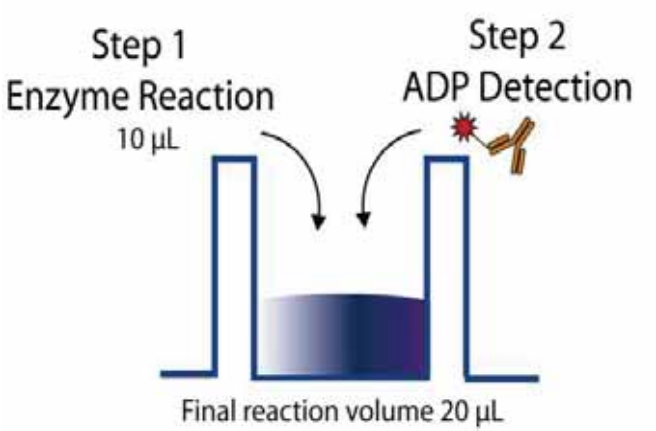
Transcreener ADP^2 FP Assay:
Overnight Reagent and Signal Stability



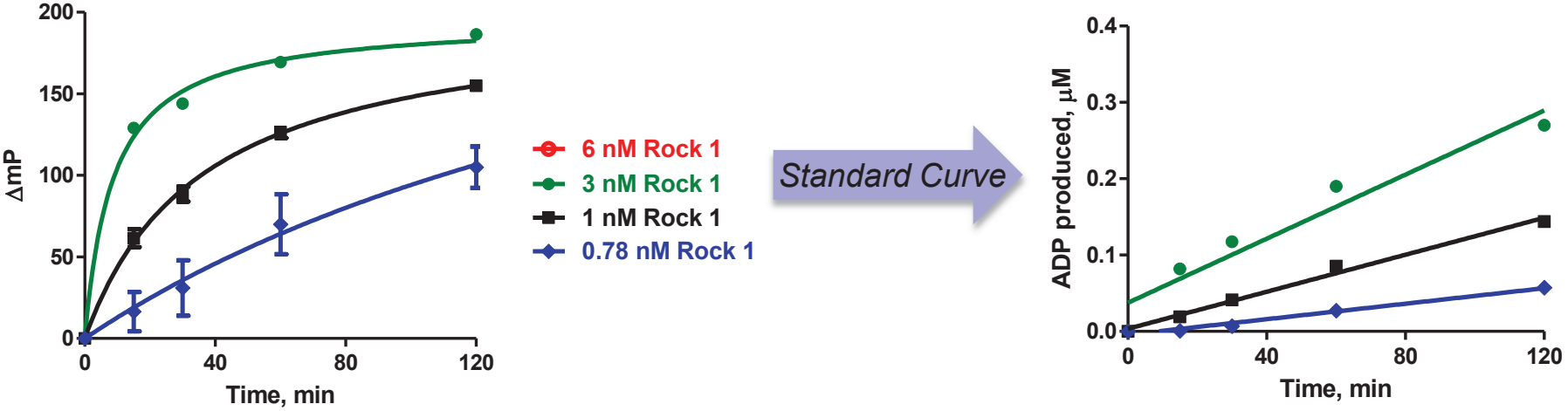


Standard curves for conversion of 10uM ATP to ADP demonstrate the outstanding stability of Transcreener detection reagents prior to addition to reaction and the stability of the signal following addition to kinase reactions. Data is for the FP assay, the FI and TR-FRET assays also have at least overnight reagent and signal stability. This provides outstanding flexibility for automated HTS platforms, especially with large numbers of plates, where there may be a lag between addition of detection reagent and plate-reading.

True mix and read detection format, endpoint or continuous detection.



Transcreener assays are true mix-and-read format, with the enzyme quenching and ADP detection components added as a single reagent for endpoint assays. The assay can also be used in a continuous detection mode, which makes optimization of enzyme reactions simpler and reduces reagent consumption.



Reaction is linear for 2 hrs with up to 1nM Rock 1; higher concentrations exceed initial velocity conditions.

Transcreener ADP^2 Assay:

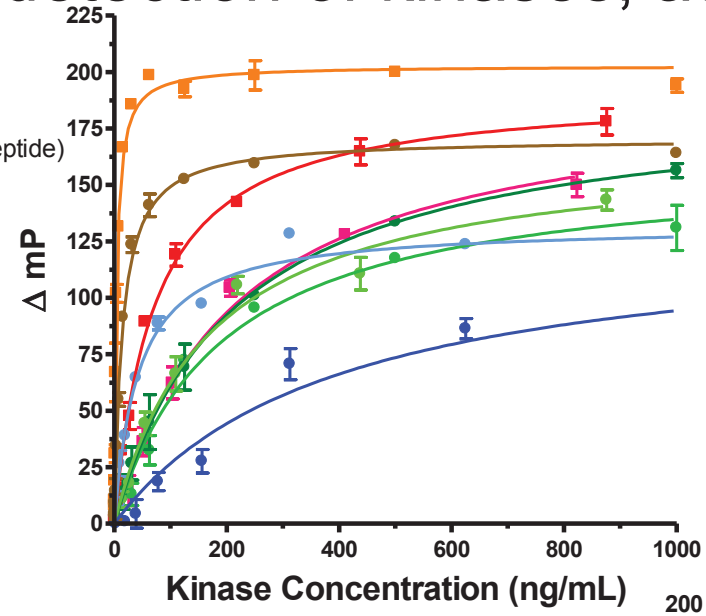
Universal detection of kinases, acceptors

Peptide Substrates

- Abl1 (Abltide)
- AKT/PKB (Akt/SKG peptide)
- PKA (kemptide)

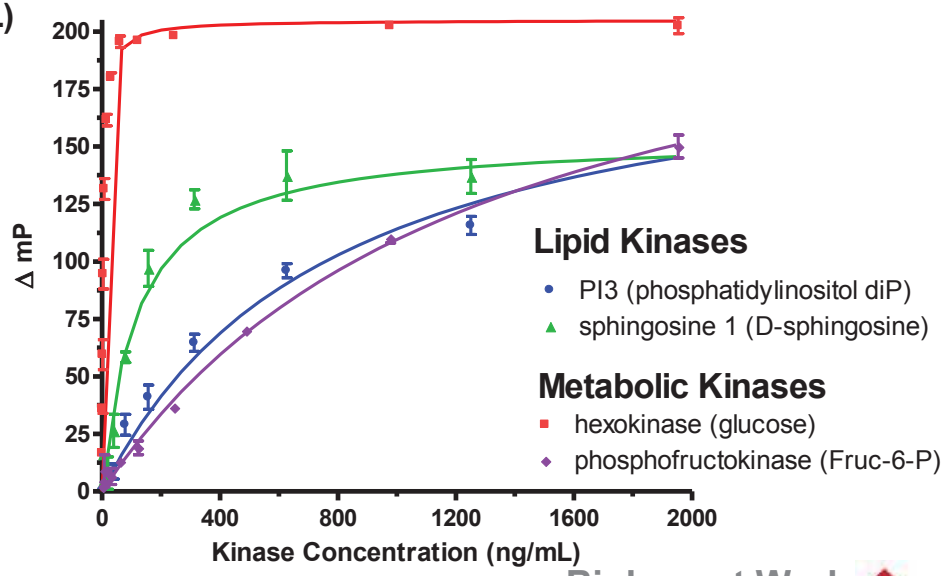
Protein Substrates

- cdk5/p35 (Histone 1)
- COT (MEK1)
- p38alpha (MBP)
- PKA (histone H1)
- PKA (MBP)
- RAF1 (MEK1)



Universal detection means you can use any kinase and any acceptor substrate, including native proteins , which provide a more physiologically relevant measure of kinase activity.

Universal detection means straightforward detection of lipid and carbohydrate kinases in addition to protein kinases.



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Dynamic Range can be Tuned for Robust Initial Velocity Detection at any ATP Concentration

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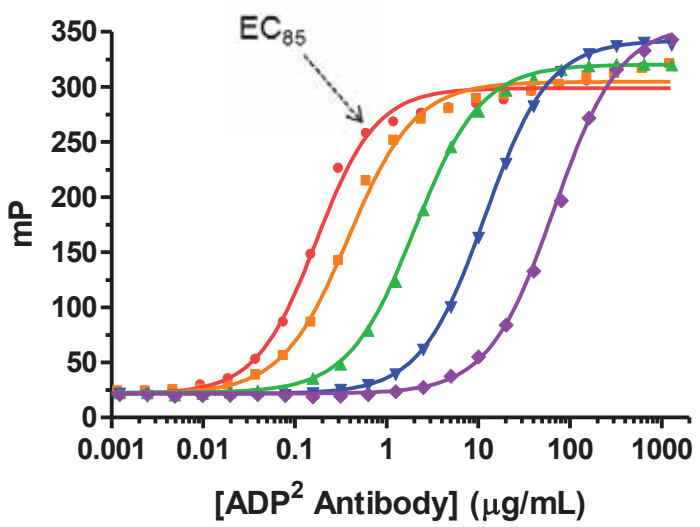
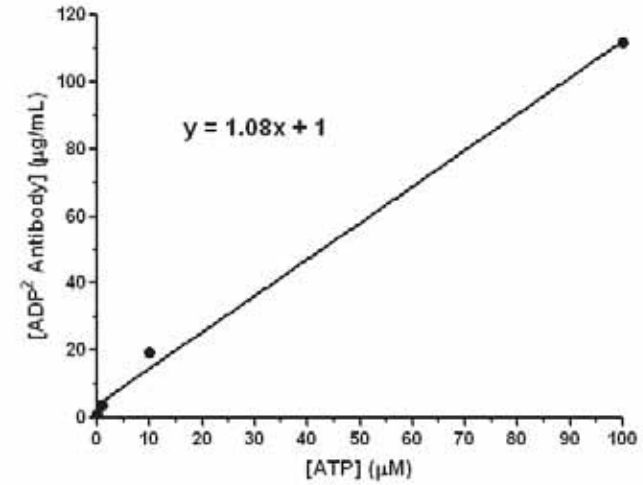
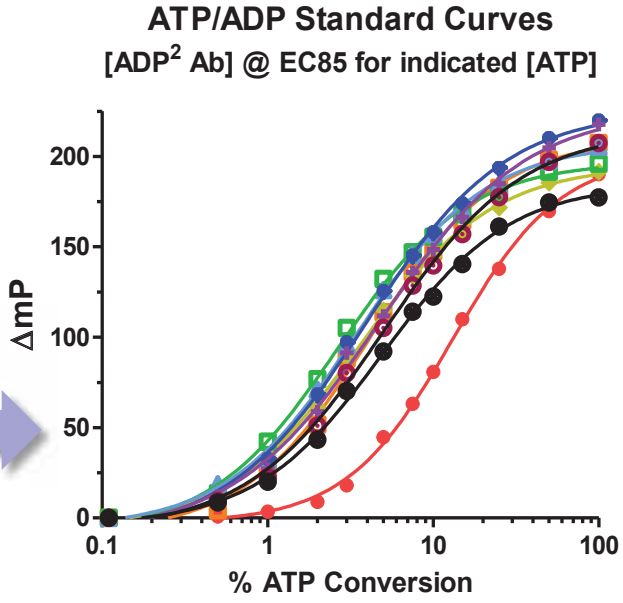
Ab titrations in the presence of ATP



