Transcreener® ADP² TR-FRET Red Assay: Homogeneous ADP Detection

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Introduction

High-throughput screening (HTS) is a cornerstone of drug discovery research enabling rapid identification of high-value, pharmacologically important compounds. HTS groups bring to bear a wide selection of tools and methods to this effort efficiently bringing robust, biologically useful, information to light. As these groups strive to improve efficiency and biological relevance, nucleotide detection has become an important part of the HTS arsenal as a means to monitor the activity of multiple drug targets using native, unlabeled-substrates with a single set of reagents.

In this study the utility of ADP detection as a means to screen inhibitor potency using a far-red, time-resolved Förster resonance energy transfer (TR-FRET) format is described. This assay uses the widely adopted TR-FRET technique to take advantage of low compound interference common to this method and owed to its time-gated nature as signal is measured after most background fluorescence has subsided. This assay has proven both sensitive and robust with Z´>0.7 at low substrate conversion levels enabling enzymology during initial velocity conditions and is an important addition to the HTS toolkit.

The Transcreener® ADP² TR-FRET Red Assay

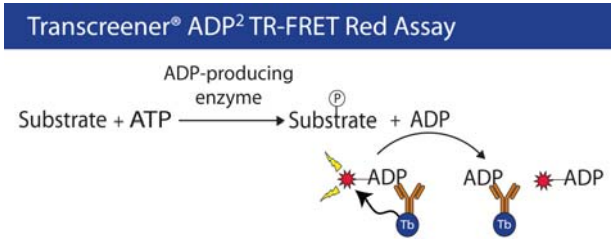

Figure 1. The Transcreener ADP² TR-FRET Red Assay. The Transcreener ADP² TR-FRET Red Assay was developed to follow the progress of any enzyme that produces ADP. The Detection Mixture comprises ADP HiLyte647™ Tracer bound to ADP² Antibody-Tb, which is displaced by ADP, the product generated by multiple enzyme families. 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 of this assay and use of a far-red tracer minimizes interference from fluorescent compounds and light scatter.

Conclusions

- The Transcreener ADP² TR-FRET Red Assay enables rapid and facile pharmacological studies of ADP producing enzymes using native, unlabeled substrates under initial velocity conditions.
- One set of reagents detected nanomolar levels of ADP with initial ATP concentrations ranging from 0.1 to 100 µM.
- Robust assay signal is stable from 1 to 24 hours.
- Enzyme and substrate dependent signal were demonstrated.
- Dose-dependent inhibition was observed.

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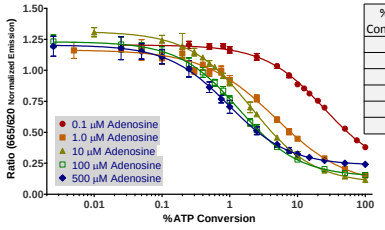
Facile and Sensitive ADP Assay

Adenosine Concentration µM	Recommended ADP HiLyte647 Tracer Concentration nM
0.1 to 1.0	4
10	20
100	100
500	250

Table 1. EZ Protocol Enables Out of the Box Use. The predictable and reproducible relationship between ADP HiLyte647 Tracer and ATP concentrations provided the opportunity to develop a tracer selection table. This table provides researchers with the means to begin use of this assay immediately upon receipt by cross-referencing their ATP requirement with the related tracer concentration. This table is accurate over a wide range of assay conditions, however in the case of extreme assay requirements a simple one-step tracer optimization procedure is available.



% ATP Conversion	Adenosine Concentration (µM)				
	0.1	1	10	100	500
1	N/A	N/A	0.53	0.48	0.19
2.5	N/A	0.29	0.77	0.65	0.51
5	0.09	0.54	0.83	0.77	0.62
10	0.58	0.67	0.80	0.80	0.68
25	0.79	0.74	0.89	0.82	0.71
100	0.90	0.79	0.90	0.84	0.70

Figure 2. Excellent Z´ Values Achieved at Low ATP Conversion. Standard Curves (ADP/ATP) were performed across a range of adenosine concentrations including: 0.1, 1.0, 10, 100, and 500 µM. In all cases Z´ > 0.7 were achieved with Z´ > 0.5 generated at conversion levels typical of initial velocity conditions.

