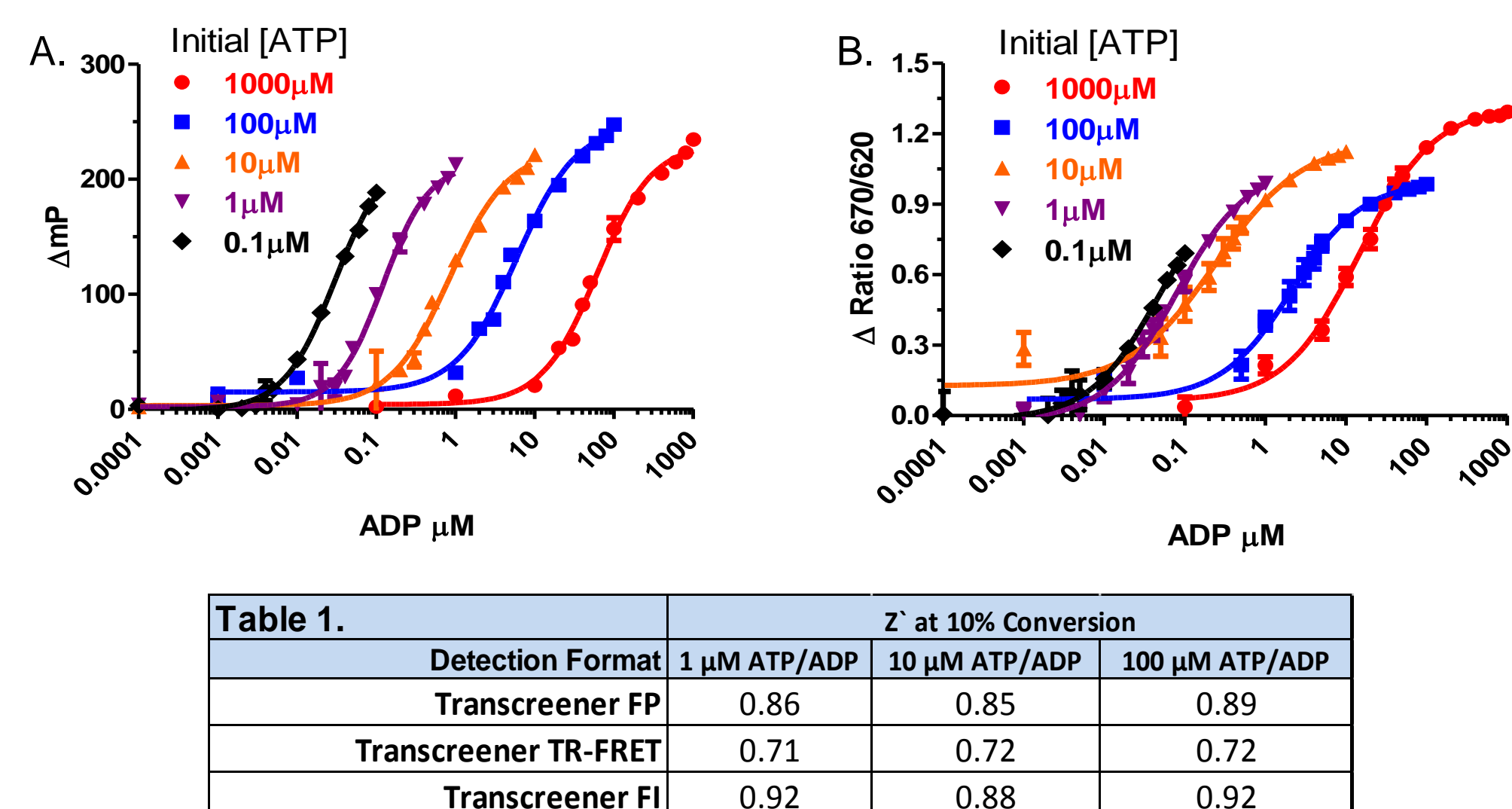


## Overview

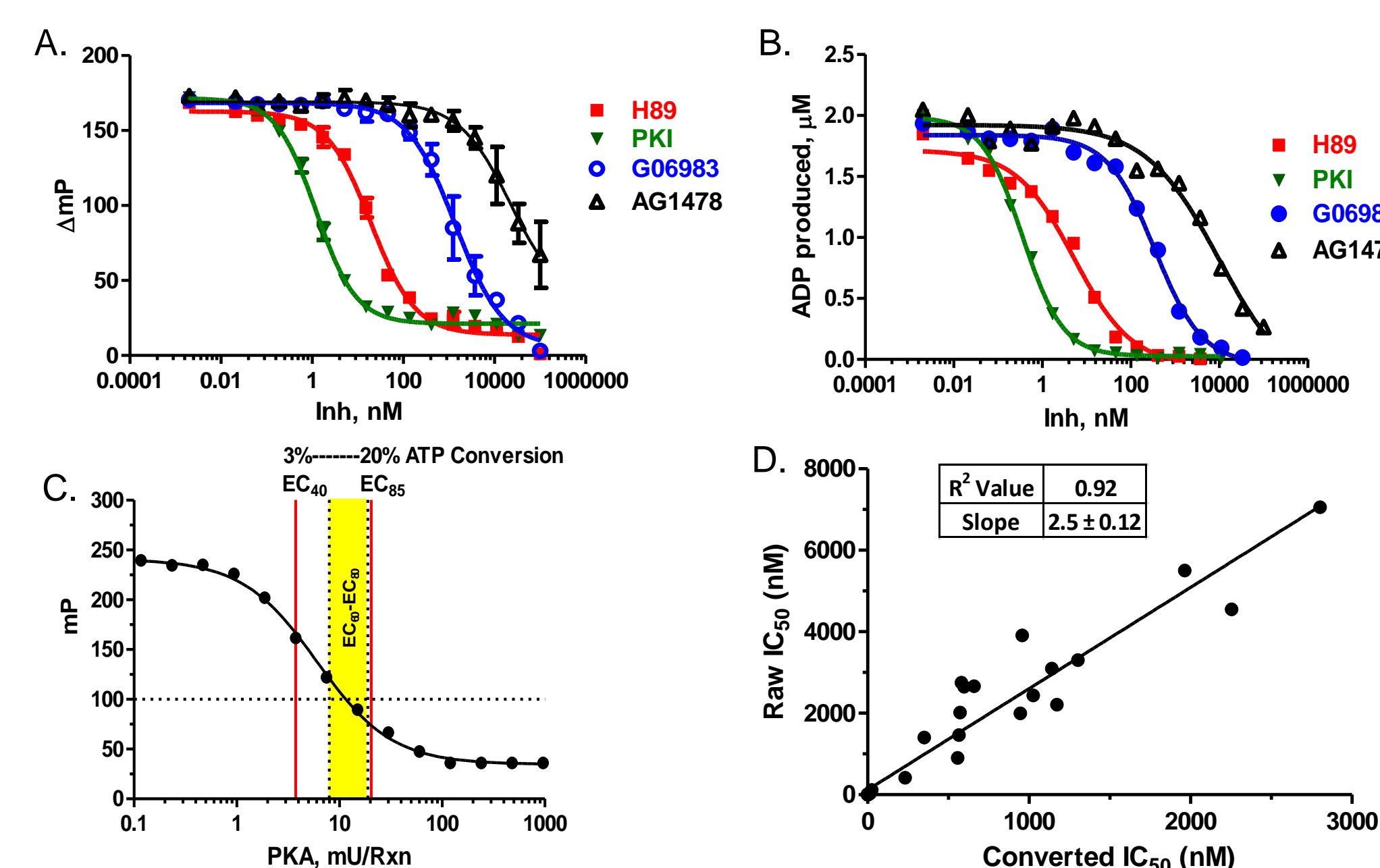
Biochemical HTS assays are a critical component of drug discovery programs focused on kinases and other types of ATP-dependent enzymes, such as chaperonins and helicases. Assembling, evaluating and optimizing high quality enzymes and assay reagents can be a costly and time consuming undertaking. The shift toward screening focused libraries against multiple targets in parallel can add to the assay development burden substantially. BellBrook's Transcreener ADP Assay provides an extensively validated, generic HTS platform for ATPase and kinase assays based on homogenous immunodetection of ADP with a choice of FP, TR-FRET or FI readouts. In this study, we outline a streamlined approach for optimizing conditions to measure enzyme initial velocity for kinases and ATPases that leverages some of the differentiating characteristics of the Transcreener assay including direct detection of ADP without the use of coupling enzymes and low nanomolar sensitivity. Key steps include tuning the dynamic range to accommodate enzymes with different ATP concentrations, optimizing enzyme concentration to produce a good signal, and determining IC<sub>50</sub> values and residence times for inhibitors. We illustrate how monitoring ADP production in real time allows optimization of multiple assay parameters using a single reaction and show that IC<sub>50</sub> values can be determined directly from raw fluorescence data, eliminating the need to use a standard curve to quantify product formation. These approaches reduce the number of experiments required to incorporate new enzymes into a screening or profiling campaign, saving time and reducing reagent costs. Further, we demonstrate a straightforward approach for minimizing enzyme usage by finding the right balance between ATP concentration and enzyme velocity. Taken together, these methods provide a rapid, user friendly solution for screening or profiling kinases and other types of ATP dependent enzymes with minimal pilot experimentation and reagent usage.