- 0.1 µM ATP
- 1 µM ATP
- 10 µM ATP
- 100 µM ATP
- 1000 µM ATP

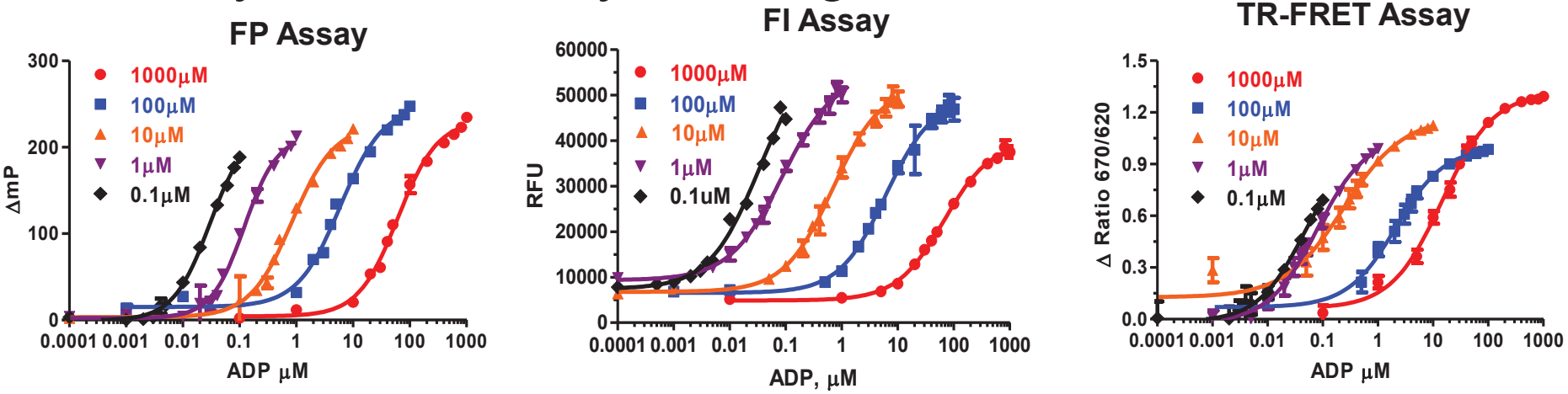
The ADP antibody concentration sets the dynamic range of the assay. EC_{85} is optimal for initial velocity detection; ie, a good signal @ 10% conversion of ATP.





- 0.1 µM ATP
- 0.33 µM ATP
- 1 µM ATP
- 3.3 µM ATP
- 10 µM ATP
- 33 µM ATP
- 100 µM ATP
- 333 µM ATP
- 1000 µM ATP

Transcreener ADP^2 Assay: Greater sensitivity reduces enzyme usage



	1 μM ATP/ADP standard curve		10 μM ATP/ADP standard curve		100 μM ATP/ADP standard curve	
	Z' at 10% Conv	LLD (μM)	Z' at 10% Conv	LLD(μM)	Z' at 10% Conv	LLD(μM)
Transcreener FP	0.86	0.02 ±0.07	0.85	0.01 ±0.12	0.89	1.0 ±0.3
Transcreener TR-FRET	0.71	0.10 ±0.06	0.72	0.10 ±0.09	0.72	1.0 ±0.3
Transcreener FI	0.92	0.03 ±0.01	0.88	0.05 ±0.04	0.92	0.5 ±0.4
Luc-ADP Detection Assay	ND	0.40 ±0.87	0.30	0.50 ±0.32	0.62	5.0 ±0.7
Luc-ATP Depletion Assay	ND	0.25 ±0.40	ND	1.50 ±0.30	0.52	7.0 ±0.6

Standard curves for conversion of ATP to ADP demonstrate robust detection at 10% conversion starting at ATP concentrations from 0.1 to 1,000 μM. By comparison, Luciferase-based ADP and ATP detection methods require greater conversion of ATP, especially at lower ATP concentrations; this translates into higher enzyme consumption.

Residence Time

What is Drug Residence Time?

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- **Drug–target residence time:** Lifetime of the binary complex between a drug molecule and a macromolecular target.
- Experimentally, the residence time is measured as the reciprocal of the rate constant for drug–target complex dissociation (1/koff).
- Drug potency is a function of drug occupancy on a target (residence time) as well as its affinity (IC_{50}).

EI formation

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The Static View of Drug-Target Interactions

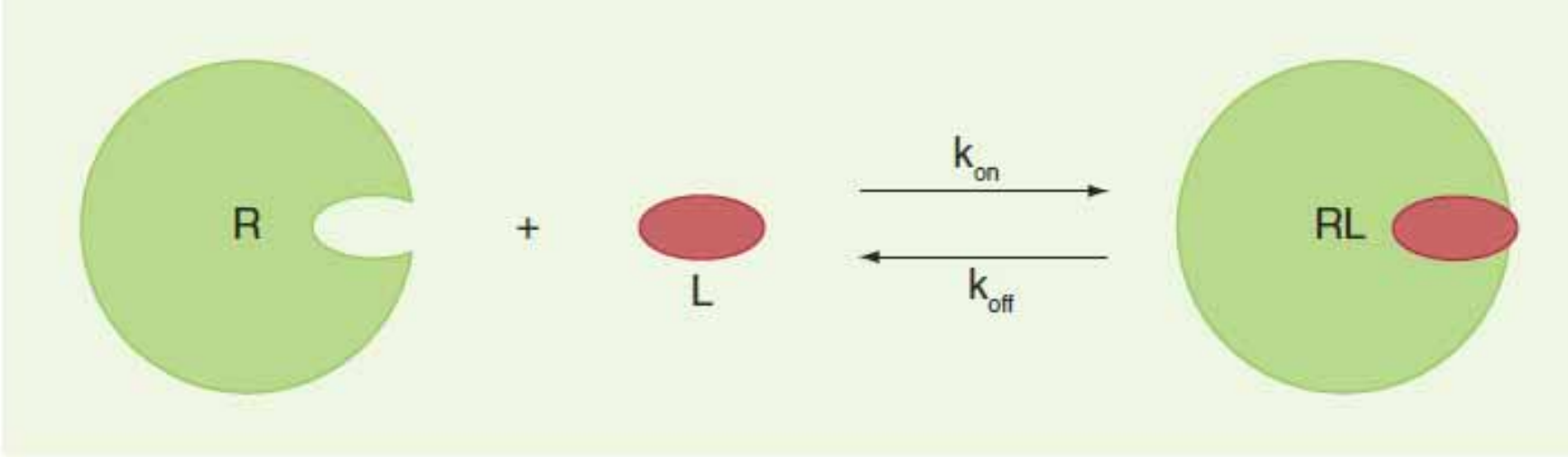
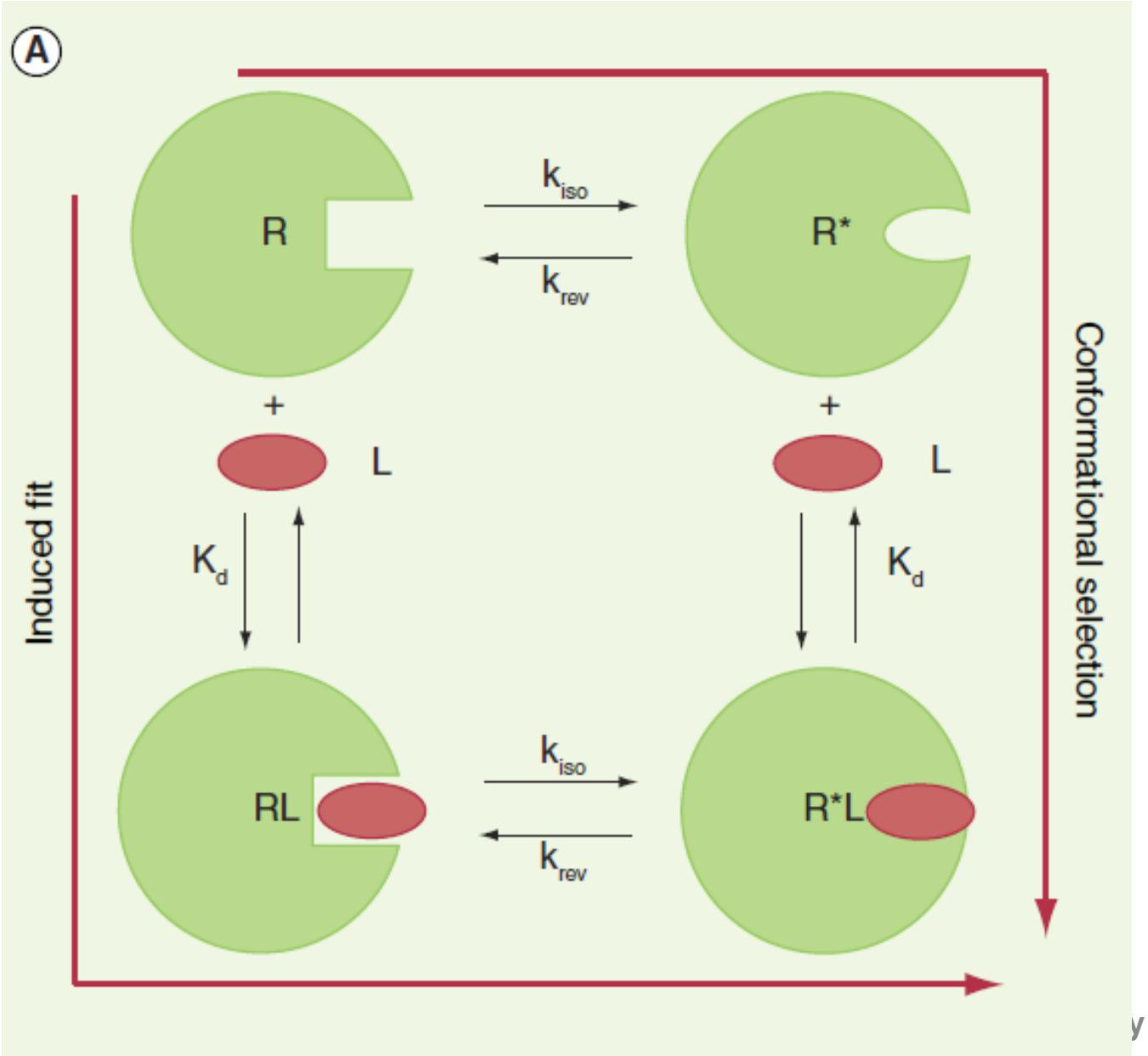


