ADP Detection in FP, TR-FRET and FI Assay Formats: Versatile Kinase/ATPase Detection without the Use of Coupling Enzymes

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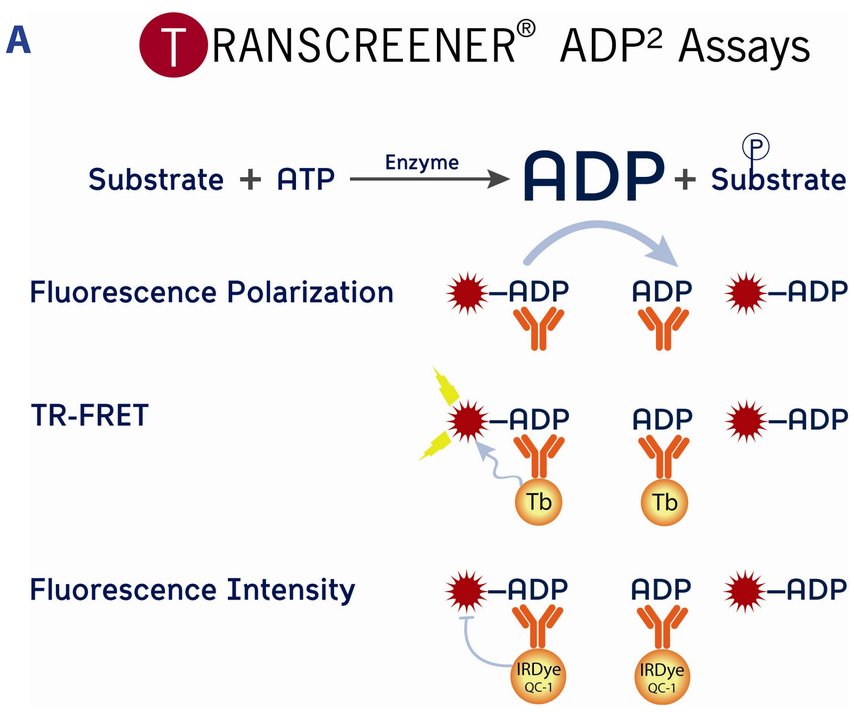
Abstract

The Transcreener ADP² Assay is a single addition, homogenous assay method that directly measures ADP formation in kinase or ATPase reactions. The first generation Transcreener ADP assay, introduced in 2006 as a far red fluorescence polarization (FP) assay, was recently improved by incorporating a new antibody (ADP²) that enables a 20-fold increase in sensitivity. Together these Transcreener ADP assays have been validated for high throughput screening with diverse targets including protein, lipid and carbohydrate kinases, carboxylases, and chaperonin ATPases. To extend the instrument compatibility for the ADP2 assay, we have now formatted it for two additional red fluorescence detection modes, time resolved Forster resonance energy transfer (TR-FRET) and fluorescence intensity (FI). Here we showcase the sensitivity and robustness of the three Transcreener assays and compare them with other nucleotide detection assay methods. With each of the Transcreener assay formats, Z' values greater than 0.7 were observed at less than 10% ATP conversion over the full range of initial ATP concentrations (0.1µM to 1mM ATP). Similar EC_{50} values and inhibitor profiles for PKA were also observed with the three Transcreener detection modes demonstrating the equivalence of the assays for inhibitor profiling.