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



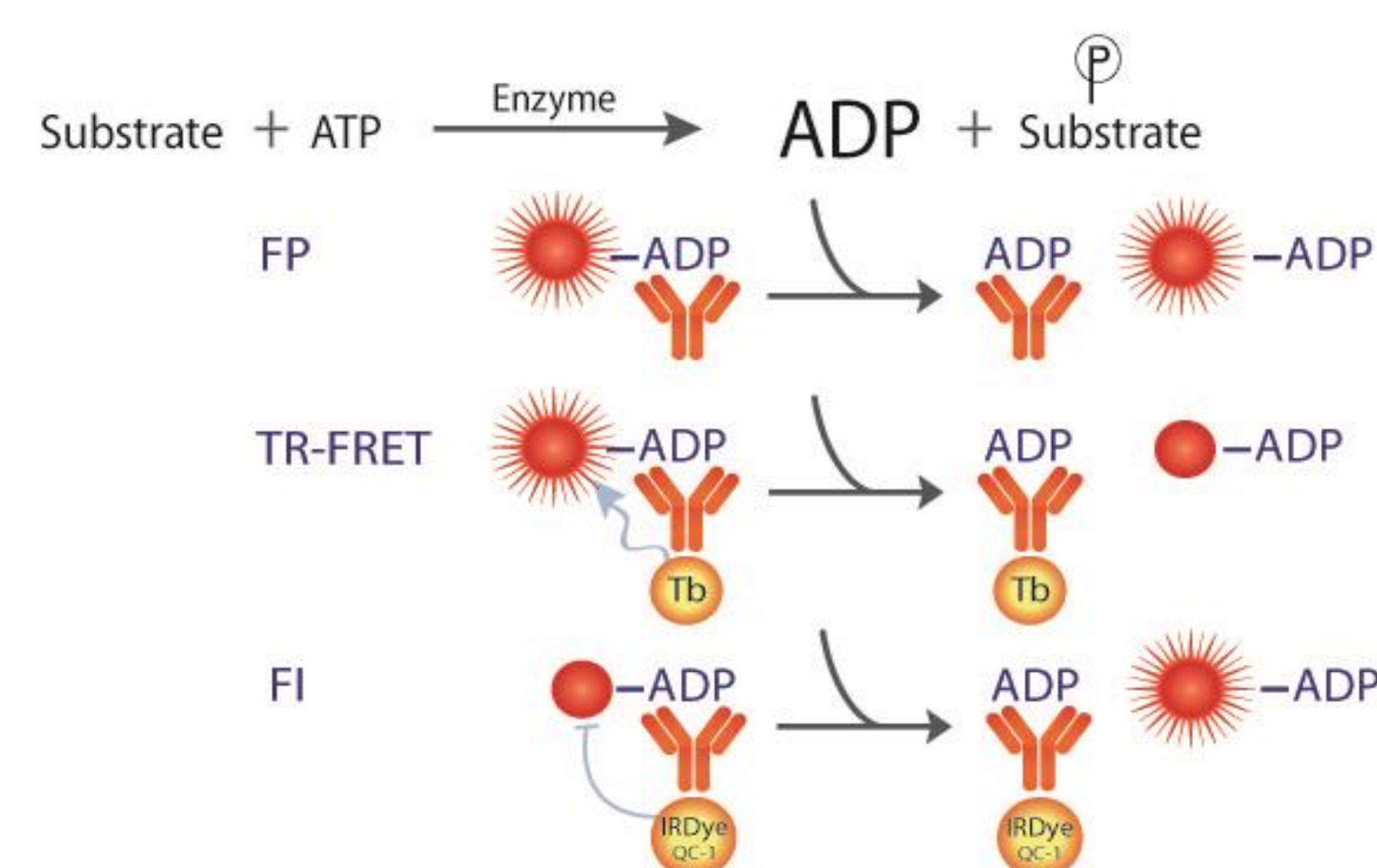
**Figure 3. Standard curves for conversion of ATP to ADP demonstrate the robust response of the Transcreener ADP<sup>2</sup> Assays at different initial ATP concentrations.** Standard curves for FP (A.) and TR-FRET (B.) assays mimic enzyme reactions: starting at the indicated concentrations of ATP, ADP is titrated and ATP is decreased proportionately, n = 24. The dynamic range of the assay is set for each initial ATP concentration by adjusting antibody concentration based on a linear equation ([Ab] (μg/ml) = 1.08 [ATP] (μM) + 1). **Table 1.** The high sensitivity of the Transcreener ADP<sup>2</sup> Assays allows detection of 10% conversion of ATP even at low levels of ATP.