Figure 1. Static lock-and-key model of receptor–ligand binding in which k_{on} and k_{off} each occur in a single kinetic step.
k_{on}: Complex association rate constant; k_{off}: Complex dissociation rate constant.

EI Formation:Conformational Adaptation in Drug–Target Interactions



Methods available to measure Drug residence times

- Surface Plasmon Resonance
  - Requires that you immobilize proteins on a sensor surface.
  - Requires Expensive and specialized instruments, accessories and consumables to record measurements
  - Low Throughput
  - Only provides binding kinetics information

- Changes in intrinsic fluorescence

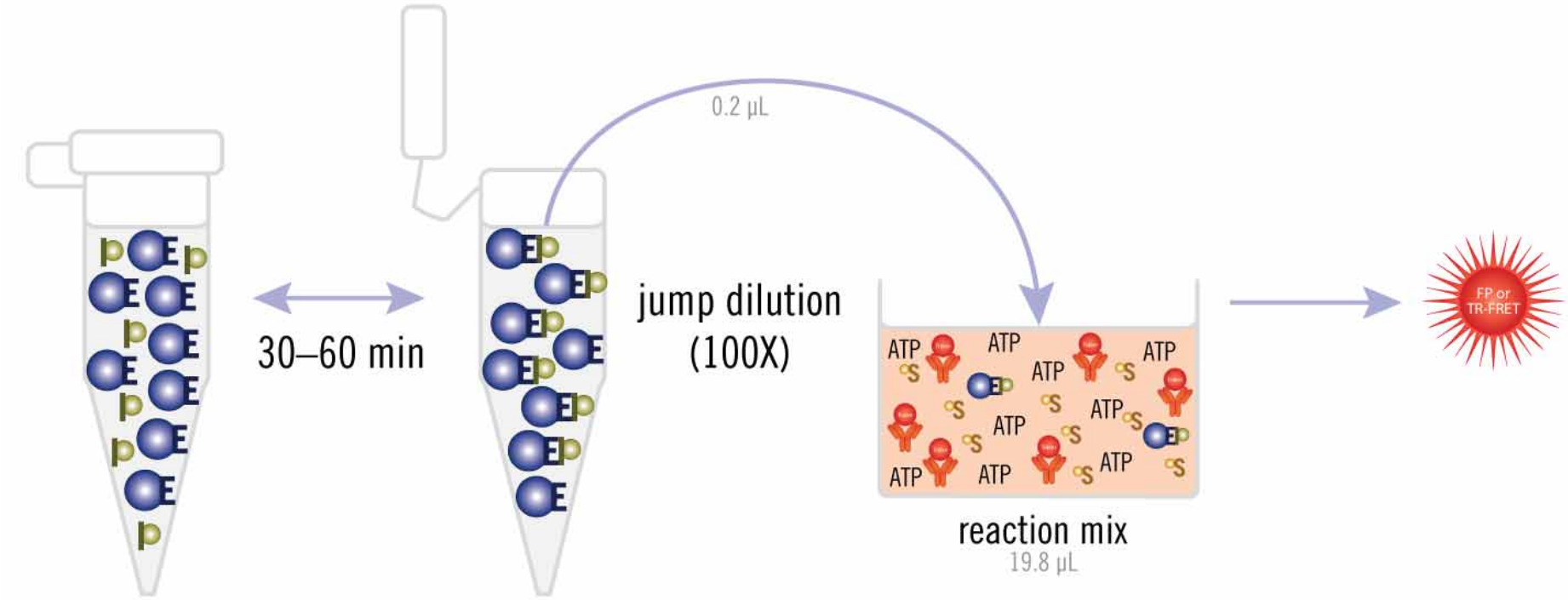
- Non Equilibrium reaction kinetics.

- Fluorescence Binding assays-Not activity based assays

Streamlined protocol for residence time determination

- Determine EC_{80} of target concentration.
- Determine IC_{50} values of the compounds.
- Preincubate 10* IC_{50} compound+100*[EC_{80}]
- Jump Dilution: Dilute 100X into enzyme reaction and Transcreener detection reagents.
- Continuously read over time and monitor the change in fluorescence.
- Data Analysis and calculate residence times.

Jump dilution protocol for determining residence times



Jump dilution

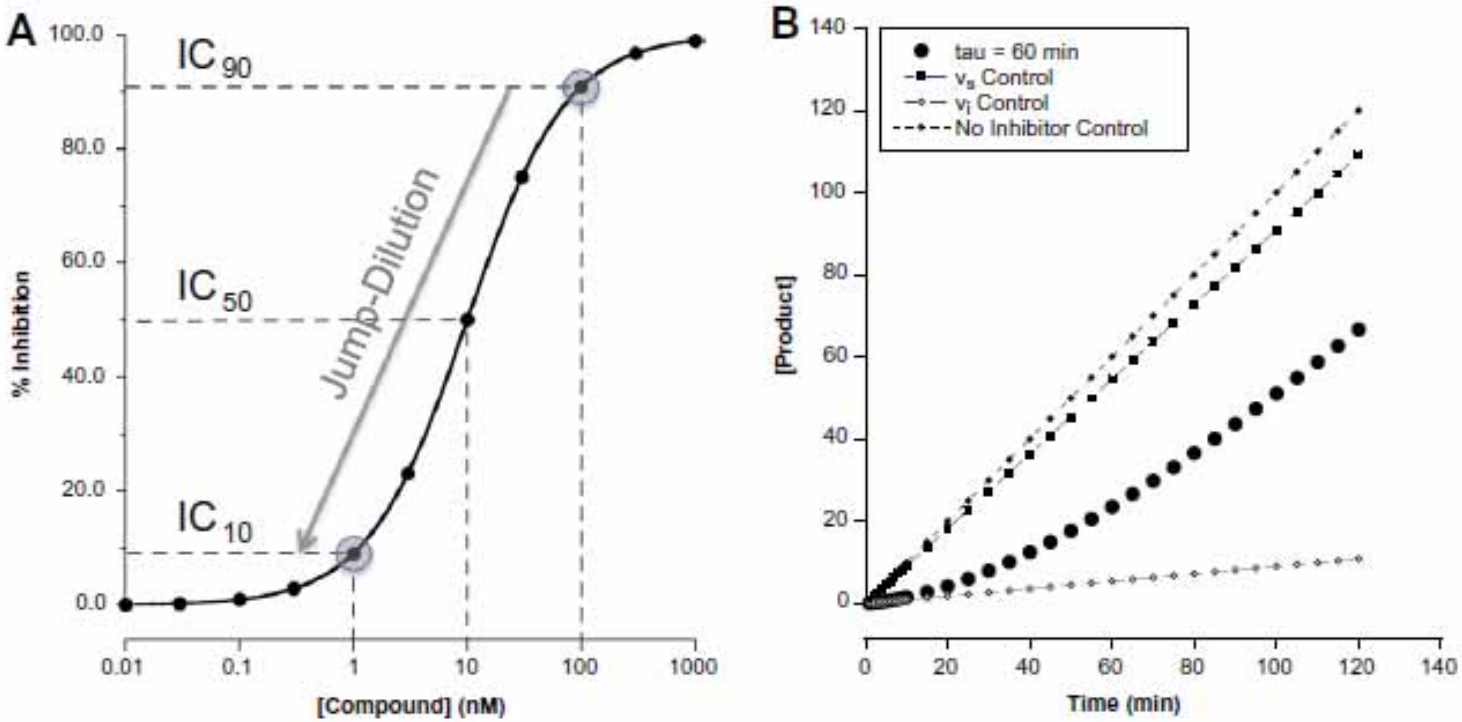
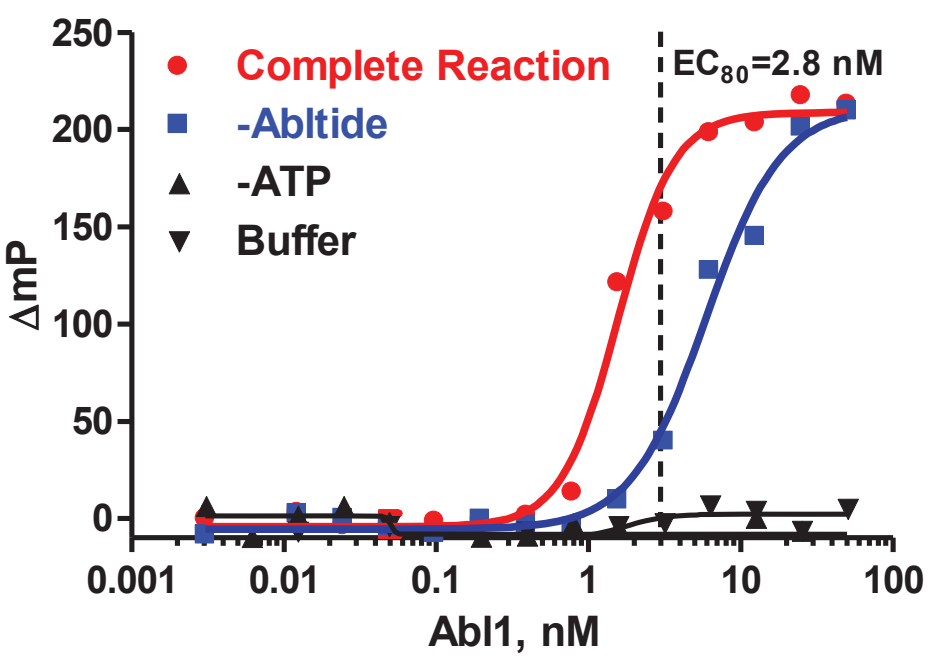