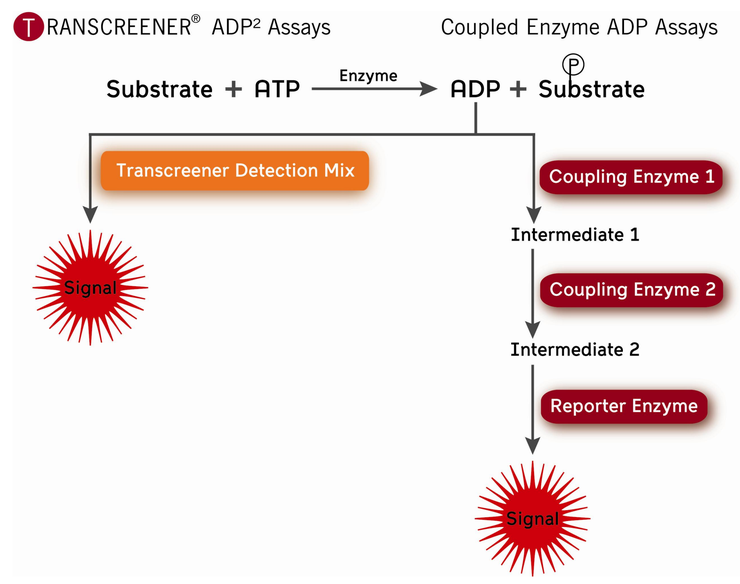


Figure 1. The Transcreener ADP² Assays are competitive immunoassays that directly measure enzymatically generated ADP. A) Transcreener is the simplest, most direct ADP detection method available: binding of ADP to antibody displaces a tracer, causing a change in its fluorescence properties . The assay has been formatted for three detection modes: Fluorescence polarization, TR-FRET and Fluorescence intensity. B) Other ADP or ATP detection methods use coupling enzymes to convert ADP to a product that can be detected as well as reporter enzymes, to generate a signal. Each coupling and reporter enzyme is a potential target for the compounds being screened, which increases the risk of false positives or of missing a hit. Some coupled methods require extra liquid addition and incubation steps, which complicates assay automation.

Sensitive Initial Velocity Detection over a Broad Range of ATP

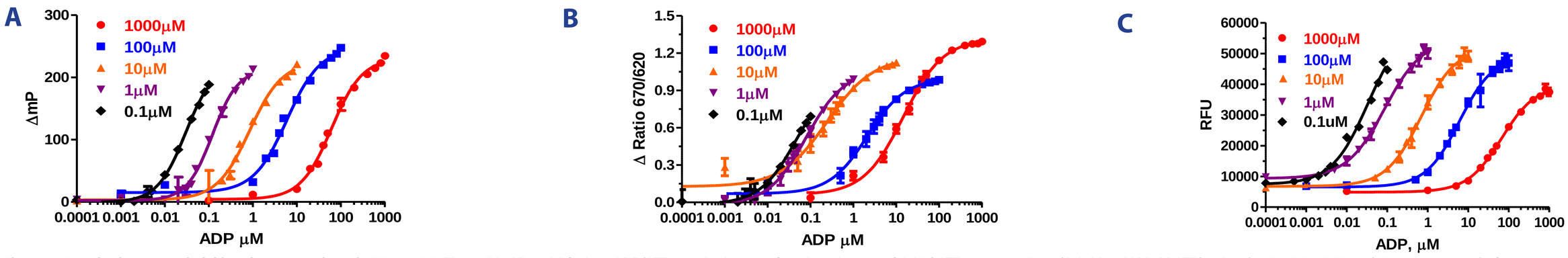


Figure 2. Standard curves mimicking the conversion of ATP to ADP. Figure 2A, 2B and 2C show ADP/ATP standard curves for a broad range of initial ATP concentrations (0.1µM to 1000µM ATP) using the FP, TR-FRET and FI assay respectively. ADP was increased as total ATP + ADP was held constant. Antibody was adjusted based on a linear relationship between EC85 and initial ATP. All assays were performed in 384-well plates (n=24) and read on the Tecan Safire2TM (FP), BMG Labtech PHERAstar Plus(TR-FRET), or the Perkin Elmer EnVision (FI) multimode plate readers.

