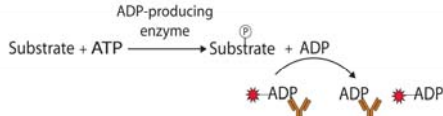


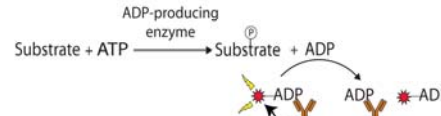
Introduction to the Transcreener® ADP² Assays

The Transcreener® ADP² Assay allows the facile detection and screening of established drug targets including protein and lipid kinases, as well as other ATPases by directly measuring ADP formation. BellBrook Labs extends the robust Transcreener technology from far red fluorescence polarization (FP) to include two other red emission spectrum detection modes including time resolved fluorescence resonance energy transfer (TR-FRET) and fluorescence intensity (FI). The result is compatibility with all major instrument platforms providing the flexibility to move from assay development to high throughput screening using a single assay platform. Validated in multiple microplate well formats, the ADP² Assays are compatible with most plate readers commonly found both in academic labs and high throughput screening settings. Each Transcreener ADP² Assay employs BellBrook Labs' monoclonal ADP² antibody which has >100-fold selectivity for ADP vs. ATP providing generic ADP detection from at least 0.1 μM to 100 μM ATP at initial velocity conditions (<10% ATP conversion). The single step assay directly detects ADP formed in the enzyme reaction which eliminates false positives seen with technologies that use coupling enzymes prior to detection of ADP. Here, we provide a new EZ protocol which shows a linear relationship between [ATP] and [ADP² Antibody]. Simply perform the enzyme titration and detect ADP in one step. Furthermore, we compare dose-dependency curves for protein kinase A (PKA) using known inhibitors for each detection mode.

Transcreener® ADP² FP Assay far red FP



Transcreener® ADP² TR-FRET Red Assay



Transcreener® ADP² FI Assay

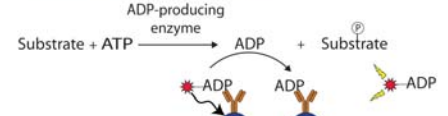


Figure 1. The Transcreener ADP² Assays are generic competitive immunoassays which follow the progress of enzymes that produces ADP. The monoclonal antibody-bound tracer is displaced by ADP, the invariant product of all kinase reactions. With each Transcreener Assay, red tracers minimize interference from fluorescent compounds and light scattering. **A)** The Transcreener ADP² FP Assay comprises an AlexaFluor 633 ADP² Tracer bound to the ADP² Antibody. The displaced tracer freely rotates in solution leading to a decrease in fluorescence polarization, relative to bound tracer. Production of ADP is proportional to a decrease in polarization. **B)** The Transcreener ADP² TR-FRET Assay comprises ADP² HiLyte647™ Tracer bound to ADP² Antibody. The displaced tracer no longer participates in FRET leading to a decrease in the 665 to 620 nm emission ratio, relative to bound tracer. Therefore, ADP production creates a concomitant decrease in the FRET dependent emission ratio. The time-gated nature further minimizes interference from fluorescent compounds and light scatter. **C)** The Transcreener ADP² FI Assay comprises a quenched Alexa594 Tracer bound to the ADP² Antibody conjugated to an IRDye® QC-1 quencher licensed from LI-COR®. The displaced tracer becomes unquenched in solution leading to a positive increase in fluorescence intensity. Therefore, ADP production is proportional to an increase in fluorescence.

An EZ Format Provides a Simple Way to Get Started With Any [ATP]