Fig. 1. (A) Typical concentration–response plot for target inhibition by a compound of interest illustrating the starting and ending concentrations of compound, relative to the IC_{50}, used in a jump dilution experiment. (B) Example of a jump dilution progress curve for control samples and for a test compound displaying a residence time of 60 min. Both panels of this figure are adapted and redrawn from Copeland [2].

Jump dilution analysis / R.A. Copeland et al. / Anal. Biochem. 416 (2011) 206–210

Case study 1: Abl1



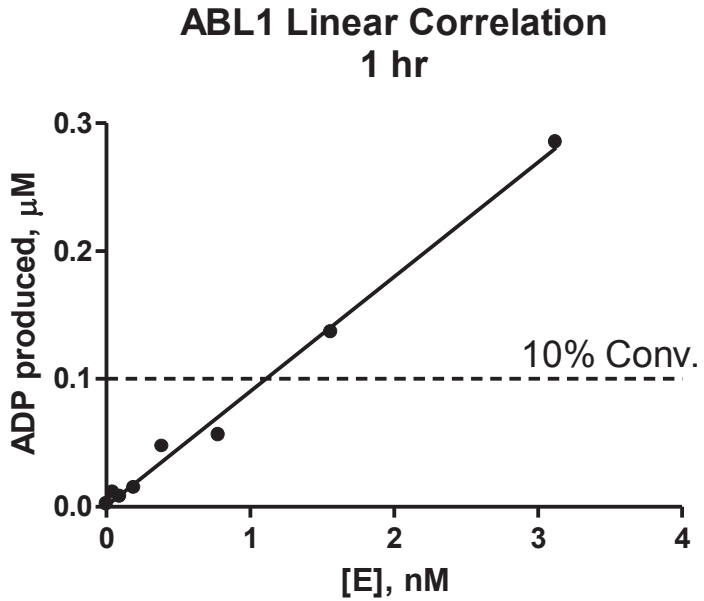
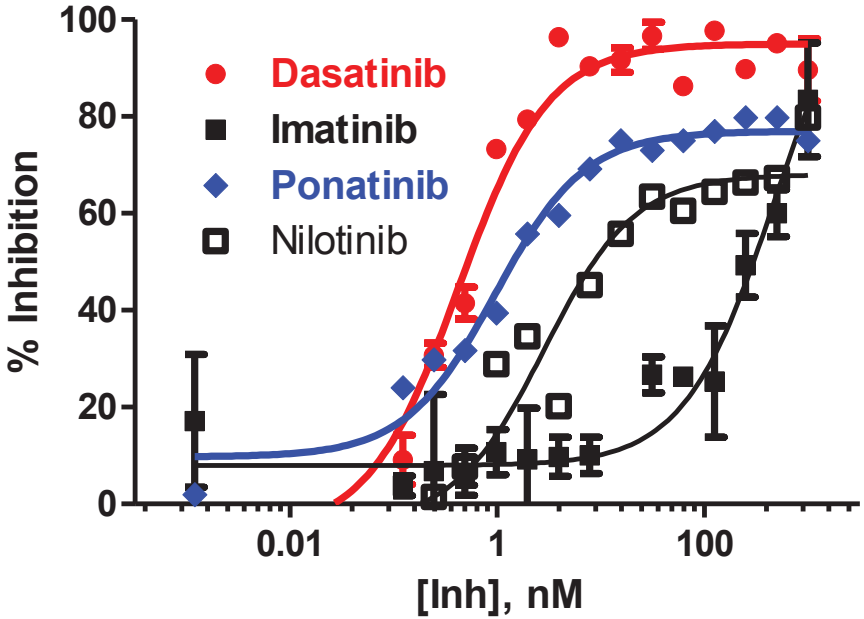


Figure 3. Determining the Optimal concentration of Abl1 and Drugs for the "Jump Dilution" Experiment. A. Titration of Abl1 enzyme, performed in the presence of 1 µM ATP and 10 µM Abltide, determined an optimal EC_{80} concentration as 2.8 nM. **B.** Linear relationship between Abl1 concentration and ADP formation. The polarization values were converted into ADP (product formed) using a standard curve set up under similar conditions.

Determining the optimal drug concentration for residence time experiments



	Dasatinib	Imatinib	Ponatinib	Nilotinib
IC_{50} (nM)	0.4209	474.9	0.9547	2.847

A dose response curve for Abl1 enzyme (2.8 nM) in the presence of 1 µM ATP and 10 µM Abltide enabled determination of IC_{50} values of 0.42 nM (dasatinib), 475 nM (imatinib), 2.8 nM (Nilotinib) and 0.95 nM (ponatinib).

Preincubation for [EI] Complex formation

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- Enzyme for [EI]
  - ➢ $100*EC_{80}$= 2.8 nM* 100 =280 nM

- Drug for [EI]
  - ➢ $10*IC_{50}$ (Dasatinib) = 45 nM
  - ➢ $10*IC_{50}$ (Imatinib) = 45 μM
  - ➢ $10*IC_{50}$ (Ponatinib) = 100 nM
  - ➢ $10*IC_{50}$ (Nilotinib) =25 μM

Preincubation Protocol. The amount of Abl1 used for preincubation is at least 50 × EC_{80}, so that it still gives a robust signal after dilution. The inhibitor concentration is 10 × IC_{50}, such that the enzyme is saturated with the inhibitor. The mix was incubated for 1 hour at room temperature to insure formation of the EI complex.

Jump dilution

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- The dilution of the EI complex is done such that the inhibitor concentration is at least 100-fold below its IC_{50} value.
- A 100-fold dilution (0.2 µL of Abl/Inhibitor mixture into 19.8 µL of Detection Mixture) was made in an LV-384 well plate.
- The Detection mixture comprised of 5 µM ATP and 10 µM Abltide in kinase buffer also contained 2 nM tracer and 3.2 µg/mL of ADP[2] antibody.
- The plate was mixed well and read kinetically every 5 minutes for 4 hours in a Tecan Safire plate reader using the fluorescent polarization mode with EXC at 630 nm and EMS at 670 nm.