## Determining IC<sub>50</sub> Values from Raw Fluorescence Data Reduces Experimental Work and Data Analysis



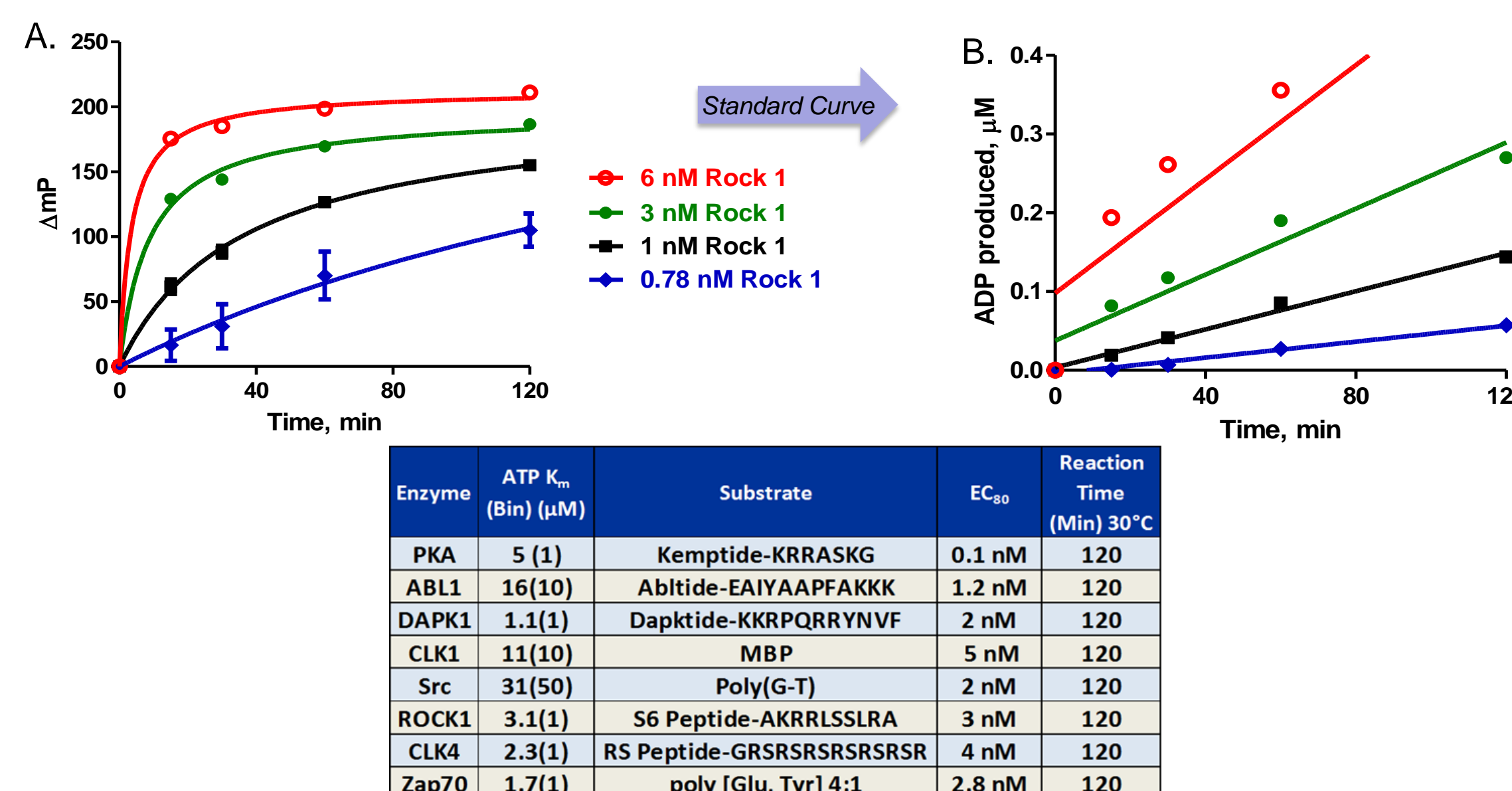
**Figure 6. Linear correlation of IC<sub>50</sub> values determined from raw polarization and ADP formation.** Inhibitor dose response curves from raw polarization data (A.) and after conversion to ADP formation (B.) are similar but not identical. To enable accurate determination of IC<sub>50</sub> values directly from raw data, we determined a conversion factor of 2.5 (D.) that can be used if kinase enzyme concentrations between EC<sub>60</sub> and EC<sub>90</sub> are used (C.). The correlation between IC<sub>50</sub> determined from raw and converted data was established using PKA, AKT2, Rock1, Clik4, Zap 70 and Abl1 with Staurosporine, K252a, H89, Tyrophostin AG1478, PKI and Gleevec.

