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

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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. Transcreener is the only assay that directly detects ADP without any secondary signaling components, making it the simplest and most flexible ADP assay available. Other ADP detection methods rely on multiple coupled enzyme steps that are inherently prone to interference and require time consuming counter screens to triage false positives. Here we provide data on some of the key competitive advantages of the Transcreener ADP² Kinase Assay for HTS and examples of its use in important hit-to-lead applications, including dose response curves (IC_{50}) and inhibitor dissociation rate (k_{off}) measurements. The Transcreener ADP² assay provides sensitive detection of initial velocity using ATP concentrations from 1 μM to 1 mM, which encompasses the full range of kinase Km values. The assay is more sensitive than other ADP detection methods, especially at lower ATP concentrations, which reduces consumption of costly enzymes in HTS. Compatibility with 96, 384 and 1536 well formats and extensive validation on all major multimode readers provide flexibility with respect to equipment and throughput. Overnight reagent and signal stability at room temperature allows maximal flexibility for liquid handling and assay protocols – an important consideration in large volume screens - without compromising data quality. The availability of three detection modes allows the use of different combinations for primary and orthogonal assays. The assay also has distinct advantages in SAR and MOA studies. The high sensitivity allows accurate dose response measurements for potent inhibitors; typically kinase concentrations less than 5 nM can be used, insuring that inhibitor depletion does not impact IC_{50} values. Transcreener can also be used in kinetic mode, which simplifies assay development and provides flexibility for MOA studies; e.g., determining drug-target residence times using jump-dilution assays. In summary, the Transcreener ADP² Kinase Assay is 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

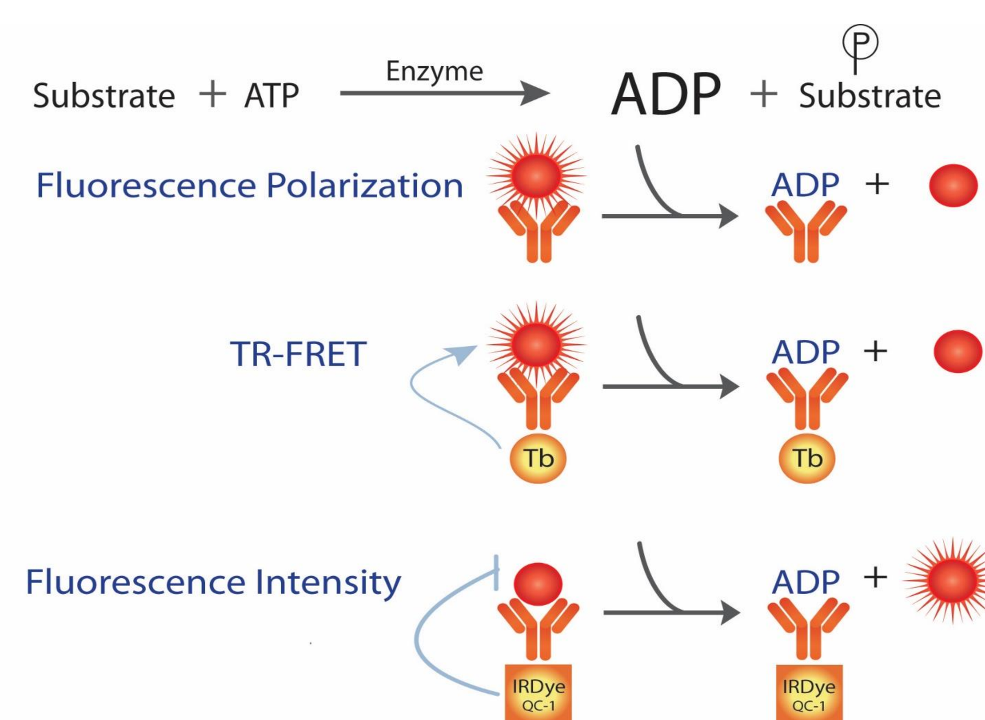


Figure 1. Transcreener Assays: the only direct detection method available for ADP. ADP displaces a fluorescent tracer from a highly specific monoclonal antibody resulting in a change in fluorescence. Three homogenous mix-and-read formats are available: fluorescence polarization (FP), time resolved FRET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

Far-Red Fluorescent Detection Exhibits Low Compound Interference in Pilot Screens