Raw data showing mP values

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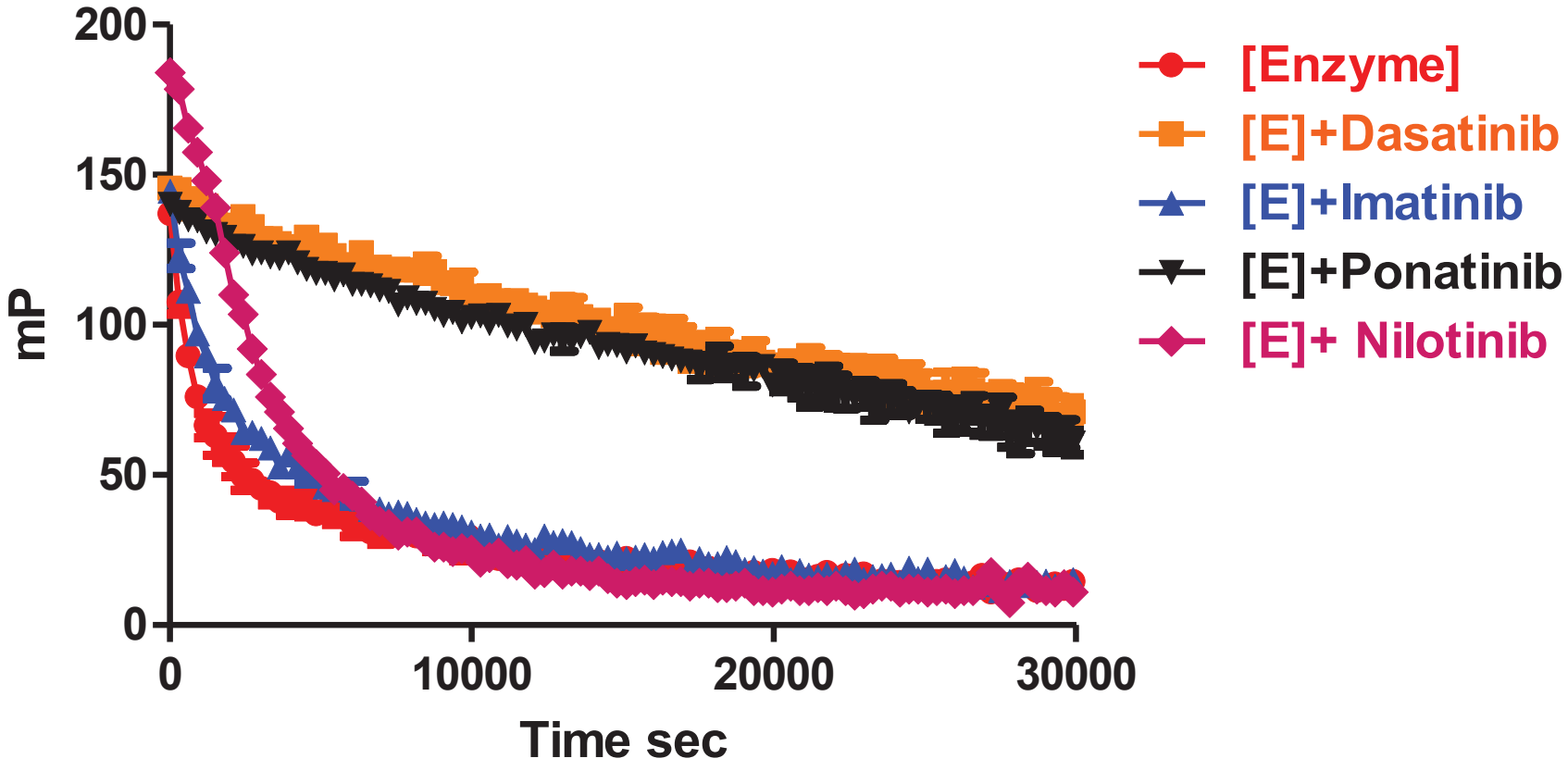
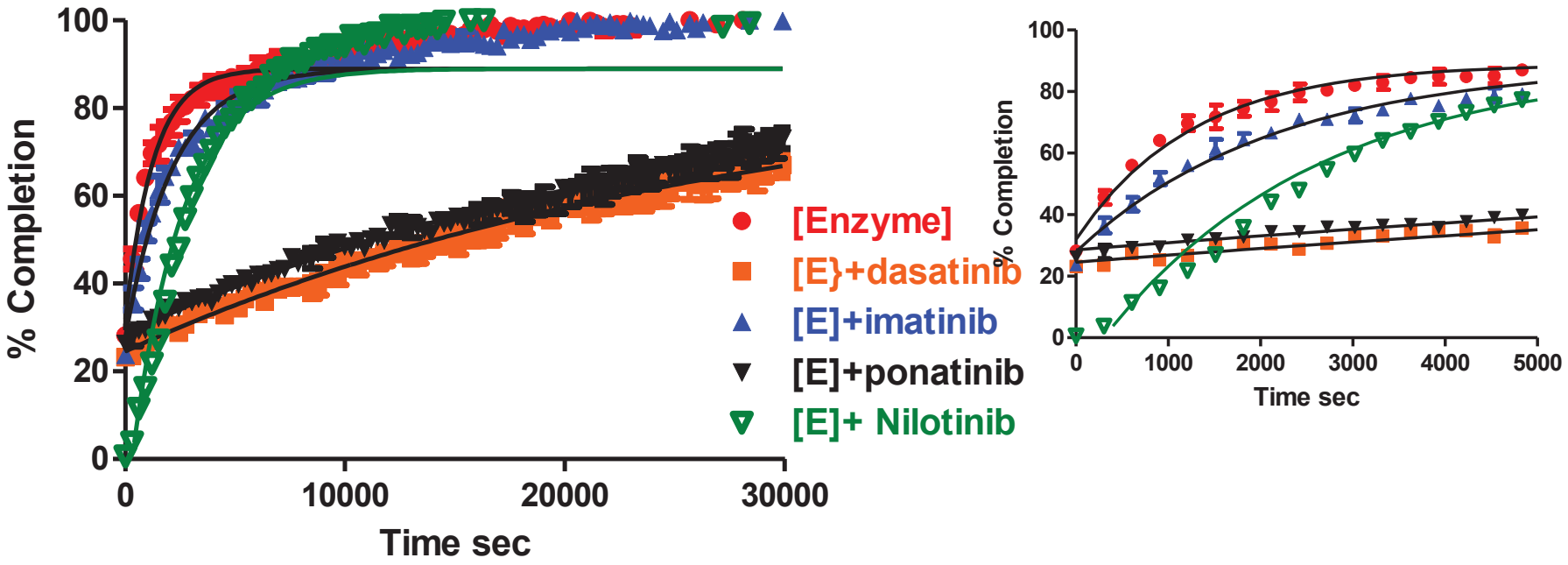
Raw data: mP decreases as ADP is produced.



Data analysis

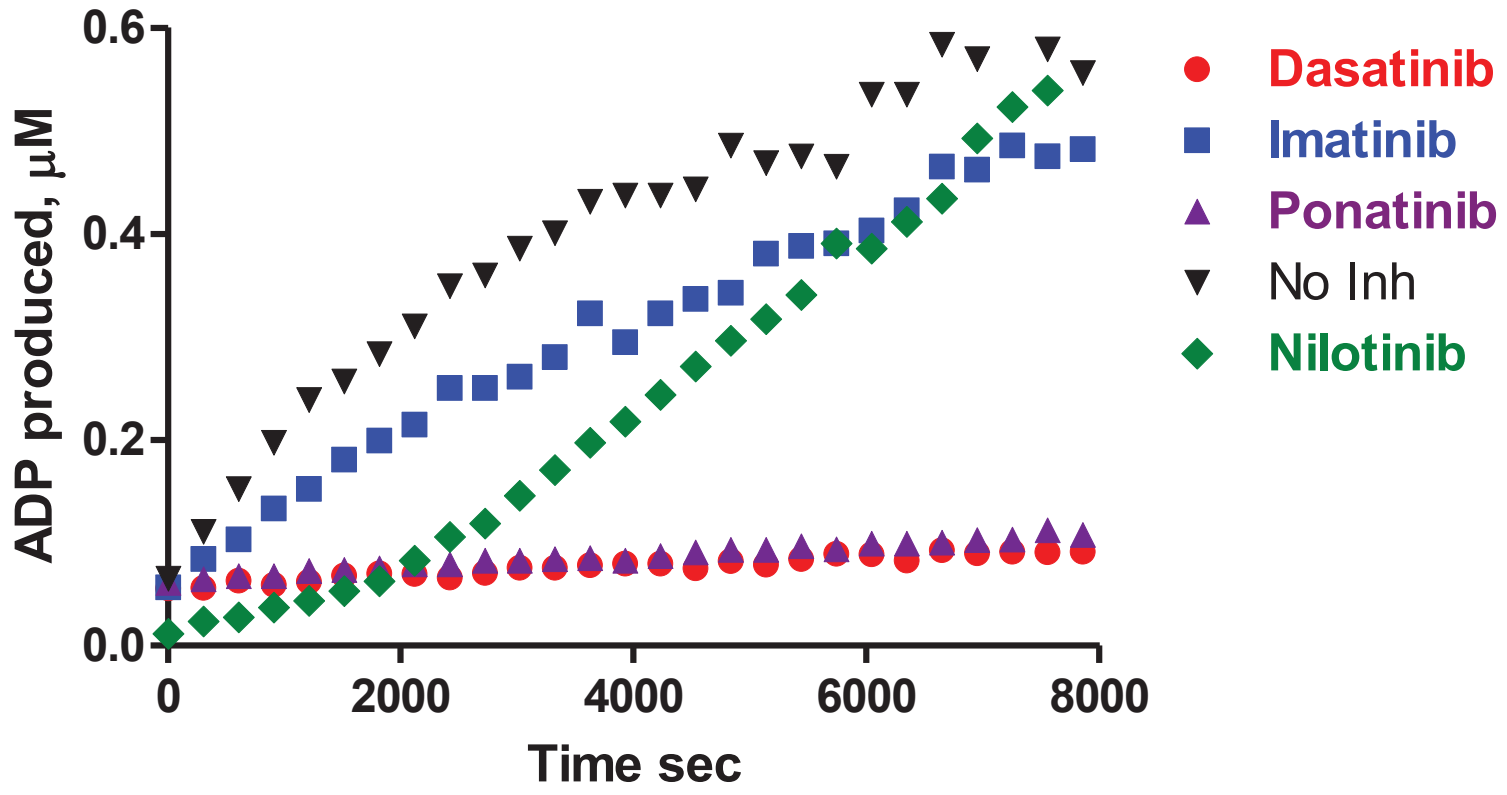


	[Enzyme]	[E}+dasatinib	[E]+imatinib	[E]+ponatinib	[E]+ nilotinib
K	0.0007879	3.561e-005	0.0004612	3.899e-005	0.0004319
Half Life	879.8	19467	1503	17776	1605
Tau	1269	28085	2168	25645	2315

Data analysis: One phase decay using raw data.
Normalized data based on the following controls-

a) Inh without enzyme =0% activity.
b) Enzyme after completion= 100% activity.

Converting mP values to ADP (product formed)

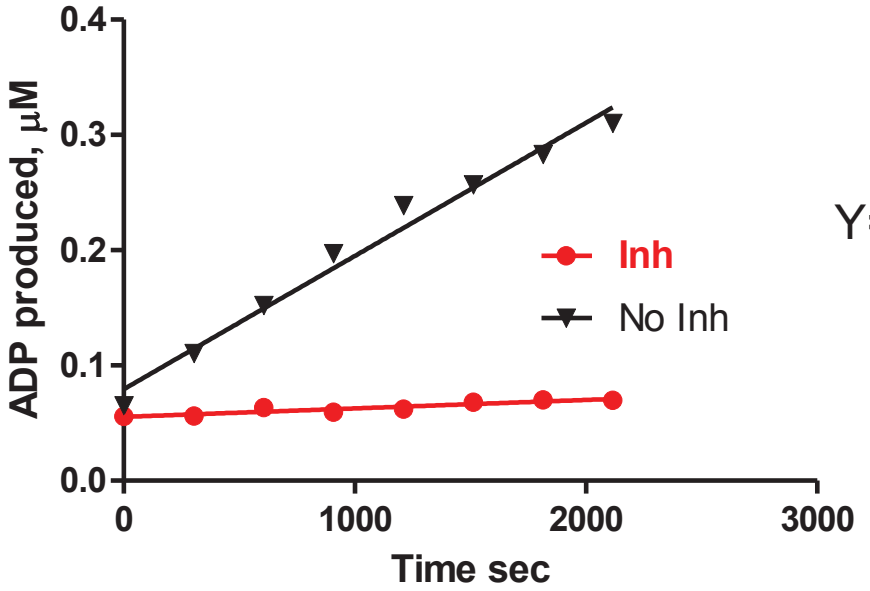


How to determine residence time from the product formed

- Determine Koff values by fitting the curve to equation-

$$P = V_S t + (V_0 - V_S)\frac{(1 - e^{-kt})}{k}.$$

- V_0 represents fully inhibited enzyme velocity, V_S represents the uninhibited enzyme velocity.

