# Demonstration of the Next Generation Transcreener® ADP² FP Assay on the Tecan Safire²™ Microplate Reader

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## Overview

We present instrument validation data and enzyme application data in 384-well format using BellBrook Labs' Transcreener® ADP² FP Assay and the Tecan Safire²™ microplate reader. The Transcreener ADP² Assay is a universal, second generation far red fluorescence polarization assay that detects the ADP nucleotide produced in most ATP-utilizing enzyme reactions. The increased sensitivity of the new ADP² antibody allows for detection of ADP at less than 10% ATP consumption, over broader initial ATP concentrations ranging from 0.1 µM to 1,000 µM. Data generated with a standard curve on the Safire² met BellBrook Labs' Instrument Validation Program criteria, which include Z' > 0.7 with Δ mP shift ≥ 95 at 10% conversion of 10 µM ATP to ADP. Optimized instrument settings are provided, allowing the researcher to quickly utilize the assay with their Tecan reader. In addition, we use the Protein Kinase A (PKA) enzyme as a model system to show utility of the assay at ATP concentrations ranging from 0.1 µM to 1,000 µM ATP using one set of reagents.

## Introduction

BellBrook Labs has developed a universal assay technology called Transcreener® HTS Assay Platform that enables seamless incorporation of hundreds of new drug targets into HTS. The Transcreener HTS platform relies on a proprietary fluorescence detection method for group transfer enzymes that enables an entire family of enzymes to be screened using the same detection reagents. The Transcreener ADP² FP Assay is a universal, second generation far red fluorescence polarization assay that detects the ADP nucleotide by-product from any ATP-utilizing enzyme reaction. The increased sensitivity of the new ADP² antibody allows for detection of ADP at less than 10% ATP consumption, or at initial velocity enzyme reaction rates, and at broader ATP concentrations ranging from 0.1 µM to 1,000 µM. The improvement in sensitivity of the ADP² antibody over the previous Transcreener ADP antibody allows the researcher to expand their high throughput screening capabilities to low activity enzymes or those possessing low ATP $K_m$.

A critical factor in achieving success with an assay signal is to ensure that the microplate reader is compatible with the assay. In this study, we evaluated the Tecan Safire² instrument's excitation and emission wavelengths, number of flashes per well, and the delay time between well read using a 10 µM ADP/ATP standard curve. From this curve we determined Z'-factor and mP shift at 10% ATP conversion. We show that data generated with a standard curve in 384-well format on the Safire² meet BellBrook Labs' Instrument Validation Program criteria, which include Z' > 0.7 with Δ mP shift ≥ 95 at 10% conversion of 10 µM ATP. The attributes of the new Transcreener Assay were demonstrated by performing PKA titrations over the range of initial ATP concentration (0.1 µM to 1,000 µM), with the convenience of using one set of Transcreener ADP² reagents.

## The Transcreener® ADP² FP Assay

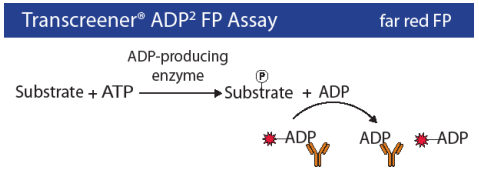


**Figure 1. The Transcreener ADP² Assay.** The Transcreener ADP² Assay was developed to follow the progress of any enzyme that produces ADP. The Transcreener ADP Detection Mixture comprises an ADP Alexa633 Tracer bound to an ADP² antibody. The tracer is displaced by ADP, the invariant product generated during an enzyme reaction. The displaced tracer freely rotates in solution leading to a decrease in fluorescence polarization. Therefore, ADP production is proportional to a decrease in polarization. The far red tracer minimizes interference from fluorescent compounds and light scattering.

## The Tecan Safire²™ Microplate Reader

**Figure 2. The Safire²™ Microplate Reader.** Safire²™ can handle microplate formats with up to 1536-wells with high speed in every detection mode. This reader is a fully modular microplate detection system based on the quad-4 monochromator quad-4 technology that offers a range of high speed detection techniques. Safire²™ eliminates the need for cumbersome filter changes. The autofocus feature automatically adjusts the optics to the assay volume, microplate height or well shape. The modular concept of Safire²™ allows upgrades to new detection modes at any time. Detection modules are available for top and bottom fluorescence intensity measurements, fluorescence polarization studies and multi-channel absorbance and luminescence measurements. Quasi-simultaneous readouts in FP allows high speed measurements. For FP, we used the following optimized wavelengths: excitation 635 nm, emission 670/20 nm bandwidth.