Greater Sensitivity than Luciferase Based ATP or ADP Detection

	1µM ATP/ADP standard curve		10µM ATP/ADP standard curve		100µM ATP/ADP standard curve	
	Z' at 10% ATP Conv	*LLD (µM)	Z' at 10% ATP Conv	*LLD(µM)	Z' at 10% ATP Conv	*LLD(µM)
Transcreener ADP² FP	0.86	0.020 ± 0.07	0.85	0.01 ± 0.12	0.89	1 ± 0.3
Transcreener ADP² TR-FRET	0.71	0.03 ± 0.06	0.72	0.1 ± 0.09	0.72	1 ± 0.25
Transcreener ADP² FI	0.92	0.030 ± 0.01	0.88	0.05 ± 0.04	0.92	0.5 ± 0.37
Luc- ADP Detection Assay	ND	0.4 ± 0.87	0.3	0.5 ± 0.32	0.62	5 ± 0.67
Luc-ATP Depletion Assay	ND	0.25 ± 0.4	ND	1.5 ± 0.3	0.52	7 ± 0.56

Table 1. Z` values and Lower Limits of Detection for ADP in the Presence of Excess ATP
Each of the Transcreener ADP² Assays show robust Z' at low ADP concentrations (10% ATP conversion) with a 20-fold better lower limit of detection to luciferase ADP detection methods. The Transcreener ADP² Assays consistently show Z' > 0.7 at 10% ATP conversion for 1µM, 10µM and 100µM ATP/ADP standard curves (shown highlighted). A Z'> 0.5 is considered a robust assay. *LLD=Lower Limit of Detection, defined as the concentration of ADP that generates Z'>0.

More Sensitive ADP Detection Minimizes Enzyme Usage

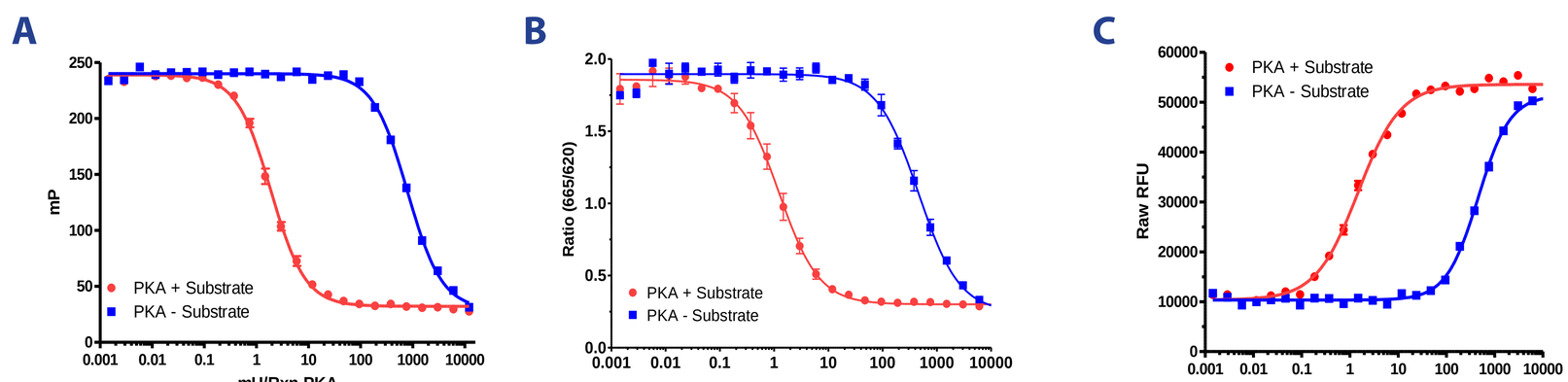


Figure 3. PKA Enzyme Titrations. Excellent signal is demonstrated at low [enzyme] for each of the assay readouts. PKA generates 400-times more ADP in the presence of substrate(productive phosphorylation) than in its absence (intrinsic ATP hydrolysis). A) FP EC_{50}values = 1.9 and 793 mU/RXN with and without substrate. B) TR-FRET EC_{50} values = 1.2 and 458 mU/RXN with and without substrate. C) FI EC_{50} values = 1.5 and 454 mU/RXN with and without substrate. Detection Mixture (10 µL) was added and reactions equilibrated for one hour before reading the final 20 µL assay volume.