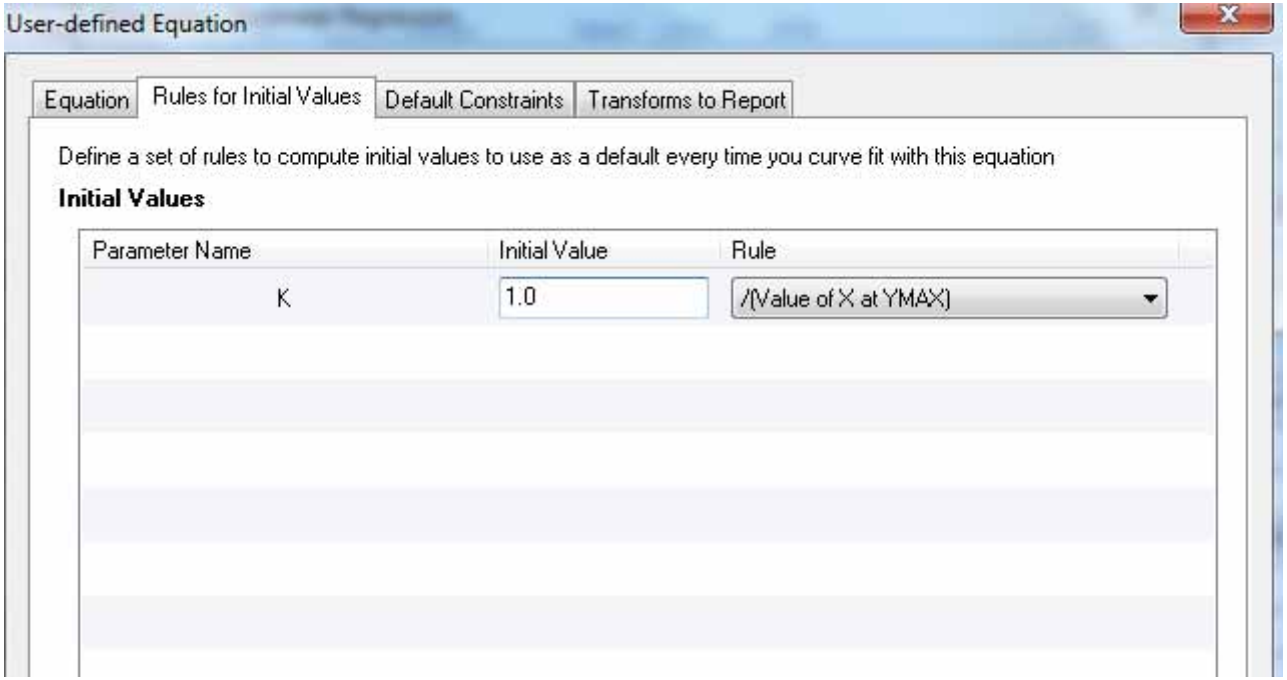


Y=0.0001156X-0.000108243/K(1-exp-K*X)

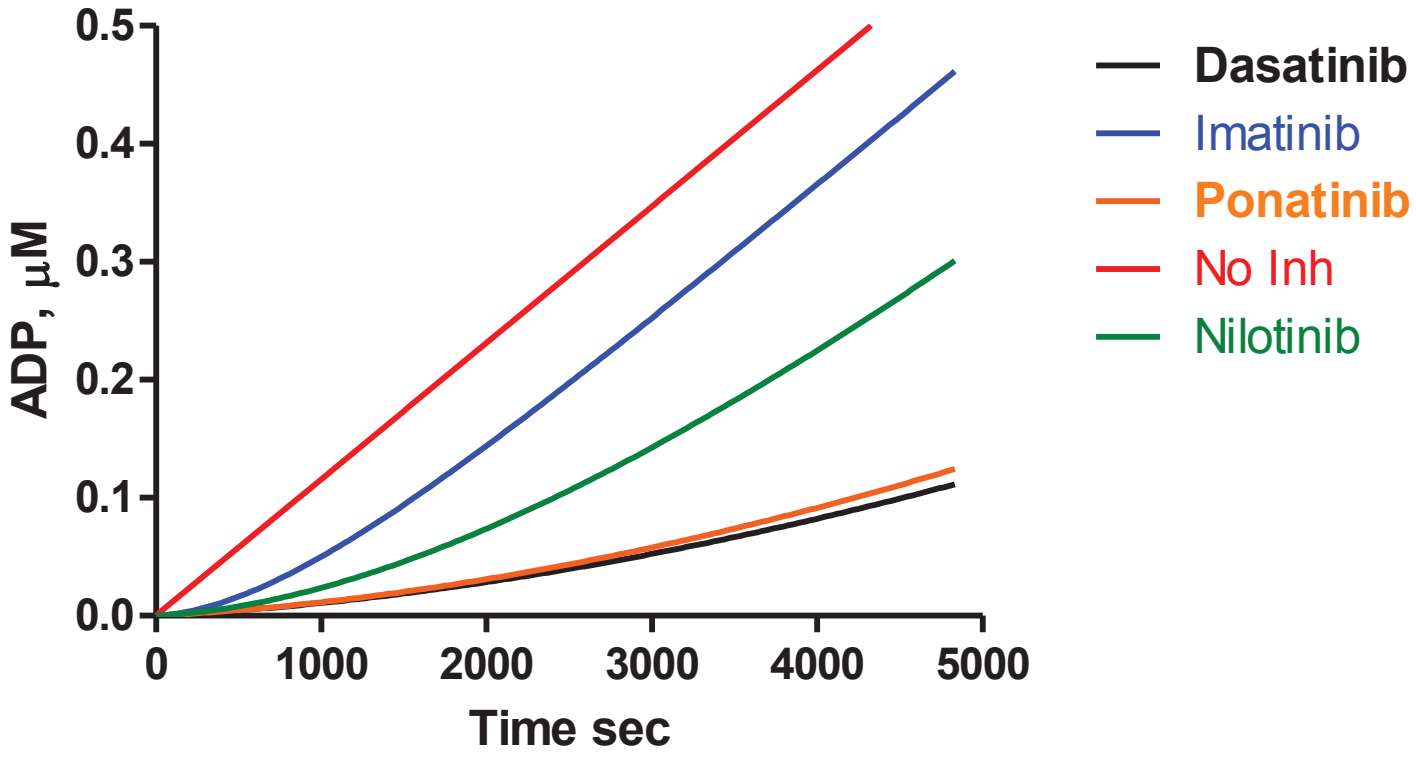
V_s	0.000007357 ± 0.000001187
V_0	0.0001156 ± 0.000006600

Curve fitting to this equation in Graph Pad Prism

- Open the analysis window in Graph Pad prism and select new equation.
- Enter the new equation.
- Set “rules”- Define K as 1/Value of X at Ymax



Determining residence time

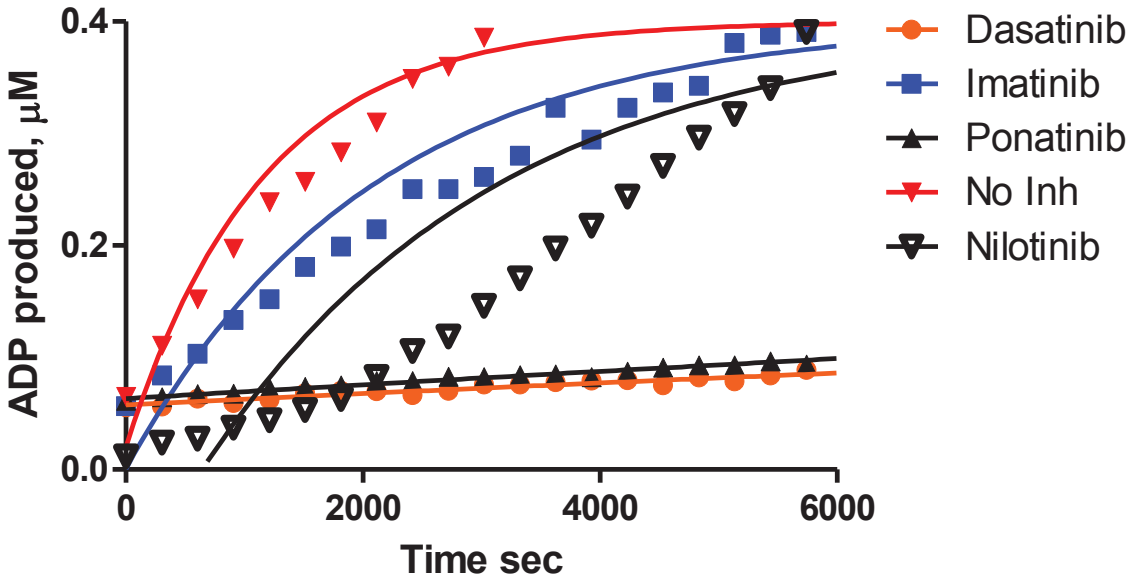


	Dasatinib	Imatinib	Ponatinib	Nilotinib
K	0.00006669	0.001106	0.00007959	0.0003377
Tau, min	250	15	209	50

