

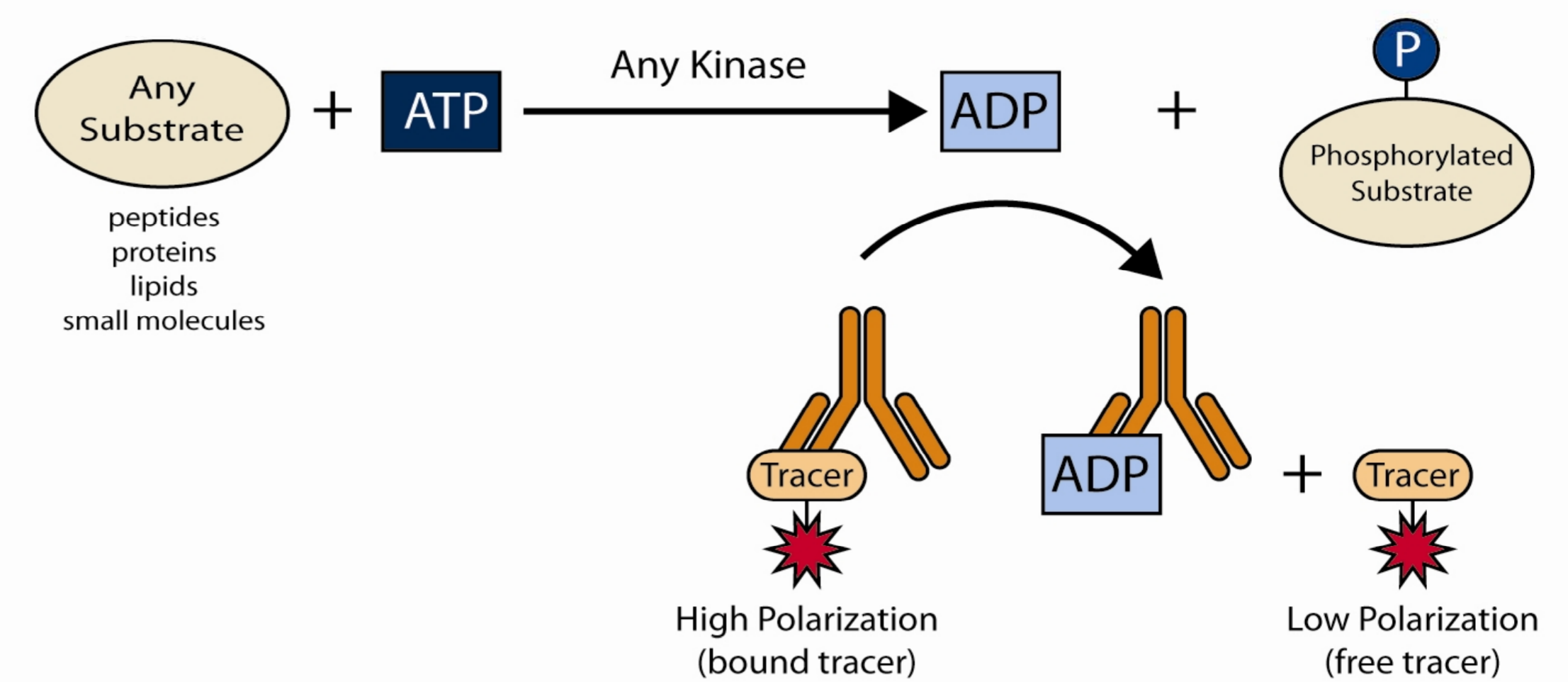
# Robust Detection of ATP-Utilizing Enzymes with an Improved Fluorescence Polarization ADP Detection Assay