Accurate Inhibitor Pharmacology with All Three Readouts

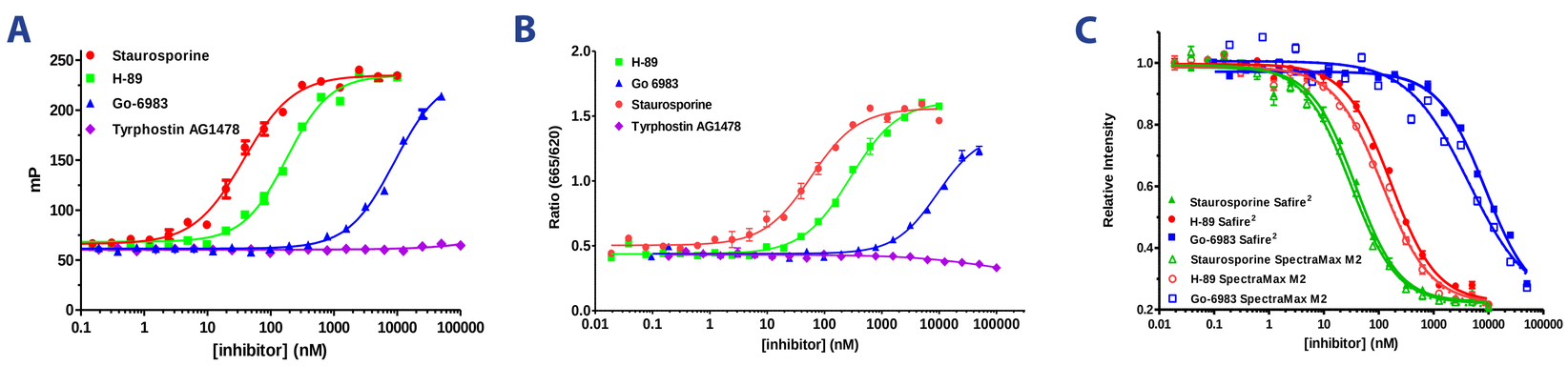


Figure 4. Inhibition curves with known inhibitors and PKA. Dose dependency curves were generated for Staurosporine, H-89, and Go-6983 (known PKA inhibitors) by serially titrating into the PKA reaction conditions. A) For FP, IC_{50} values for staurosporine, H-89 and Go-6983 were 37 nM, 179 nM and 9.0 µM respectively. B) For TR-FRET, IC_{50} values for staurosporine, H-89 and Go-6983 were 58 nM, 283 nM and 9.3 µM respectively. C) Similar IC_{50} values are shown using both instruments (Safire2/SpectraMax M2) for staurosporine, H-89 and Go-6983 were 37/32 nM, 165/124 nM and 9.0/4.7 µM respectively.

Conclusions

1) All three ADP2 Assays are capable of robust, initial velocity ADP detection (≥ 9:1 ATP:ADP ratio) with Z' values greater than 0.7 using total (ATP + ADP) concentrations ranging from 0.1µM to 1,000µM.

2) The lower limits of detection for ADP in the presence of 1µM total (ATP + ADP) are 20nM, 30nM and 30nM for the FP, TR-FRET and FI assays, respectively.

3) Compared to luciferase-based ATP and ADP detection, the Transcreener ADP² Assays exhibited significantly higher Z' values for initialvelocity detection and greater sensitivity (LLD) at all (ATP + ADP) levels tested.

4) Sensitivity of detection of PKA enzyme activity was similar with the three Transcreener ADP2 Assays: EC_{50} values of 1.9, 1.2, and 1.5 mU per 10µl enzyme reaction were observed for the FP, TR-FRET and FI assays respectively.

5) Similar potencies were observed for known inhibitors of PKA using all three ADP² assays.

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Transcreener HTS Assays- The direct route to drug discovery.

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