The Transcreener® ADP² Assay: A Universal Kinase Assay for Both HTS and Lead Discovery

Meera Kumar, Justin Brink, and Robert G. Lowery
BellBrook Labs, Madison, WI, USA

Overview

Kinases are one of the most highly validated target families, yet only a small fraction of the kinome has been exploited therapeutically. ADP detection enables a generic assay method that has been broadly adopted for HTS efforts targeting kinases. The Transcreener ADP² Kinase assay uses homogenous detection of ADP with a choice of FP, FI, or TR-FRET readouts. Although other methods of ADP detection are available, Transcreener is the only with direct detection of ADP. Alternative assays use complex coupling mechanisms making them prone to assay interference that generate false positives and thus require time consuming counter screens to triage. The assay provides sensitive detection of kinase initial velocity over a broad range of ATP concentrations, critical for screening such a diverse class of targets. Compatibility with 1536 well-miniaturized formats along with outstanding overnight and reagent signal stability provide flexibility in liquid handling while producing data that yields dependable results. And while Transcreener's flexibility and simplicity make it an excellent fit for HTS, the assay also has distinct advantages in SAR and MOA studies. Sensitivity affords use of the assay at low enzyme concentrations and helps determine accurate IC_{50} measurements for potent inhibitors. Transcreener can also be used kinetic mode, which simplifies early stage assay development and enables high throughput assessment of drug-target residence time. Here we demonstrate the Transcreener ADP² Kinase Assay as a powerful HTS approach for discovering, evaluating, selecting, and improving small molecule kinase inhibitors.

Transcreener ADP² Assays: Direct, Homogenous ADP Detection with FP, FI, or TR-FRET Signal

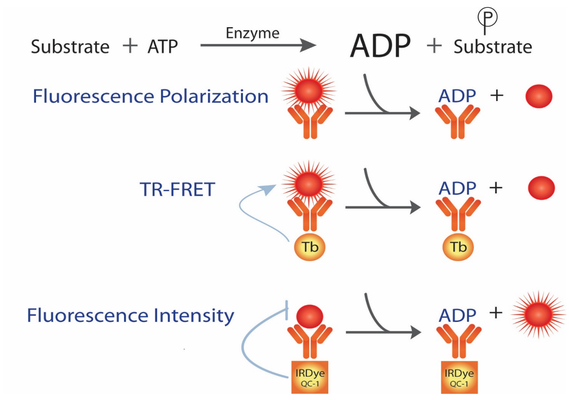


Figure 1. Transcreener Assays: the only direct detection method available for ADP. ADP displaces a fluorescent tracer from a highly specific monoclonal antibody resulting in a change in fluorescence. Three homogenous mix-and-read formats are available: fluorescence polarization (FP), time resolved FRET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

Coupled Enzyme Assay Methods are Complex and Prone to Interference

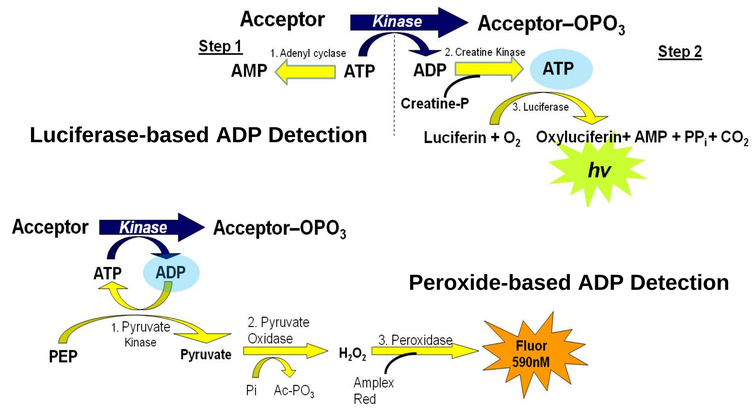


Figure 2. Coupled enzyme assay methods for detecting ADP. A. ADP Glo™ assay (Promega): Residual ATP is first converted to AMP by adenyl cyclase; ADP is converted to ATP and detected by luciferase. **B. ADP Quest™ assay (DiscoverX):** Two enzymatic steps are used to generate hydrogen peroxide, which reacts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