Lit values

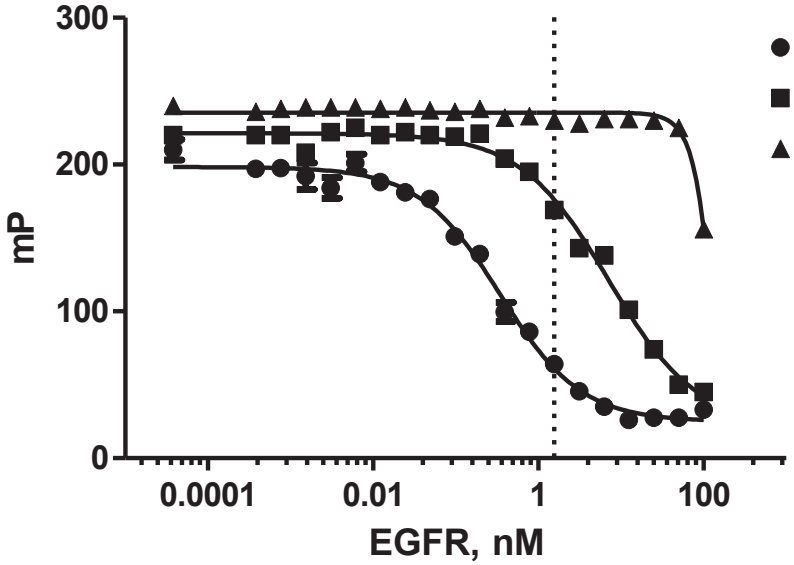
	Tau, min-1
Dasatinib	837
Ponatinib	500
Imatinib	14

Determining residence time using one phase decay (product formation)



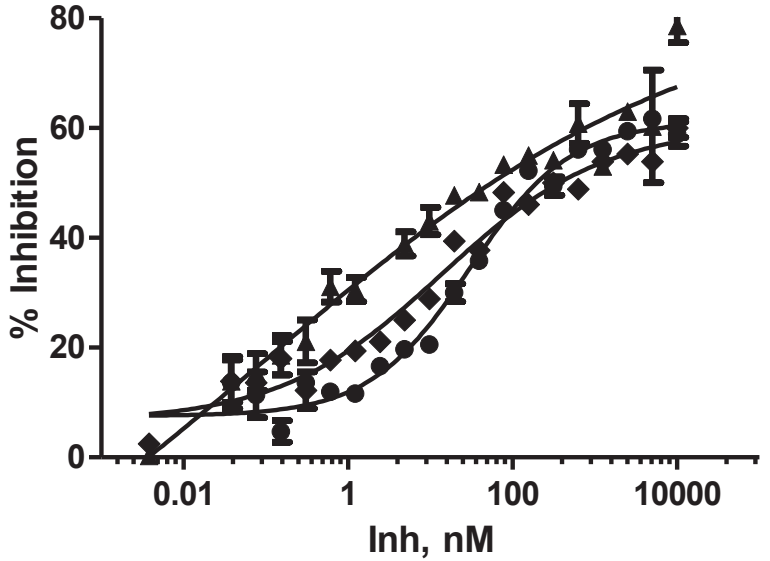
	Dasatinib	Imatinib	Ponatinib	Nilotinib
K	0.00003881	0.0004834	0.00001867	0.0004061
Tau, min	429	34	892	41

Case Study 2: EGFR



- Complete reaction
- ATP alone
- Srctide alone

	ATP+Srctide	ATP	Srctide
$EC_{80\ (nM)}$	1.655	38.42	~ 1679



- Erlotinib
- Gefitinib
- Lapatinib

	Erlotinib	Gefitinib	Lapatinib
$IC_{50\ (nM)}$	30.12	<1	13.75

Preincubation for [EI] Complex formation

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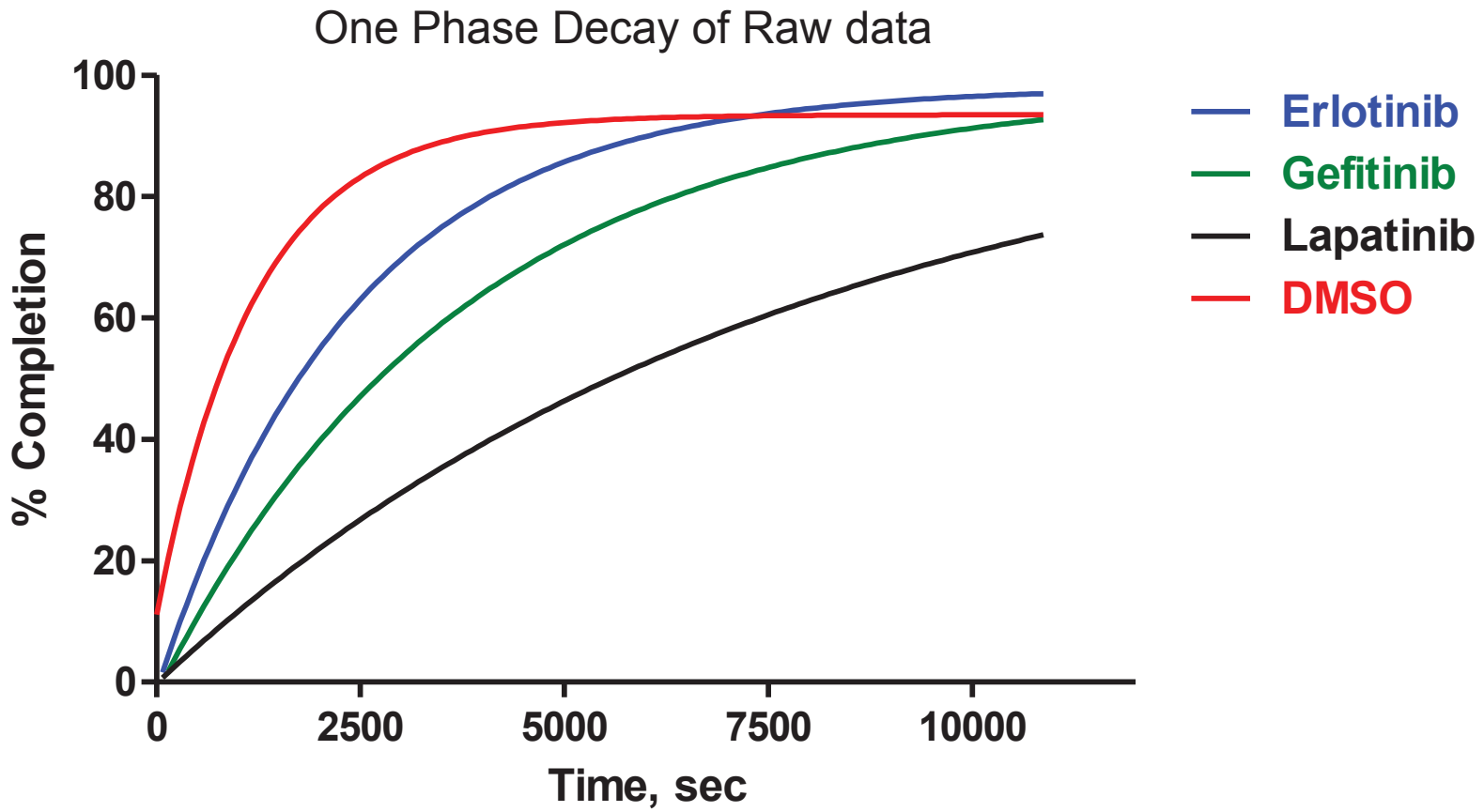
- Enzyme for [EI]
  - ➢ $100*EC_{80}$= 1.5 nM* 100 =150 nM

- Drug for [EI]
  - ➢ $10*IC_{50}$ (Erlotinib) = 300 nM
  - ➢ $10*IC_{50}$ (Gefitinib) = 100 μM
  - ➢ $10*IC_{50}$ (Lapitinib) = 140 nM

Preincubation Protocol. The amount of EGFR used for preincubation is at least 100 × EC_{80}, so that it still gives a robust signal after dilution. The inhibitor concentration is 10 × IC_{50}, such that the enzyme is saturated with the inhibitor. The mix was incubated for 1 hour at room temperature to insure formation of the EI complex.

Residence time for EGFR inhibitors using Transcreener assay (Normalization of raw data)



	Erlotinib	Gefitinib	Lapatinib
K	0.0004181	0.00027	0.0001284
Tau(min)	40	62	130

Residence time for EGFR inhibitors using product formed

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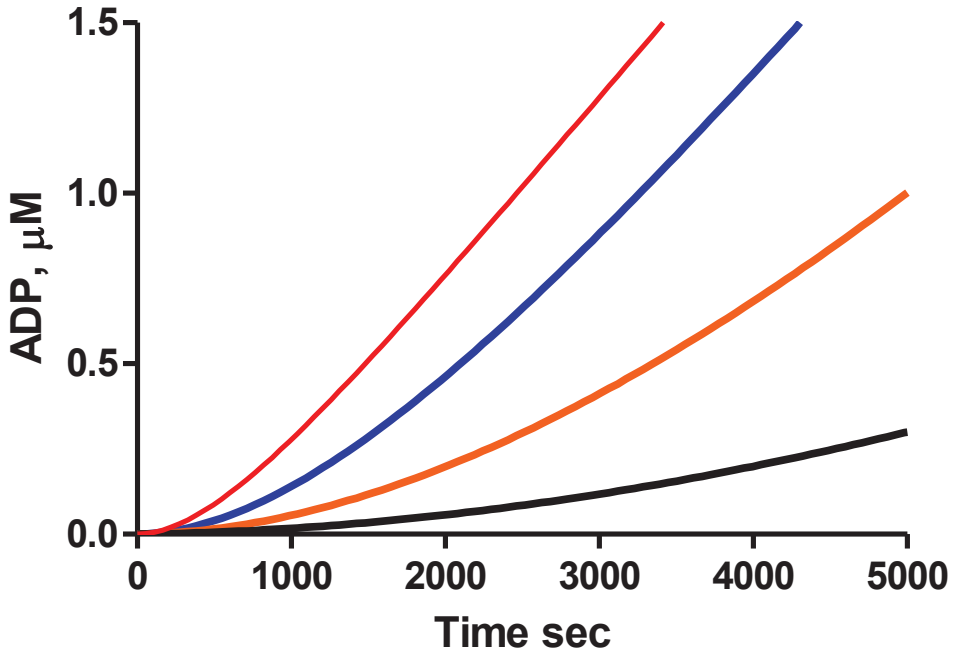
$$Y=0.0005288X-(0.000523/K)*(1-\exp(-K*X))$$