## Transcreener ADP<sup>2</sup> Assays: Direct, Homogenous ADP Detection with FP, TR-FRET or FI Readouts



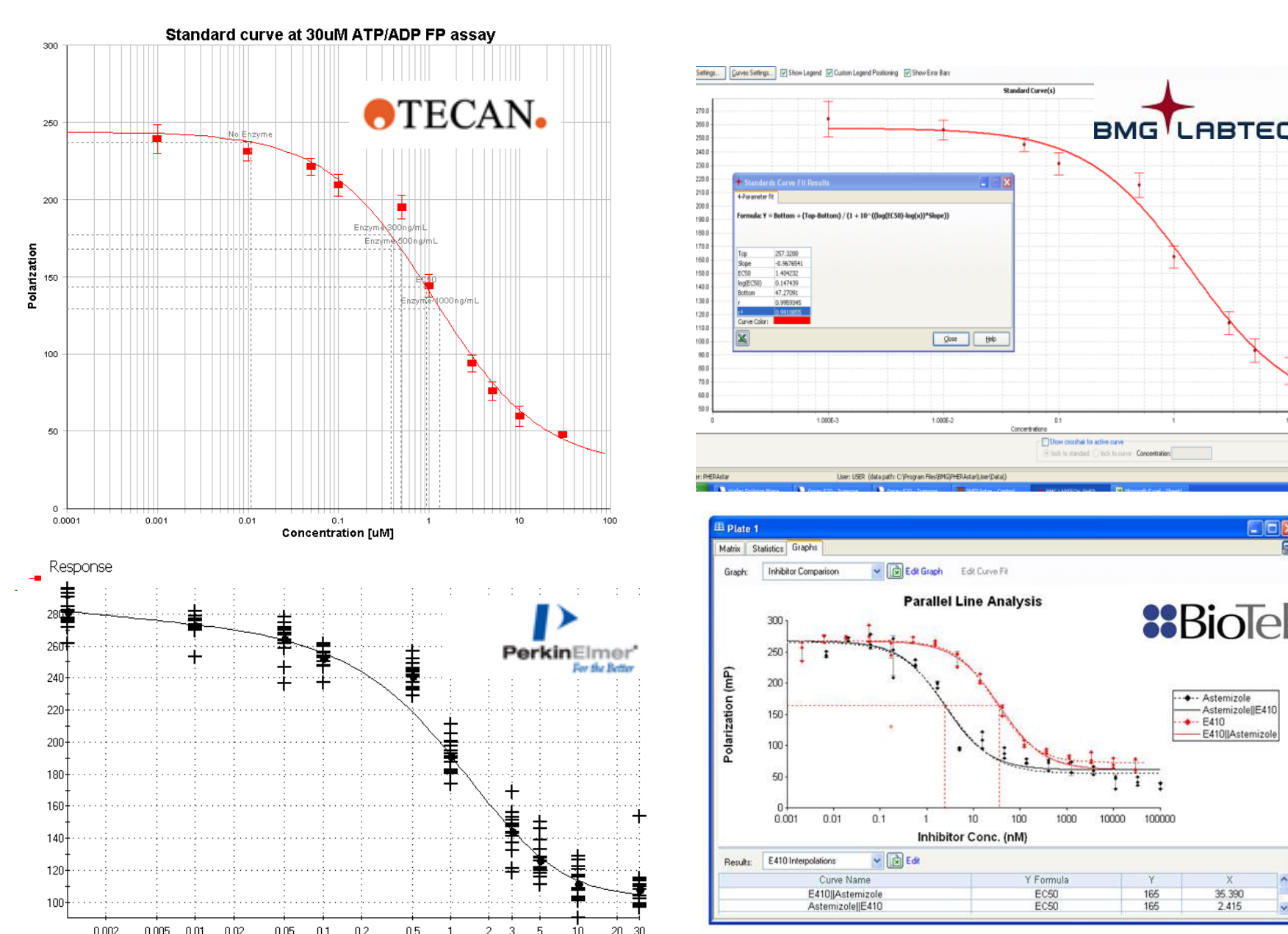
**Figure 1. Transcreener assays are the only direct ADP detection method available.** ADP displaces tracer from a highly specific monoclonal antibody resulting in a change in fluorescence, with fluorescence polarization (FP), time resolved FRET (TR-FRET) and fluorescence intensity (FI) formats available. All three are homogenous, mix and read assays and use a red-shifted tracer to minimize compound interference.