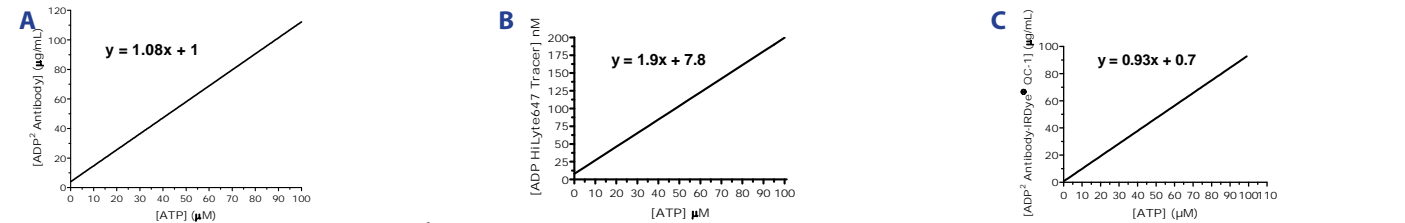


Figure 2. The EZ protocol simplifies getting started. The Transcreener ADP² Assay allows detection of ADP in the presence of excess ATP (assuming initial velocity enzyme reaction conditions) using an antibody with a finite selectivity for the diphosphate vs. the triphosphate. However, the linear relationship shown here provides an easy method to determine [Detection Reagent] at any [ATP]. **A)** For FP, the relationship between [ATP] and [ADP² Antibody] is linear, the quantity of ADP² Antibody for any [ATP] can be determined using equation of line ($y = 1.08x + 1$; where $x = [ATP]$ in the enzyme reaction, $y = [ADP^2 \text{ Antibody}]$ in the ADP Detection Mixture). **B)** For TR-FRET, the relationship between [ATP] and [ADP² HiLyte647™ Tracer] is linear, the quantity of ADP Tracer for any [ATP] can be determined using equation of line ($y = 1.9x + 7.8$; where $x = [ATP]$ in the enzyme reaction, $y = [ADP \text{ HiLyte647™ Tracer}]$ in the ADP Detection Mixture). **C)** For FI, the relationship between [ATP] and [ADP² Antibody-IRDye® QC-1] is linear, the quantity of ADP² Antibody-IRDye® QC-1 for any [ATP] can be determined using equation of line ($y = 0.93x + 0.7$; where $x = [ATP]$ in the enzyme reaction, $y = [ADP^2 \text{ Antibody-IRDye}^{\circledR} \text{ QC-1}]$ in the ADP Detection Mixture).

Excellent Sensitivity and Signal Stability at Initial Rate Conditions

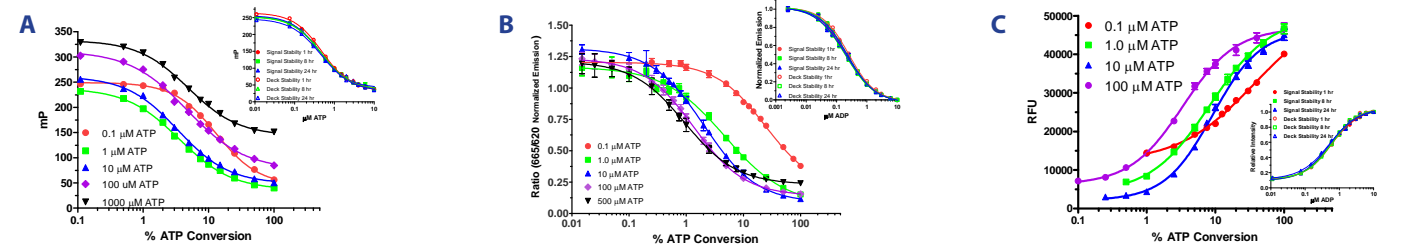


Figure 3. The robust assay provides a large signal at low percent ATP conversion with excellent Z'. Also, the assays provide excellent reagent and signal stability (inset) shown for both signal and deck stability of up to 24 hours at room temperature using a 10 μM ATP/ADP standard curve. A) Standard curves for FP readout shown for 0.1, 1.0, 10, 100, and 1000 μM ATP. Excellent Z' values of >0.7 were achieved at 3% ATP conversion for 1 μM, 10 μM, 100 μM, and 1000 μM ATP and 5% conversion for 0.1 μM ATP. **B)** Standard curves for TR-FRET readout shown for 0.1, 1.0, 10, 100, and 500 μM ATP. Excellent Z' values of >0.5 were achieved at 2.5% ATP conversion for 10 μM, 100 μM, and 500 μM ATP; 5% ATP conversion for 1 μM ATP; 10% ATP conversion for 0.1 μM ATP. **C)** Standard curves for FI readout shown for 0.1, 1.0, 10, and 100 μM ATP. Excellent Z' values of >0.7 were achieved at 1% ATP conversion for 10 μM, and 100 μM ATP and 5% conversion for 0.1 μM and 1 μM ATP.