Sensitive Assay for Diverse Kinases Compatible on Any Plate Reader

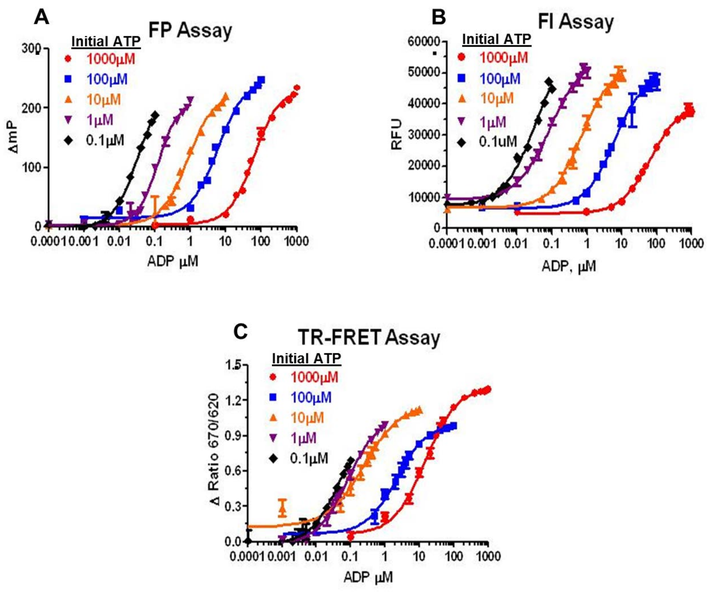


Figure 3. Standard curves for conversion of ATP to ADP demonstrate the outstanding response of the Transcreener ADP² Assays at different initial ATP concentrations. Standard curves are used to mimic enzyme reactions. Starting at the indicated concentrations of ATP, ADP is titrated and ATP is decreased proportionately. All experiments were run in 384 well format with 24 replicates. **A. FP reactions** were read in the Tecan Safire². **B. The TR-FRET reactions** were read in BMG Labtech's PHERAstar Plus **C. The FI assay** was measured using Perkin Elmer's EnVision.

High Z' Values at Low ATP Conversion

Assay	1 µM ATP/ADP Standard Curve		10 µM ATP/ADP Standard Curve		100 µM ATP/ADP Standard Curve	
	Z' at 10% Conversion	LLD (µM)	Z' at 10% Conversion	LLD (µM)	Z' at 10% Conversion	LLD (µM)
Transcreener FP	0.86	0.02 ± 0.07	0.85	0.01 ± 0.12	0.89	1.0 ± 0.3
Transcreener TR-FRET	0.71	0.10 ± 0.06	0.72	0.10 ± 0.09	0.72	1.0 ± 0.3
Transcreener FI	0.92	0.03 ± 0.01	0.88	0.05 ± 0.04	0.92	0.5 ± 0.4
Luc-ADP Detection Assay	ND	0.40 ± 0.87	0.30	0.50 ± 0.32	0.62	5.0 ± 0.7
Luc-ATP Depletion Assay	ND	0.25 ± 0.40	ND	1.50 ± 0.30	0.52	7.0 ± 0.6