Standard Assay Procedure: Signal is reported as the emission ratio of the ADP HiLyte647 Tracer (665 nm) over the ADP² Antibody-Tb emission (620 nm). Standard Curve reactions were designed to mimic a 10 µL enzyme reaction (40 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, and 1% DMSO) stopped with 10 µL Detection Mixture (1X Stop and Detect Buffer C, 8 nM ADP² Antibody-Tb, 2X ADP HiLyte647 Tracer concentrations from tracer selection table). The resulting 20 µL ADP detection reaction signal was measured in BMG LABTECH's PHERAstar *Plus* configured as follows: 100 flashes; 50 µs integration delay; 50 µs integration; and optic module: version D, $Ex_{337}/Em_{665}/Em_{620}$ resulting in 3 minute plate reads. Corning 384-well, white, round bottom, low volume plates were used (Part# 3673, sealed or covered except during measurement typically with minimal light exposure).

Robust ADP Detection

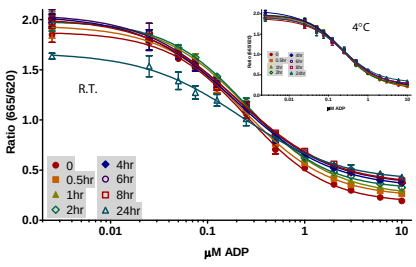


Figure 3. Excellent Deck Stability. Reproducible signal was demonstrated when using Detection Mixture (1X Stop and Detect Buffer C, 8 nM ADP² Antibody-Tb, 34 nM ADP HiLyte647 Tracer) incubated at room temperature from 1 to 8 hours prior to use in translucent 1.5 mL tubes exposed to ambient light (10 µM ADP/ATP Standard Curves were used for this evaluation). Inset demonstrates Detection Mixture stability at 4°C. Procedure and conditions were the same as figure 2 except where noted.

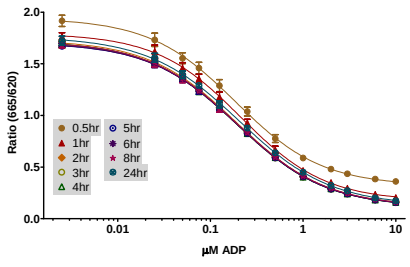


Figure 4. Signal is Stable for 24 Hours. Signal, 10 µM ADP/ATP Standard Curve, is stable from 1 to 24 hours after addition of Detection Mixture (Stop and Detect Buffer C, ADP² Antibody-Tb, ADP HiLyte647 Tracer) when held at room temperature in a high humidity chamber to reduce evaporation. Time points beyond 24 hours were not tested. Procedure and conditions were the same as figure 2 except where noted.

PKA Enzyme Titrations and Dose-Dependent Inhibitor Curves

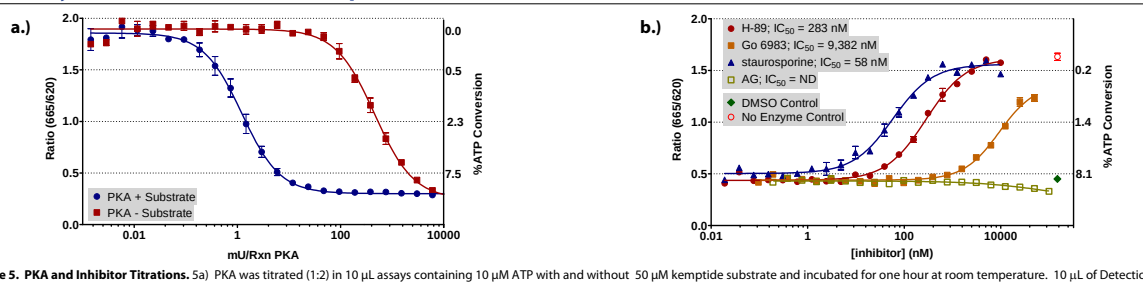


Figure 5. PKA and Inhibitor Titrations. 5a) PKA was titrated (1:2) in 10 µL assays containing 10 µM ATP with and without 50 µM kemptide substrate and incubated for one hour at room temperature. 10 µL of Detection Mixture was added with the final 20 µL ADP detection reaction incubated at R.T. for 1 hour prior to signal measurement. This final ADP detection reaction consisted of 30 nM ADP HiLyte647 Tracer, 0.5X Stop & Detect Buffer C, 4 nM ADP² Antibody-Tb with the enzyme reaction contributing: 25 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.005% Brij-35, 5 µM ATP and 25 µM kemptide. 5b) Dose dependency curves were generated using 1 mU/µL PKA. Staurosporine, H-89, and GO-6983 (known PKA inhibitors) were serially titrated into PKA reactions using the assay conditions noted in figure 5a. PHERAstar *Plus* was used to measure fluorescence as in figure 2.