	Erlotinib	Gefitinib	Lapatinib
K	0.00063	0.0002031	0.00004458
Tau(min)	27	82	374

Lit values from Lanthascreen assay

	Tau, min
Gefitinib	14
Erlotinib	10
Lapitinib	52

Residence time for EGFR inhibitors using Transcreener assay

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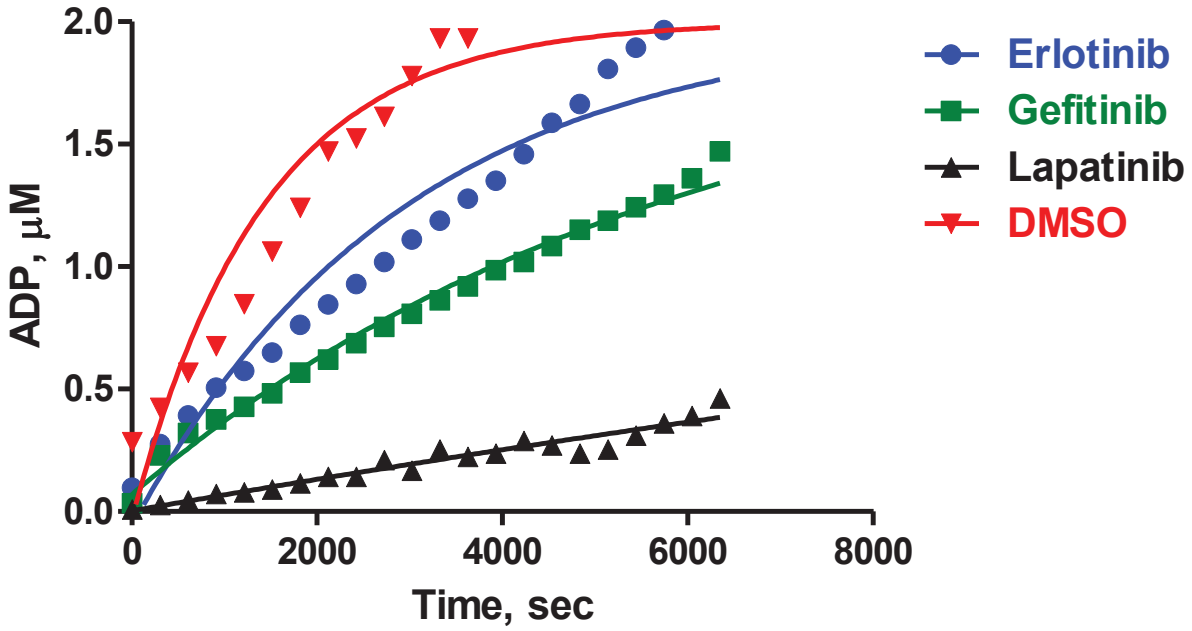
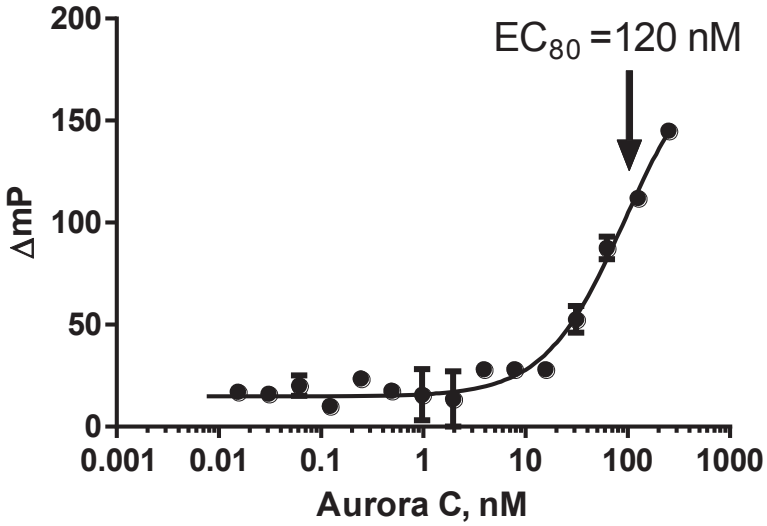
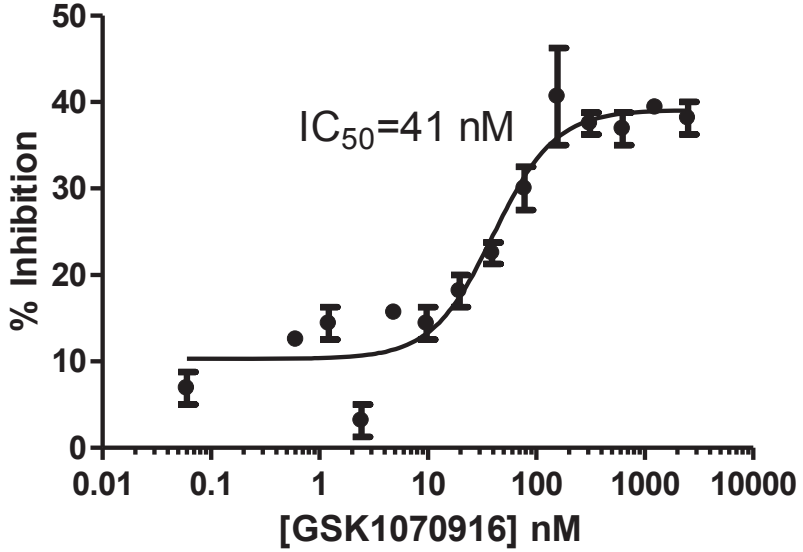
One phase Decay of product formed

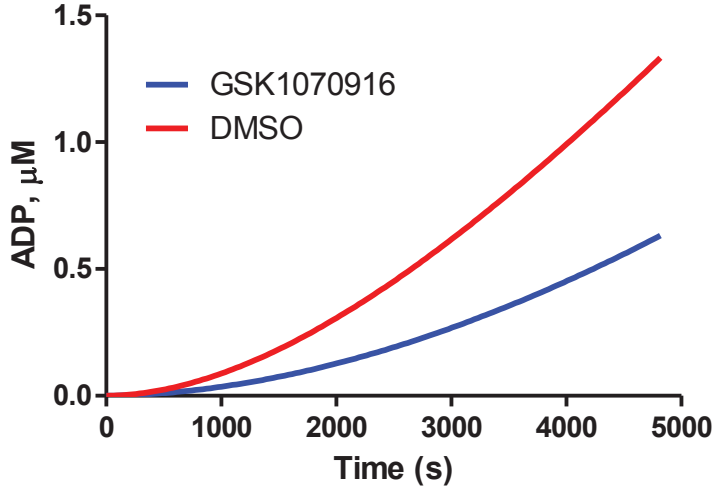


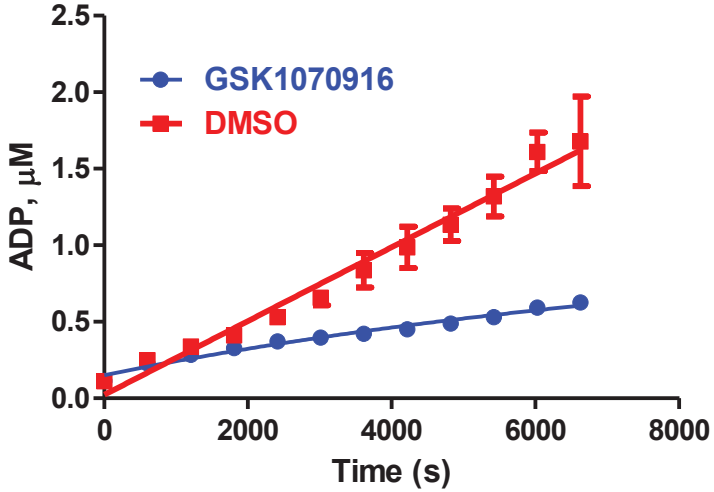
	Erlotinib	Gefitinib	Lapatinib
K	0.0003412	0.0001693	0.00003341
Tau(min)	49	98	498

Aurora C: Residence time for GSK-1070916









	GSK1070916
K	0.0001193
Tau (min)	140

	GSK1070916
K	0.0001
Tau (min)	154

Lit value-280 min

Biochem. J. (2009) **420**, 259–265

Conclusions

- The Transcreener® ADP^2 Assay relies on highly selective immunodetection of ADP with FP, TR-FRET, and FI readouts. Direct detection has advantages over enzyme-coupled assays in terms of simplicity of use and resistance to compound interference.
- The assays can be tuned for detection of kinases or ATPases at any ATP concentration from 0.1 to 1000 µM, making it well suited for profiling inhibitors with enzymes that have diverse ATP requirements.
- The ability to run reactions in kinetic mode enables determination of inhibitor residence times using a jump dilution method.
- The residence times determined for Abl1, EGFR and Aurora C inhibitors using the Transcreener ADP Assay in jump dilution experiments were consistent with literature values.

References and Acknowledgements

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- Impact of enzyme concentration and residence time on apparent activity recovery in jump dilution analysis. R.A. Copeland et al. / Anal. Biochem. 416 (2011) 206-210.
- Biochemical characterization of GSK1070916, a potent and selective inhibitor of Aurora B and Aurora C kinases with an extremely long residence time. K. Anderson et al. Biochem. J. (2009) 420, 259–265
- Evaluation of enzyme inhibitors in drug discovery.Robert A. Copeland.

- Acknowledgements:
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  - **Tim Wigle-Warp Drive Bio**
  - **BellBrook Labs team**.

Transcreener® Platform

Four assays, thousands of targets