PKA Titrations: Minimize Enzyme Requirements

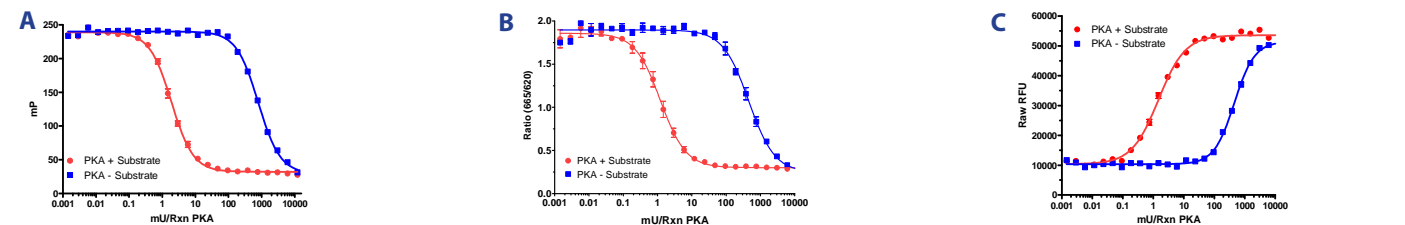


Figure 4. The high sensitivity of the Transcreener ADP² Assays minimizes enzyme costs. Graphs show PKA is capable of catalyzing ATP hydrolysis with 400-fold less efficiency, relative to productive phosphorylation of substrate. PKA was titrated (1:2) in a 10 μL assay volume with 10 μM ATP and 50 μM kemptide substrate (with or without substrate) for one hour. Detection Mixture (10 μL) was added and reactions equilibrated for one hour before reading. Enzyme EC₅₀ values show that excellent signal at low [enzyme] for each of the readouts. **A)** For FP, EC₅₀ values = 1.9 and 793 mU/RXN with and without substrate. **B)** For TR-FRET, EC₅₀ values = 1.2 and 458 mU/RXN. **C)** For FI, EC₅₀ values = 1.5 and 454 mU/RXN.

Inhibitor Dose Dependency Curves: Consistent Data for Each Transcreener ADP² Assay

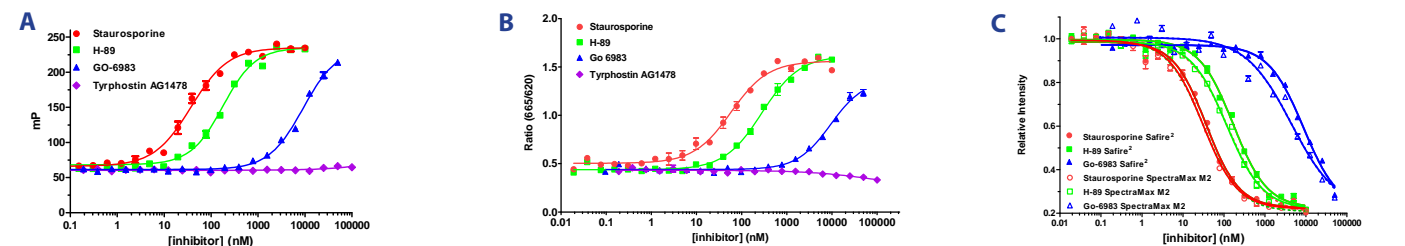


Figure 5. Accurate pharmacology is shown for each Transcreener ADP² Assay. Dose dependency curves were generated for known PKA inhibitors by serially titrating into the PKA reaction conditions. **A)** For FP, IC₅₀ values for Staurosporine, H-89 and GO-6983 were 37 nM, 179 nM and 9.0 μM respectively. **B)** For TR-FRET, IC₅₀ values for Staurosporine, H-89 and GO-6983 were 58 nM, 283 nM and 9.3 μM respectively. **C)** For FI, similar IC₅₀ values are shown using commonly used HTS and academic plate reader 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

Transcreener ADP² Assays: Three detection modes. All major instrument platforms. Generic ADP assay for easy development to HTS. The Transcreener ADP² Assay is now offered in red emission spectra readouts for FP, TR-FRET, and FI readouts allowing compatibility with all instrument platforms. Transcreener assays directly detect ADP formed in the enzyme reaction in a single step, which eliminates false positives seen with technologies that use coupling enzymes prior to ADP detection. The new monoclonal ADP² antibody has increased sensitivity and greater than 100-fold selectivity over ATP which enables its use from 0.1 μM to 1 mM ATP.