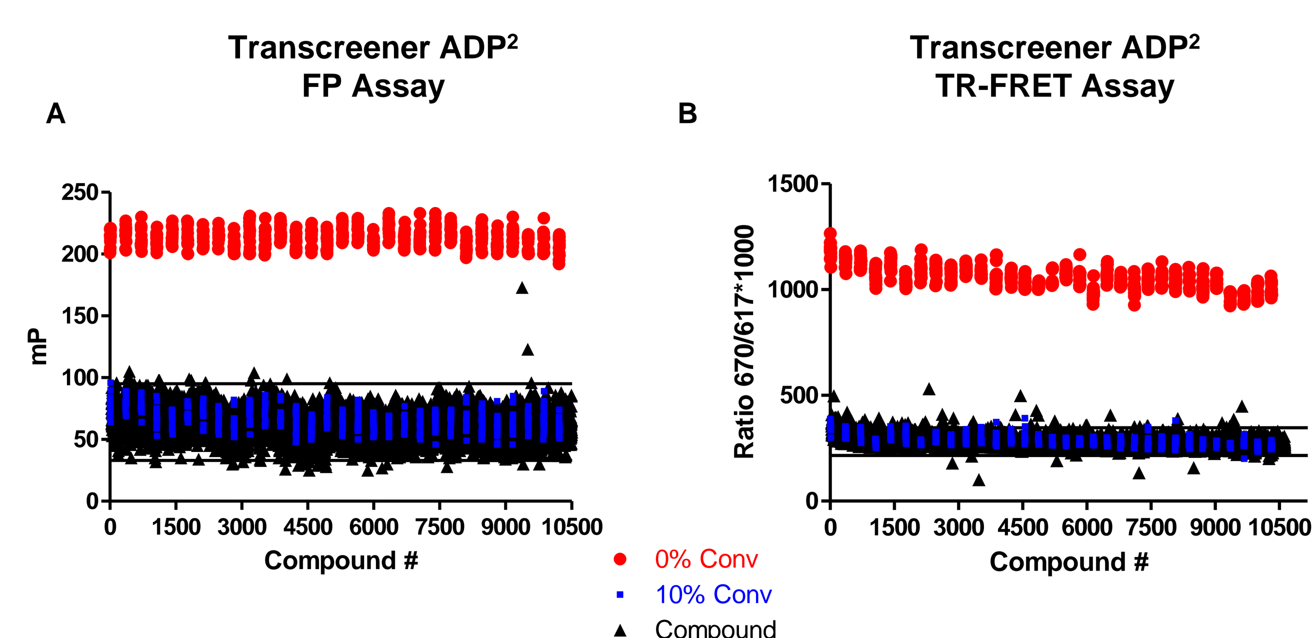


Figure 2. Compound interference from pilot screens. 9,920 lead-like diversity compounds from the ChemBridge DIVERSet were screened at 10 μM. Assay conditions for the **A. Transcreener ADP² FP** and **B. Transcreener ADP² TR-FRET Assays** were 20mM Tris (pH 7.5), 5 mM $MgCl_2$ 0.01% Brij. 10 μL mock reaction +/- compound was followed by adding 10 μL Transcreener Detection Mix and incubating for 60 minutes. Negative control mimics 0% conversion with no ADP and 10 μM ATP. The positive control mimics 10% conversion of ATP to ADP.