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## Introduction

The Transcreeper ADP<sup>2</sup> FP Assay allows the facile detection and screening of established drug targets including protein and lipid kinases as well as emerging targets such as heat shock proteins and other ATPases by directly measuring ADP formation. This homogenous, single-step competitive fluorescence polarization immunoassay employs a selective ADP antibody and a red-shifted ADP-Alexa Fluor<sup>®</sup> 633 tracer. Here we describe the recent development of an improved monoclonal antibody with >100-fold selectivity for ADP vs. ATP, which enables robust detection of initial velocity rates ( $Z' > 0.7$  at  $\leq 20\%$  substrate consumption) at ATP concentrations ranging from 0.1  $\mu\text{M}$  to 1,000  $\mu\text{M}$ . This generic ADP detection system enables robust detection of enzymes with low activity or low ATP requirements, saving on reagents and improving data quality, especially relative to ATP depletion methods.

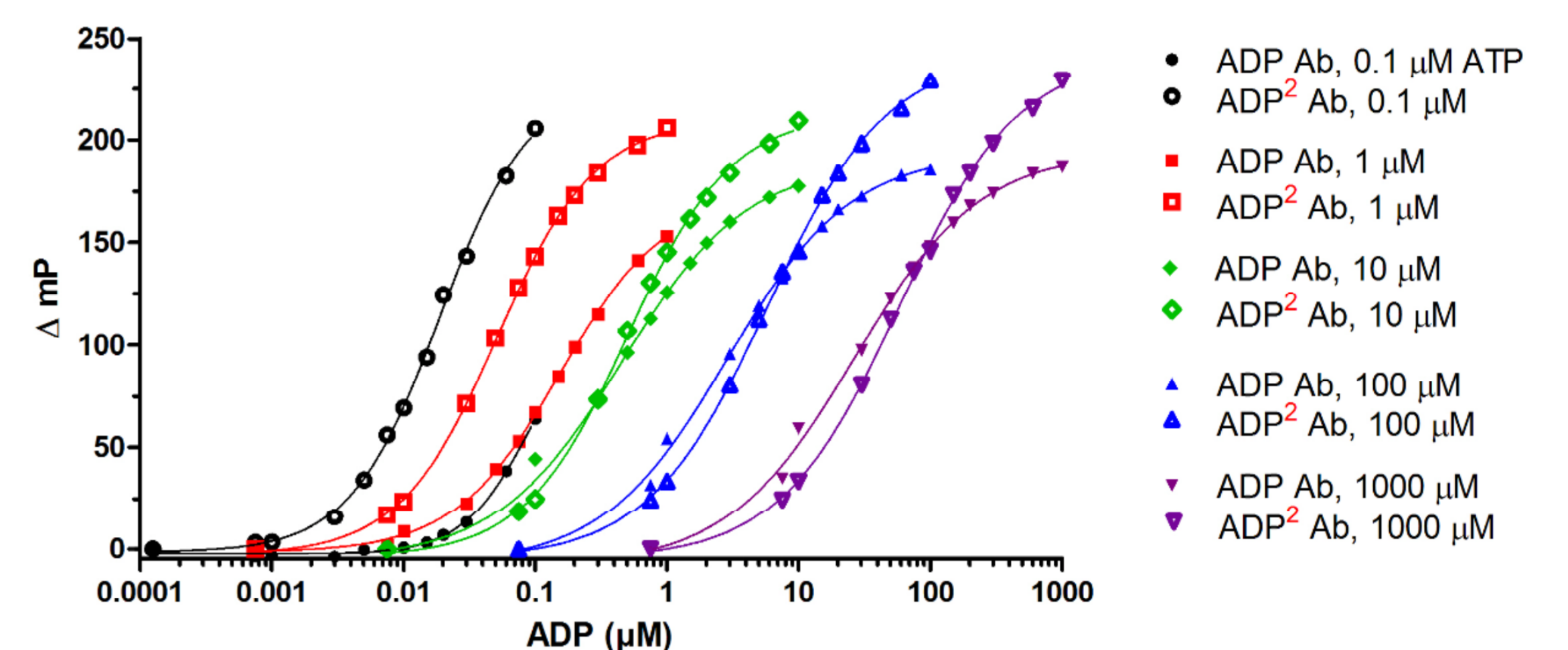


**Figure 1. The Transcreeper ADP<sup>2</sup> FP Assay enables direct detection of ADP using a competitive fluorescence polarization immunoassay.** ADP produced by kinases or other ATP-utilizing enzymes displaces a far red fluorescent tracer from a highly selective mAb against ADP, reducing its polarization. The method is a single addition, homogenous format that has been extensively validated in pharmaceutical HTS laboratories with more than 40 million data points.