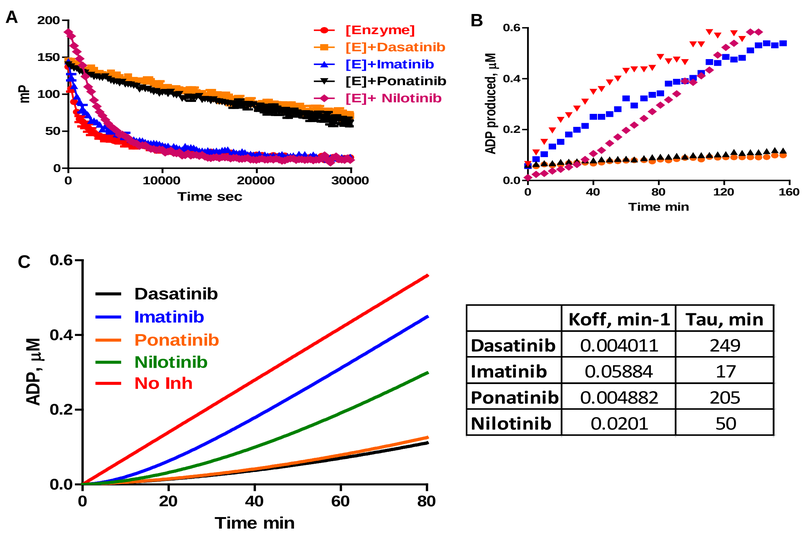
Table 1. The high sensitivity of the Transcreener ADP² Assays allows detection of 10% conversion of ATP even at low levels of ATP. Assay statistics, including Z' values and lower limits of detection (LLD) were calculated and compared with ADP-Glo and Kinase-Glo (Promega) reactions run under identical conditions.

Use of Low Enzyme Concentrations Ensures Accurate IC_{50} Values for Potent Inhibitors

Enzyme	ATP K_m	ATP (µM)	Substrate	Enzyme Concentration
PKA	5.0	1	Kemptide-KRRASKG	0.1 nM
ABL1	16.0	10	Abltide-EAIYAAPFAKKK	1.2 nM
DAPK1	1.1	1	Dapktide-KKRPQRRYNVF	2 nM
CLK1	11.0	10	MBP	5 nM
Src	31	50	Poly(G-T)	2 nM
ROCK1	3.1	1	S6 Peptide-AKRRLSSLRA	3 nM
CLK4	2.3	1	RS Peptide-GRSRSRSRSRSRSR	4 nM
Zap70	1.7	1	poly [Glu, Tyr] 4:1	2.8 nM

Table 2. Optimal reaction conditions for a panel of protein kinases. To ensure sensitive detection of ATP competitive inhibitors, kinases were used with 1, 10 and 50 µM ATP reactions concentration such that ATP ≤ K_m. In some cases, this resulted in ATP concentrations several-fold below the K_m, which slows kinase reactions proportionately. However, in all cases, an enzyme concentration of less than 5 nM enzyme (EC_{80}) produced a robust signal in the Transcreener ADP² FP Assay, insuring accurate IC_{50} measurements even for potent inhibitors.

Use the Assay in Kinetic Mode: Determining K_{off} Rates in a Jump Dilution Experiment



	Koff, min-1	Tau, min
Dasatinib	0.004011	249
Imatinib	0.05884	17
Ponatinib	0.004882	205
Nilotinib	0.0201	50

Figure 4. Determination of residence time for inhibitors. A. EI complexes were formed by pre-incubating with Abl (280 nM) with saturating inhibitor concentrations (10 x IC_{50}): 45 nM (Dasatinib), 45 µM (Imatinib), 25 µM (Nilotinib), or 100 nM (Ponatinib). EI mixtures were diluted 100-fold by adding into reactions containing 10 µM Abltide, 5 µM ATP and Transcreener ADP² detection reagents; plates were read at 5 min intervals. **B.** Raw polarization was converted into product formed (ADP) using a standard curve. **C.** Progress curves were fit to the integrated rate equation (not shown) and K_{off} values were determined using Graph Pad Prism. Residence times (Tau values) are reciprocal of K_{off} values.