## Optimizing Reactions in Kinetic Mode Reduces Experimental Work and Enzyme Usage



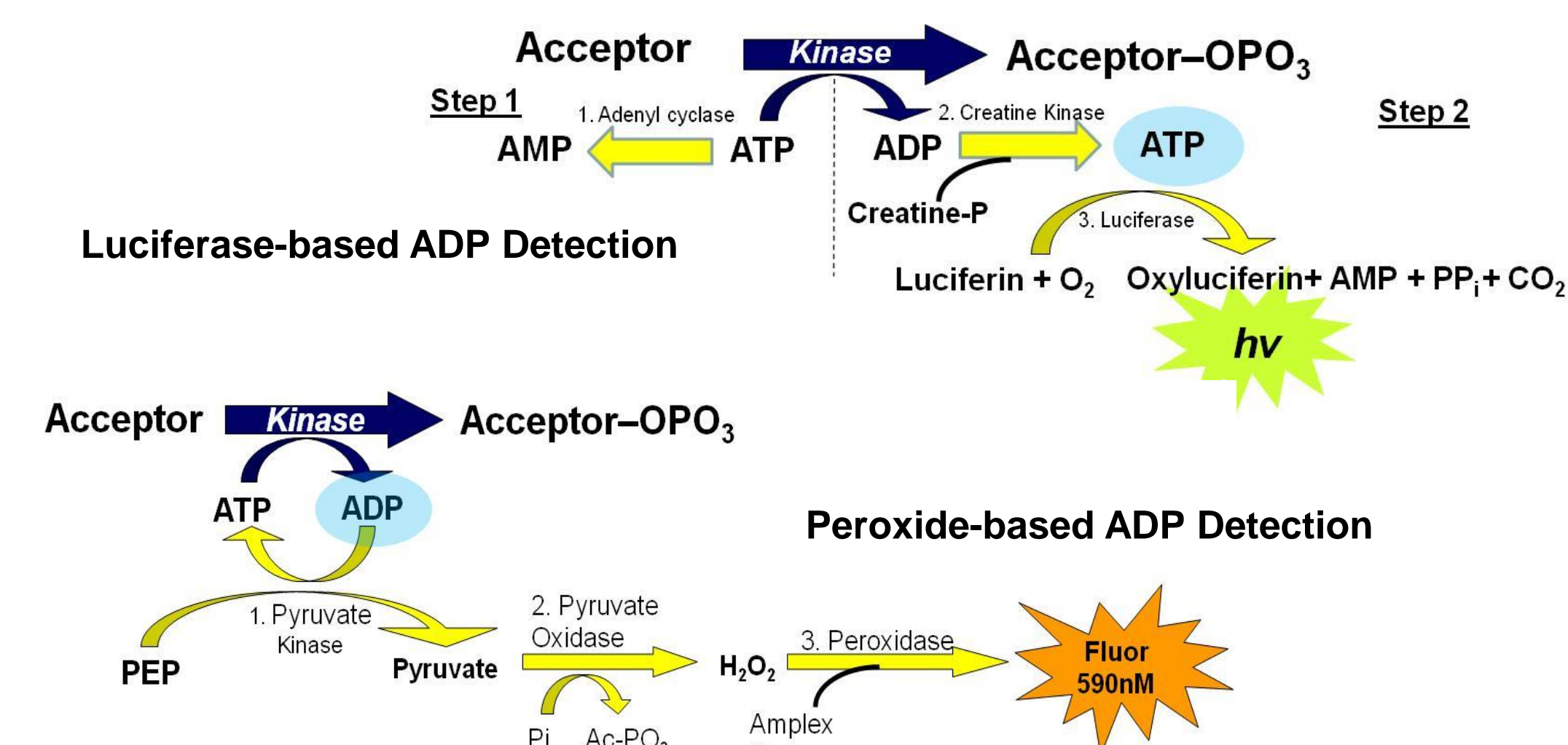
**Figure 4. Detection of kinase activity in real time allows selection of optimal concentration and reaction time in a single experiment.** Transcreener detection reagents were added to kinase reactions prior to starting reactions by addition of ATP and plates were read at 15 minute intervals. ATP concentration was 1 μM, and S6 peptide substrate was present at 10 μM. Polarization values (A.) were converted to ADP formed (B.) using a standard curve as in Fig. 3. The equilibration time for displacement of tracer from the ADP antibody is approximately 10 minutes. This approach can be used to streamline assay development for diverse kinases. (C.)