Nucleotide	IC <sub>50</sub> (nM)		NDP Affinity Improvement
	Old ADP Ab	New ADP Ab	
ADP	180	7.9	23x
ATP	17,300	915	
ATP/ADP	96	92	
GDP	50	10.7	14x
GTP	1,500	1,400	
GTP/GDP	30	131	
UDP	ND	214	ND
UDP-Glc	ND	744,000	
UDP-Glc/UDP	ND	3,476	

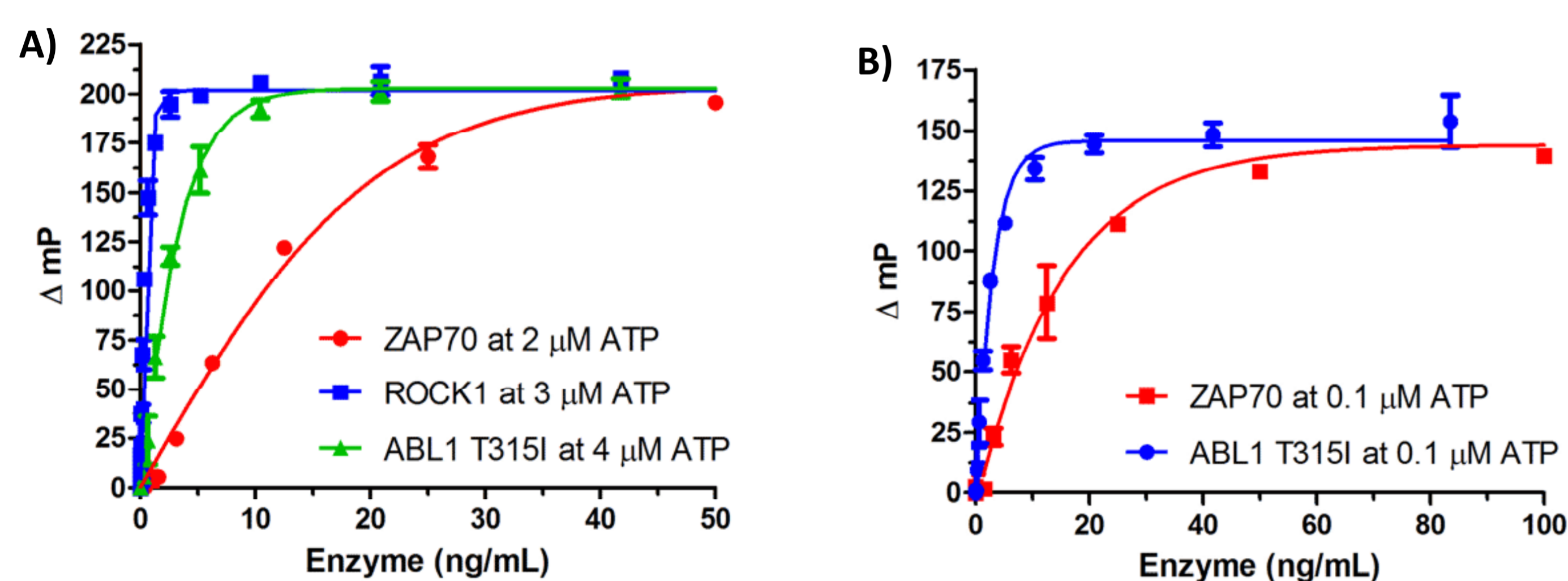
**Table 1. The new ADP Ab increases sensitivity of ADP detection more than 20-fold and enables detection of other NDPs.**

Nucleotides were titrated in the presence of ADP antibodies and an Alexa-Fluor<sup>®</sup> 633-ADP tracer in 384 well plates, fluorescence polarization measurements were made on a Tecan Safire<sup>™</sup>, and the resulting competition curves were used to generate IC<sub>50</sub> values. Solution conditions were optimized separately for each antibody.