Tunable Dynamic Range Allows Full Range of Physiological ATP Concentrations

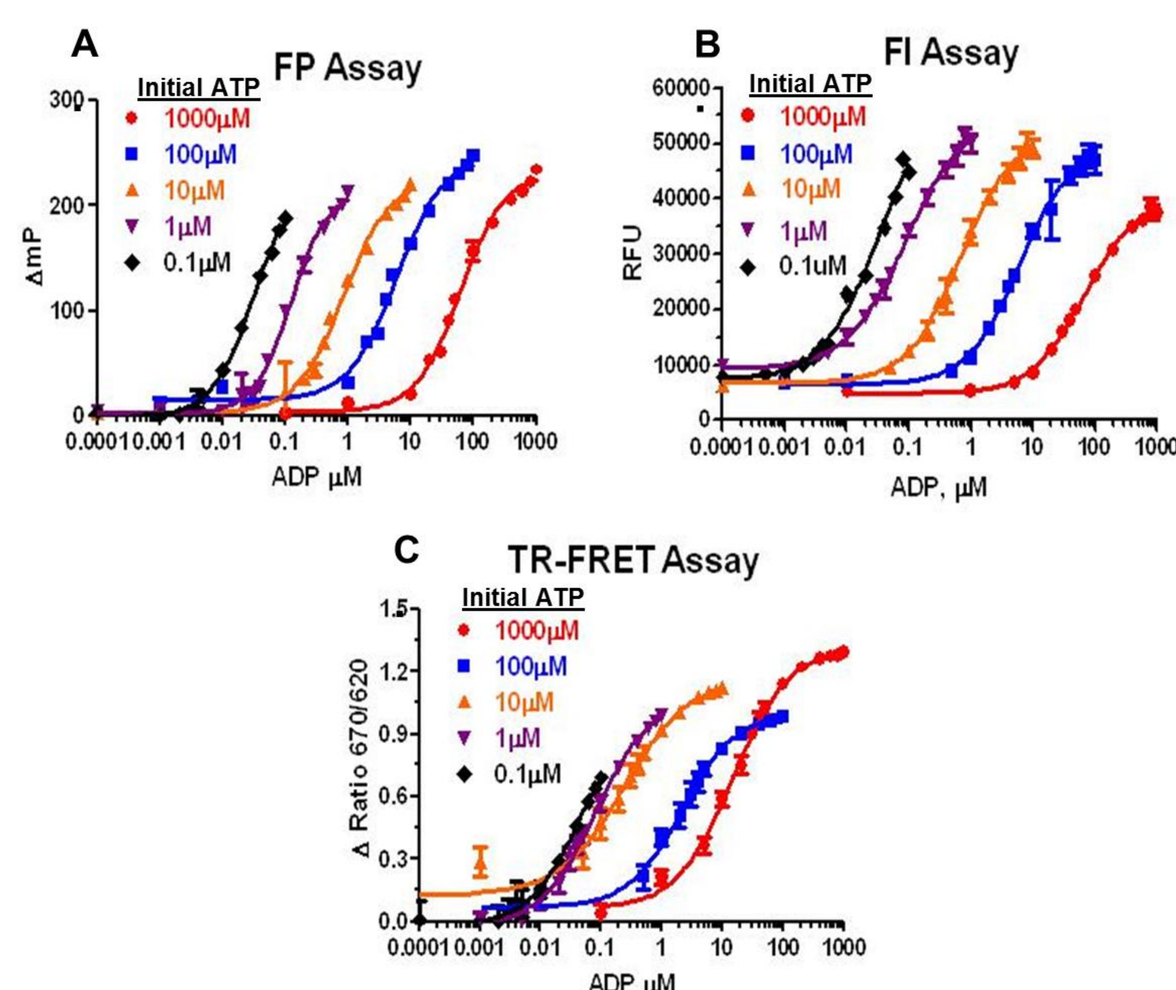


Figure 3. Standard curves for conversion of ATP to ADP demonstrate the outstanding response of the Transcreener ADP² Assays at different initial ATP concentrations. Standard curves are used to mimic enzyme reactions. Starting at the indicated concentrations of ATP, ADP is titrated and ATP is decreased proportionately. All experiments were run in 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