Assays Compatible with 1536 Well Format for HTS

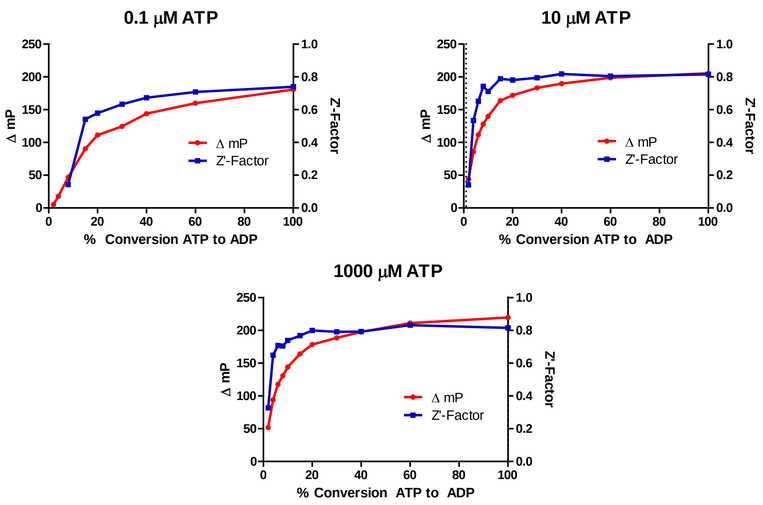


Figure 5. Z' and signal values in 1536 well-plate formats. The conversion of ATP to ADP at 0.1 µM, 10 µM, and 1000 µM ATP demonstrate the outstanding consistency of the Transcreener ADP² Assays in a miniaturized 1536 well format. Standard curves are used to mimic enzyme reactions. Starting ATP concentrations are indicated, ADP is then titrated and ATP is decreased proportionately. Z' factor and ΔmP values are shown at the respective conversion rates.

Overnight Reagent and Signal Stability

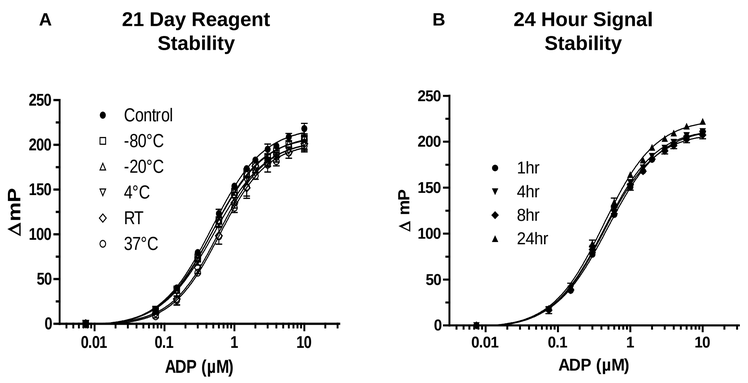


Figure 6. Overnight reagent and signal stability. Standard curves for conversion of 10 µM ATP to ADP were used to measure reagent and signal stability. **A. Stability of Transcreener detection reagents prior to addition to reactions** and **B. The stability of the signal following addition to kinase reactions.** Data is for the FP assay, the FI and TR-FRET assays also have at least overnight reagent and signal stability.

Conclusions

- **The Transcreener ADP² Assay is the simplest ADP detection method available, relying on direct immunodetection instead of coupled enzyme assays. This results in advantages over other methods with respect to compound interference, sensitivity, reagent and signal stability, and ease of use.**
- **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 greater sensitivity of the Transcreener ADP² Assay allows the practical use of ATP concentrations as low as 100 nM and reduces screening costs by minimizing enzyme consumption.**
- **The ability to run reactions in kinetic mode streamlines assay development and allows informative kinetic analyses such as measurement of inhibitor residence times.**
- **The Transcreener ADP² Assay has been can be adapted to a 1536 well format for ultra-high throughput screens.**
- **The overnight reagent and signal stability of the Transcreener ADP² Assay provides flexibility for automated HTS protocols with large numbers of plates.**

BellBrook Labs, 5500 Nobel Drive, Suite 230, Madison, WI 53711
866.313.7881 or 608.443.2400