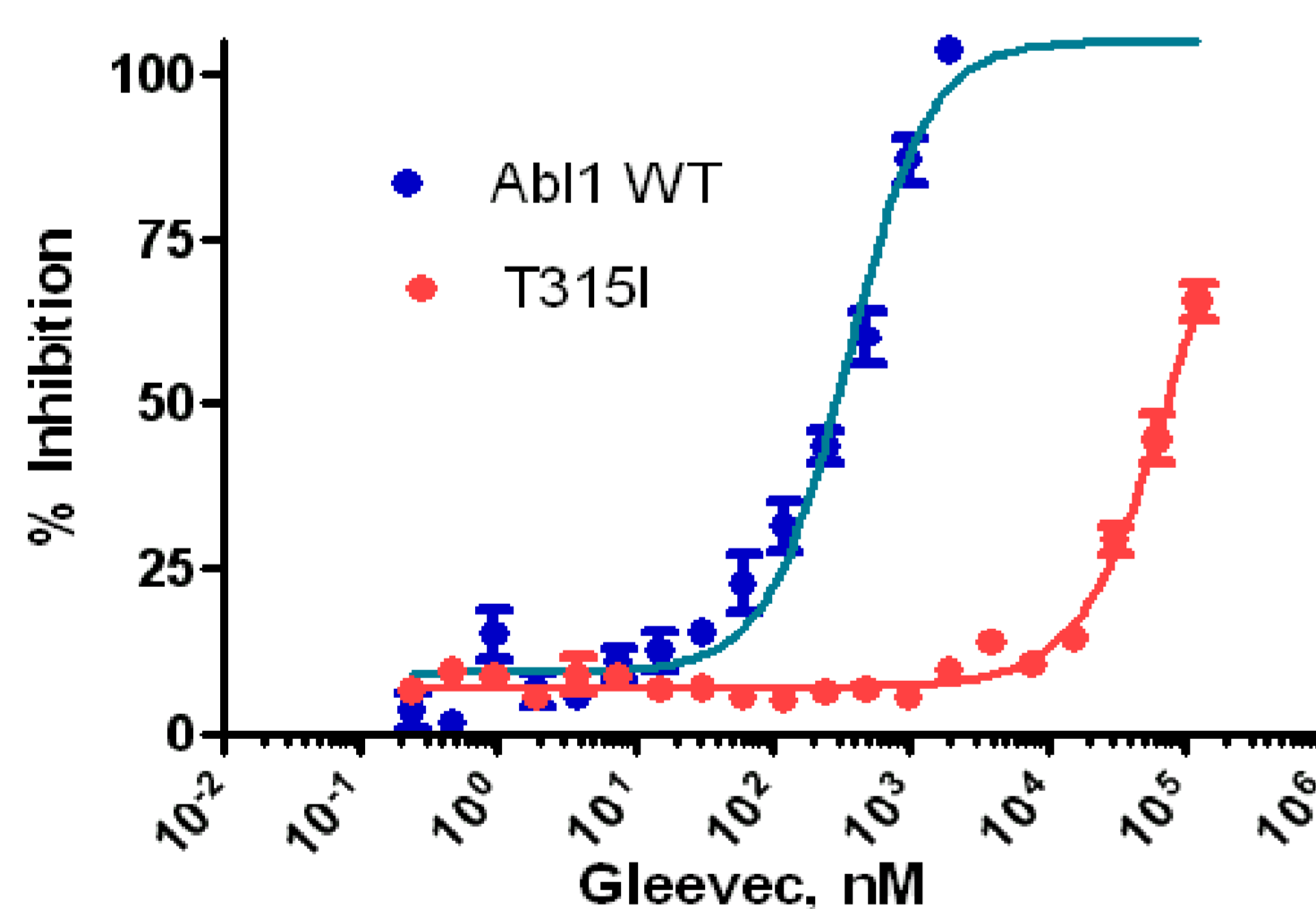