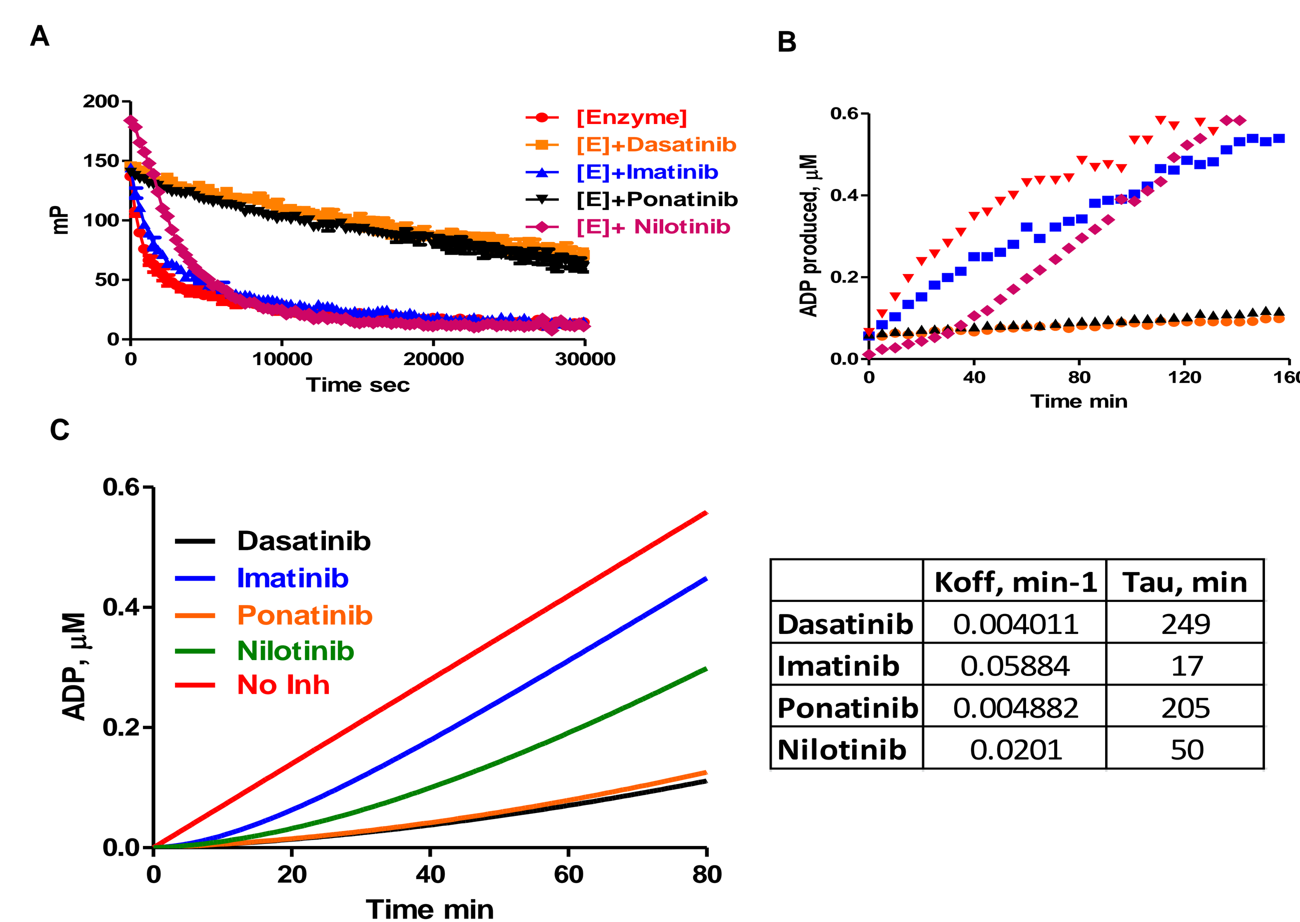
Table 1. The high sensitivity of the Transcreener ADP² Assays allows detection of 10% conversion of ATP even at low levels of ATP. Assay statistics, including Z' values and lower limits of detection (LLD) were calculated and compared between assays.

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.

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 inhibitor residence times. A. EI complexes were formed by pre-incubating with Abl (280 nM) with saturating inhibitor concentrations (10 x IC_{50}): 45 nM (Dasatinib), 4.5 μM (Imatinib), 25 nM (Nilotinib), or 10 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; residence times (Tau values) are reciprocal of K_{off} values.