## Assay Validation at 10% ATP Conversion

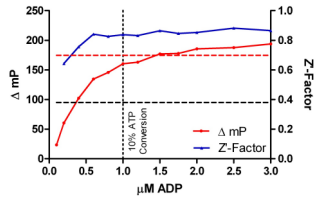


**Figure 3. The Safire²™ meets BellBrook Labs' Instrument Validation Criteria.** The validation criteria include: Z´ ≥ 0.7 at 10% ATP conversion of 10 µM ATP, a Δ mP shift ≥ 95 mP at 10% conversion and read time ≤ 5 minutes. The assay passed validation under the following conditions: 1 flash per well, 50 ms delay between move and read, resulting in a Z´= 0.84, Δ mP of 161 in 57 seconds.

## ADP² Antibody Titrations at Various ATP Concentrations

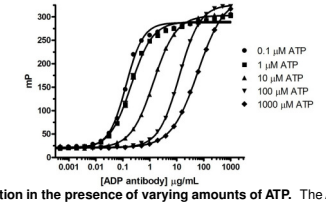


**Figure 4. Antibody titration in the presence of varying amounts of ATP.** The ADP² antibody has a finite selectivity for the nucleotide diphosphate (ADP) vs. the nucleotide triphosphate (ATP). The concentration of antibody determines the total assay window and the ADP detection range, and the amount needed is dependent on the ATP concentration in the assay. As a result, antibody must be titrated in the reaction system and ATP concentration desired in the assay. ADP² antibody was titrated as shown. The final 20 µL volume consisted of 2 nM ADP Alexa633 tracer, 0.5X Stop & Detect Buffer B, 0.5X enzyme reaction mixture (50 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.01% Brij-35, 0.5X ATP), and ADP² antibody. The $EC_{85}$ antibody concentration is determined, and that concentration used in an ADP/ATP standard curve. $EC_{85} = ((85/(100-85))^{1/hillslope})*EC_{50}$.

## ADP/ATP Standard Curves

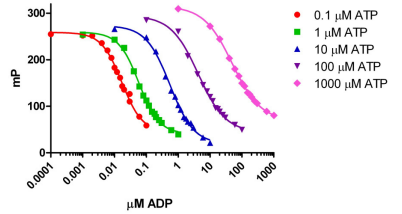


**Figure 5. ADP/ATP standard curves mimic an enzyme reaction.** In an enzyme reaction, ATP concentration decreases as ADP concentration increases; adenine concentration remains constant. Standard curves were run using the $EC_{85}$ antibody concentration. The final concentration in the 20 µL reaction consisted of 2 nM ADP Alexa633 tracer, 0.5X Stop & Detect Buffer B, and 0.5X enzyme reaction mixture (25 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.005% Brij-35, 0.5X ATP). The $EC_{85}$ antibody concentrations for each ATP concentration are as follows: 0.4 µg/mL ADP² antibody (0.1 µM ATP); 1 µg/mL ADP² antibody (1 µM ATP); 9 µg/mL ADP² antibody (10 µM ATP); 58 µg/mL ADP² antibody (100 µM ATP); 535 µg/mL ADP² antibody (1,000 µM ATP).

## PKA Titrations

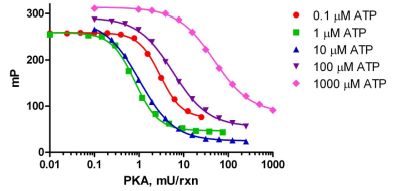


**Figure 6. PKA titrations at varying concentrations of ATP.** PKA serial titrations (1:2) were performed in a 10 µL reaction with the ATP concentrations indicated above and kemptide substrate at 5X ATP concentration. Reactions were incubated for one hour at room temperature. An equal volume of detection reagent was then added and reactions equilibrated for one hour before reading. The concentration of components in the final 20 µL reaction were as noted previously. Enzyme concentrations that resulted in 10% ATP conversion (interpolated from an ADP/ATP standard curve at each ATP concentration) were as follows: 3 mU/rxn (0.1 µM ATP); 1 mU/rxn (1 µM ATP); 2 mU/rxn (10 µM ATP); 16 mU/rxn (100 µM ATP); 125 mU/rxn (1,000 µM ATP).

## Conclusions

- For optimal performance of the Transcreener ADP² Assay on the Tecan Safire² reader, we recommend the following: excitation wavelength 635 nm, emission wavelength 670 nm with a 20 nm bandwidth.
- The Safire² passes BellBrook Labs' Validation Program under the following conditions: 1 flash, 50 ms delay between move and read. These settings yielded a Z´ > 0.7 in 57 seconds.
- The Transcreener ADP² FP Assay can be used with a wide range of initial ATP concentrations, simplifying assay optimization and reducing reagent cost.
- The Transcreener Assay can detect ADP production from an enzyme reaction within initial velocity rates (≤ 10% ATP conversion) using ATP concentrations from 0.1 µM to 1,000 µM.

For more information on Transcreener validation on Tecan readers, or for a copy of this poster, please visit Tecan at booth # 205.


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