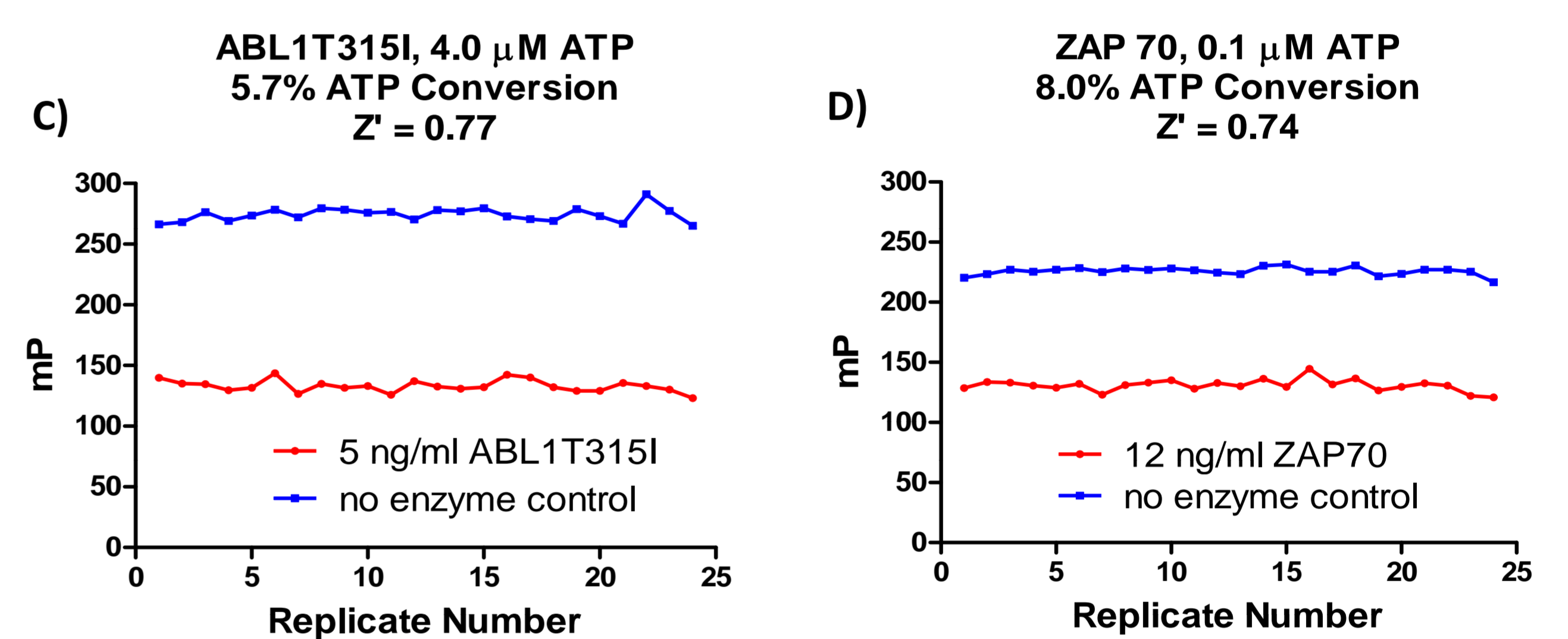