## Validation on Major Instrument Platforms Insures High Quality Results and Seamless Data Analysis.



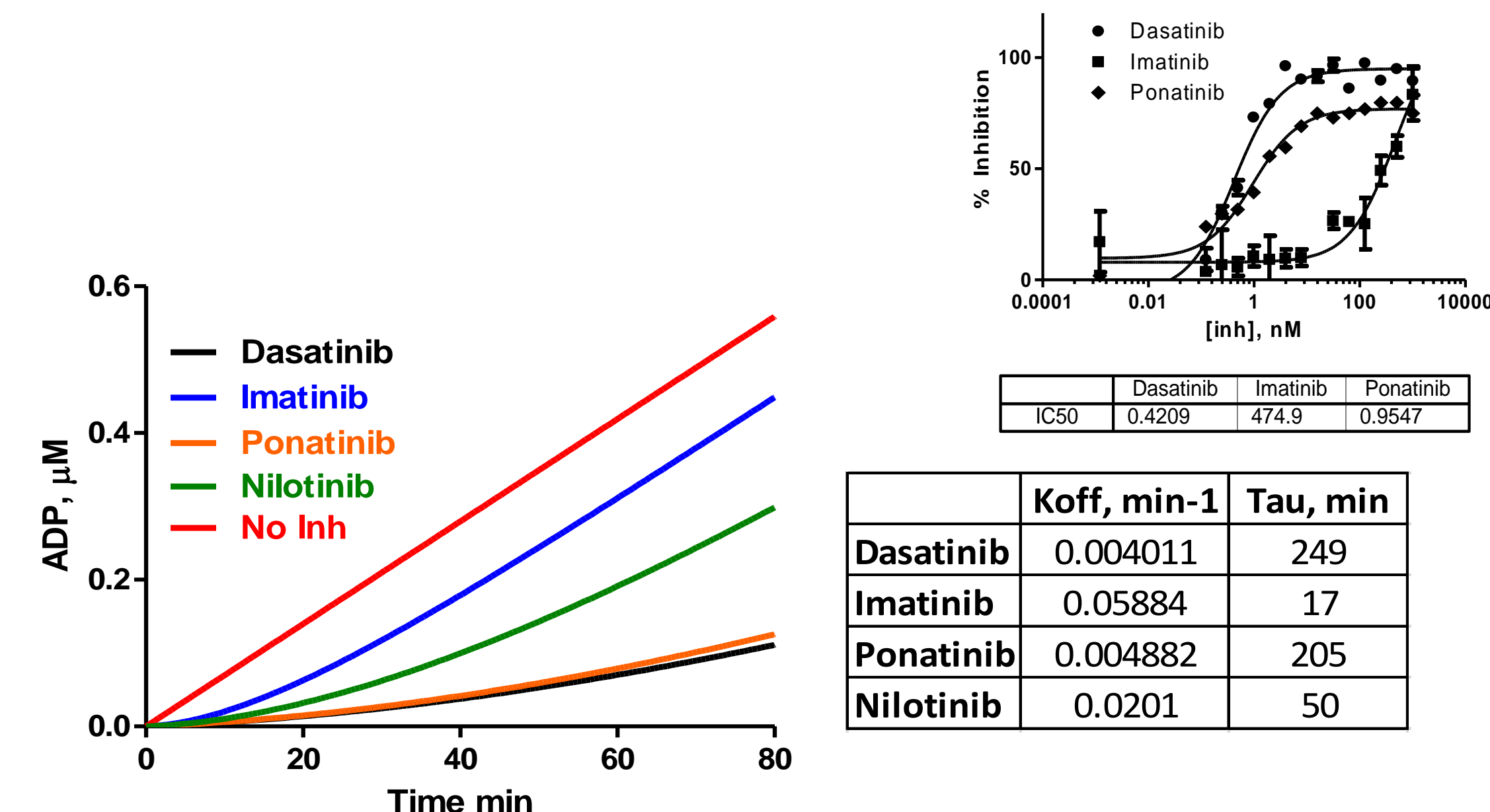
**Figure 7. BellBrook's instrument validation program** is a collaborative effort with the major suppliers of multimode readers to insure that Transcreener assays perform optimally. Scientists at BellBrook and/or the instrument vendor have tested Transcreener reagents with each instrument to determine the optimal filters and settings for maximal assay performance. The ability to determine IC<sub>50</sub> values from raw data means that all data analysis can be done with onboard software. Application notes are available with all of the important details.

## Coupled Enzyme Assay Methods are Complex and Prone to Interference



**Figure 2. Coupled enzyme assay methods for detecting ADP.** A. **ADP Glo<sup>™</sup> assay (Promega):** Residual ATP is first converted to AMP by adenyl cyclase; ADP is converted to ATP and detected by luciferase. B. **ADP Quest<sup>™</sup> assay (DiscoverX):** Two enzymatic steps are used to generate hydrogen peroxide, which reacts with Amplex Red to produce a fluorescent product in a third enzymatic step.

## Kinetic Assays Enable Measurement of Kinase Inhibitor Residence Times



**Figure 5. Determination of residence times for Abl1 inhibitors.** Residence times for Dasatinib, Ponatinib and Imatinib were determined for Abl1 in a jump dilution experiment using the Transcreener ADP<sup>2</sup> Assay. 300 nM Abl1 was preincubated with saturating concentrations of each inhibitor (see dose response curves in inset): 5 nM (Dasatinib), 5 μM (Imatinib), or 10 nM (Ponatinib). A 100-fold jump dilution was performed by adding 19.8 μL of 20 μM Abl1 and 5 μM ATP in presence of Transcreener ADP FP Assay detection reagents to 0.2 μL of the EI complex and polarization was read at intervals as shown. Using a standard curve, the raw mP was converted to product formed and fit to  $Y = V_{st} - (V_{st} - V_{s0}) / K_{off} (1 - \exp(-K_{off} t))$

## Conclusions

- The Transcreener ADP<sup>2</sup> 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 streamlines assay development and allows measurement of inhibitor residence times.
- The high sensitivity and tunability of the assay enable robust kinase detection using enzyme concentrations less than 5nM, insuring accurate IC<sub>50</sub> values for potent inhibitors and lowering costs.
- The linear correlation between IC<sub>50</sub> values determined from raw fluorescence data and from ADP formation allows accurate determination of inhibitor potencies without the use of standard curves.
- All of the Transcreener ADP Assays have been validated on the major multimode readers, including determination of optimal filters and settings, to insure seamless use and high quality results.