Assays Compatible with 1536 Well Format for HTS

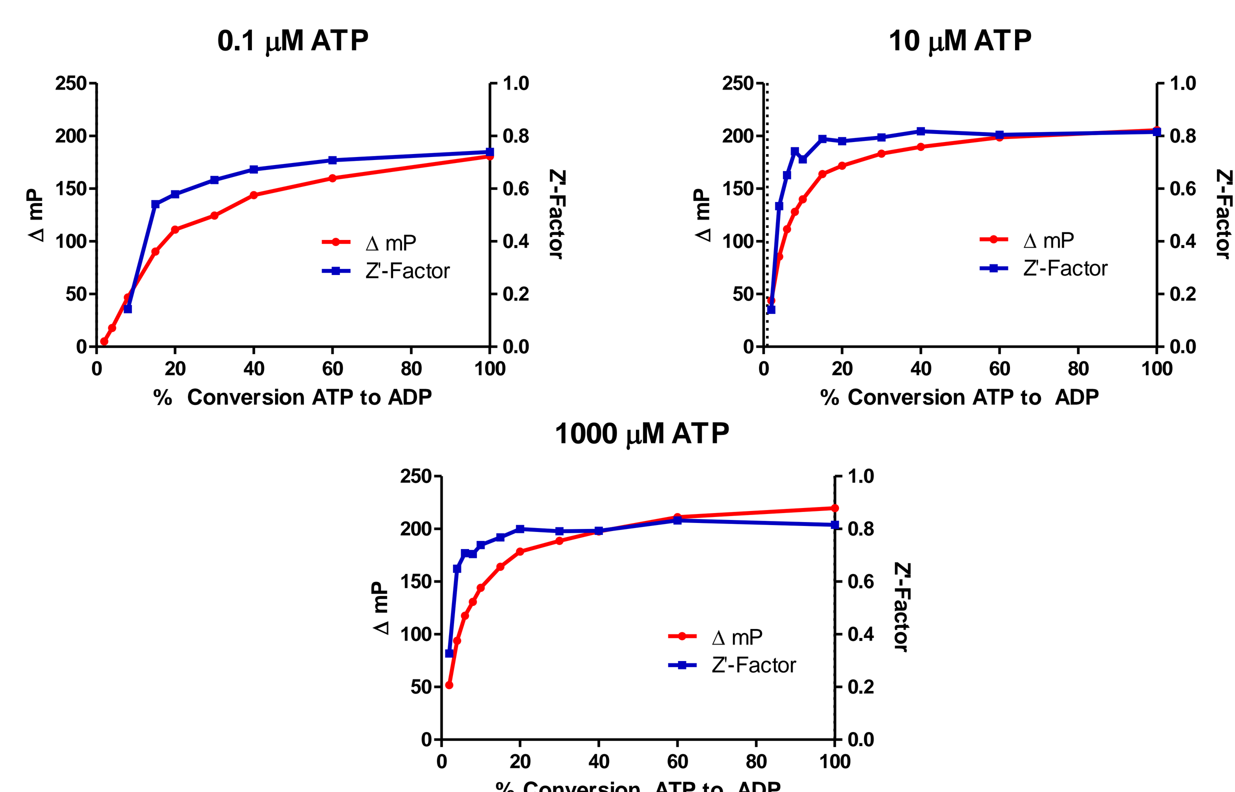


Figure 5. Z' and signal values in 1536 well-plate formats. The conversion of ATP to ADP at 0.1 μM, 10 μM, and 1000 μM ATP demonstrate the outstanding consistency of the Transcreener ADP² Assays in a miniaturized 1536 well format. Standard curves are used to mimic enzyme reactions. Starting ATP concentrations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

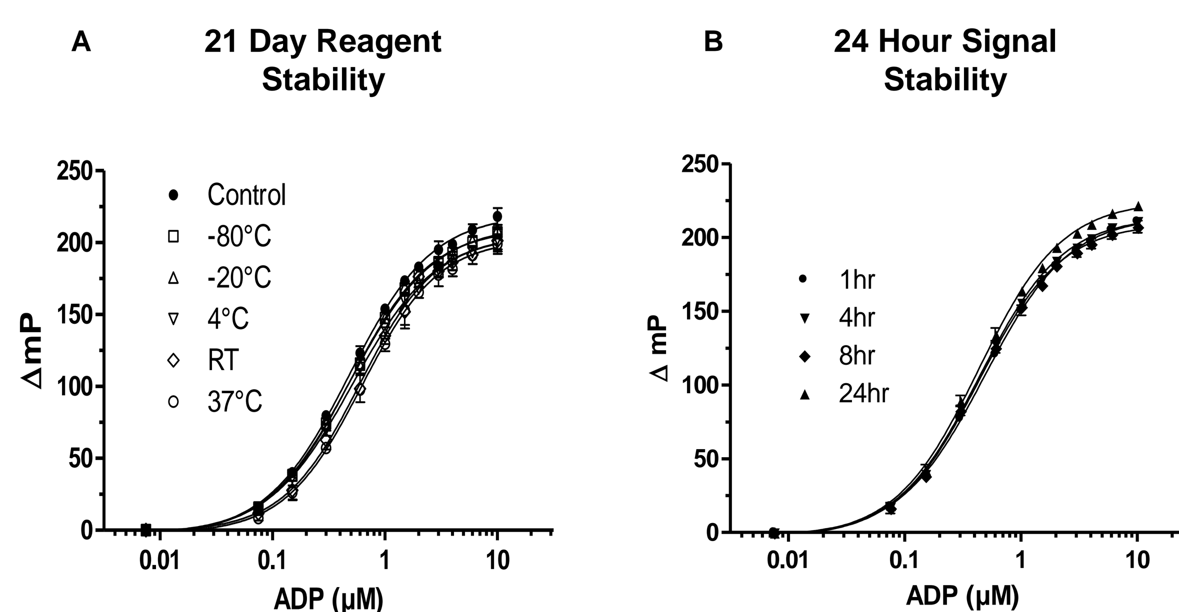


Figure 6. Overnight reagent and signal stability. Standard curves for conversion of 10 μM ATP to ADP were used to measure reagent and signal stability. **A. Stability of Transcreener detection reagents prior to addition to reactions** and **B. The stability of the signal following addition to kinase 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.**

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