**Figure 2. Standard curves for conversion of ATP to ADP show the increased sensitivity of the ADP<sup>2</sup> Ab**

Standard curves mimicking enzyme reactions were constructed for conversion of ATP to ADP using initial ATP concentrations of 0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , 100  $\mu\text{M}$ , and 1,000  $\mu\text{M}$  ATP; as ADP was added, ATP was decreased proportionately. Antibody was present at its EC<sub>50</sub> concentration for each initial ATP concentration; Alexa-Fluor<sup>®</sup> 633-ADP tracer was present at 2 nM (n=24).



**Figure 3. Enhanced ADP detection translates to robust detection of enzymes with low ATP requirements or low specific activity.** Dose response curves for kinases in the presence of A) ATP at its corresponding  $K_m$  concentration, or B) 0.1  $\mu\text{M}$ . Assay windows for initial velocity detection of C) ABL1 T315I using 4  $\mu\text{M}$  ATP and D) ZAP70 using 0.1  $\mu\text{M}$  ATP. All experiments were performed in Corning<sup>®</sup> 384 Well Black Round Bottom Low Volume Polystyrene Non-Binding Surface Microplates (Part # 3676). Fluorescence polarization was read in a Tecan Safire<sup>™</sup> or PerkinElmer EnVision multiwell reader. The free tracer reference was set to 20 mP, and buffer containing antibody was used as a blank for sample and reference wells. Kinase enzyme reactions were run in CK buffer (50 mM HEPES (pH 7.5), 0.01% Brij-35, 2 mM EGTA, 4 mM MgCl<sub>2</sub>) in 10 l final volume; final read volume after addition of Stop/Detect Mix was 20  $\mu\text{l}$ . Stop and Detect Mix contained (final assay concentrations) 10 mM EDTA, 2 nM Alexa 633-ADP and ADP Ab at concentrations appropriate for the starting [ATP]. Standard curves similar to those shown in Figure 2 were used to determine ADP formation from enzyme titrations and enzyme amounts were chosen for initial velocity conditions used for  $Z'$  determination (n=24).



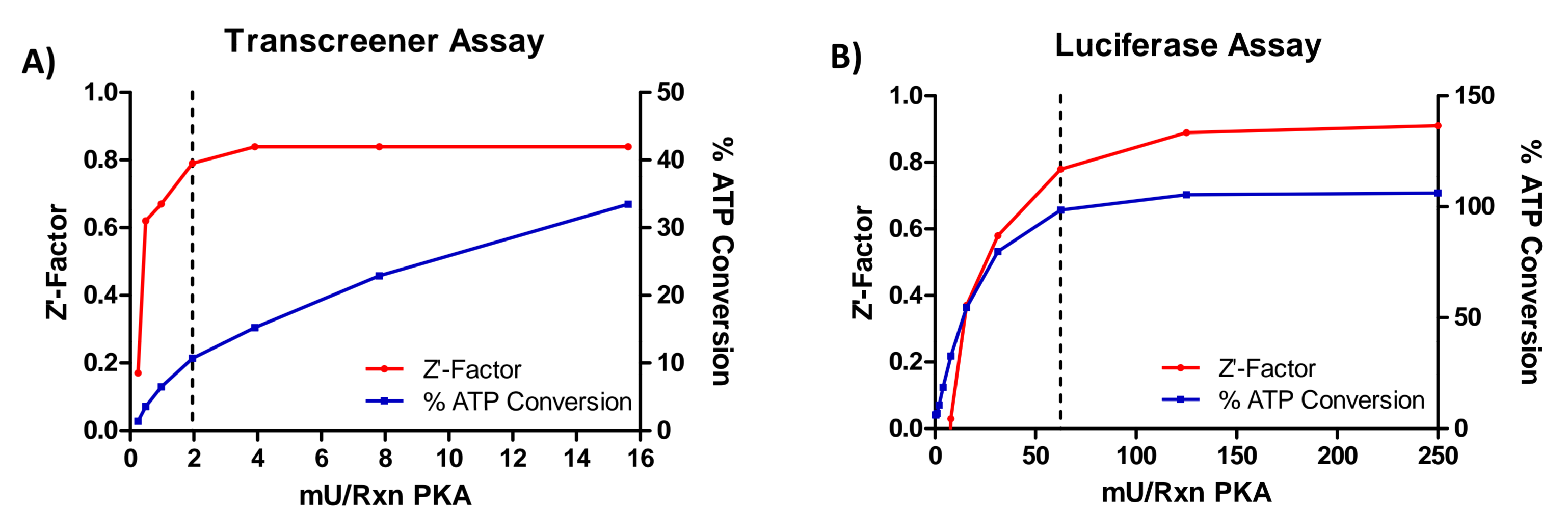
**Figure 4. Dose Response for Gleevec<sup>®</sup> with ABL1 and the ABL1 T315I shows the expected potency difference.** Enzyme reactions and detection with Transcreeper ADP<sup>2</sup> FP Assay were as described for Figure 3 in the presence of inhibitor titrations. IC<sub>50</sub> profiles for the full panel of inhibitors used is shown below.

	ABL1	ABL1 T315I
Gleevec		
GO-6983		
H-89		
Iressa		
PP2		
Ro-31-8220		
Roscovatine		
SB 202190		
Staurosporine		
Tyrphostin AG1478		
Y27632		

Legend:   
 < 1  $\mu\text{M}$   
 1 - 10  $\mu\text{M}$   
 10 -100  $\mu\text{M}$   
 No Inhibition

## Acknowledgements:

This work is supported by NIH SBIR grants R44CA110535 and R44GM073290. ©2008 BellBrook Labs. Transcreeper is a registered trademark of BellBrook Labs. AlexaFluor is a registered trademark of Molecular Probes/Invitrogen. BellBrook Labs 5500 Nobel Drive, Suite 250, Madison, WI 53711 866.313.7881 or 608.443.2400 www.BellBrookLabs.com



**Figure 5. Transcreeper ADP<sup>2</sup> FP Assay is more sensitive than Luciferase-based ATP depletion methods.**

PKA enzyme reactions were performed in 384-well plates in a 10  $\mu\text{l}$  reaction volume with 10  $\mu\text{M}$  ATP and 50  $\mu\text{M}$  Kempptide substrate for one hour at room temperature. The enzyme buffer recommended by the Luciferase Assay vendor was used for both assays and fluorescence polarization and luminescence were measured on a BMG LABTECH PHERAstar Plus plate reader. A) Transcreeper: an equal volume of ADP Detection Mixture was added to stop the reaction, followed by a 10 minute equilibration before reading. The equilibration period was shortened from the usual one hour period to be consistent with the Luciferase Assay protocol. B) Luciferase: an equal volume of Luciferase Assay reagent was added to the reaction, followed by a 10 minute equilibration before reading.

## Summary:

- A higher affinity monoclonal antibody that enhances sensitivity for ADP more than 20-fold (IC<sub>50</sub> = 7.9 nM) has been developed and incorporated into the recently introduced Transcreeper ADP<sup>2</sup> FP Assay.
- The Transcreeper ADP<sup>2</sup> FP Assay enables robust detection ( $Z' > 0.7$ ) of kinase velocity using ATP concentrations as low as 100 nM and as high as 1 mM.
- The enhanced sensitivity and flexibility for ATP allows facile screening of enzymes with low ATP requirements (<5  $\mu\text{M}$ ) or low specific activity (ABL 1 T315I).
- In direct comparisons with luciferase based ATP depletion methods, the Transcreeper ADP<sup>2</sup> FP Assay enabled robust PKA detection ( $Z' > 0.7$ ) using 30-fold less enzyme (2 mU vs. 60 mU) and much lower ATP conversion (10% vs. 100%)
- The improved ADP antibody also enables highly selective detection of GDP and UDP, allowing generic detection for GTPases and UDP-sugar dependent glycosyltransferases.