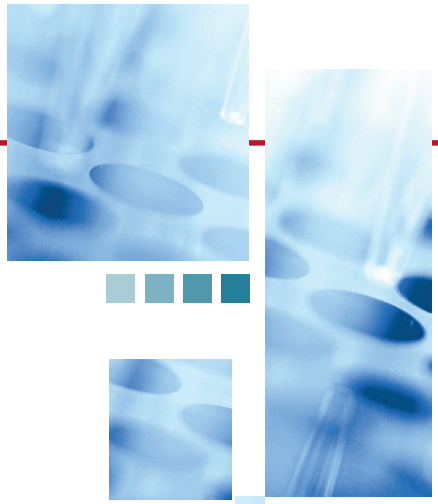
BellBrook Labs

FAR RED TR-FRET



TRANSCREENER®

ADP² TR-FRET Red Assay

Technical Manual

Instructions for Part Numbers 3011-1K and 3011-10K

TM#123 • Rev 2023-05

Transcreener® ADP² TR-FRET Red Assay Technical Manual

Contents

1.0 Introduction

The Transcreener® ADP² TR-FRET Red Assay is a competitive immunoassay for ADP with a far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) readout. Because it is highly selective for ADP, the assay can be used with any enzyme that converts ATP to ADP, regardless of what other substrates are used. Examples of enzymes include protein, lipid, and carbohydrate kinases, ATPases, DNA helicases, carboxylases, and glutamine synthetase.

The Transcreener® assay is designed specifically for high-throughput screening (HTS), with a single-addition, mix-and-read format. It offers reagent stability and compatibility with commonly used multimode plate readers. The generic nature of the Transcreener® HTS assay platform eliminates delays involved in assay development for new HTS targets and greatly simplifies compound and inhibitor profiling across multiple target families.

The Transcreener® ADP² TR-FRET Red Assay provides the following benefits:

- Accommodates ATP concentrations ranging from 0.1 µM to 1,000 µM.
- Excellent data quality (Z' ≥ 0.7) at low substrate conversion (typically 10% or less).
- Overcomes the need for time-consuming, one-off assay development for individual members within a group transfer enzyme family by using a single set of assay reagents that detect an invariant product.
- Time-gated detection method largely eliminates interference that can result from prompt fluorescence of test compounds.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

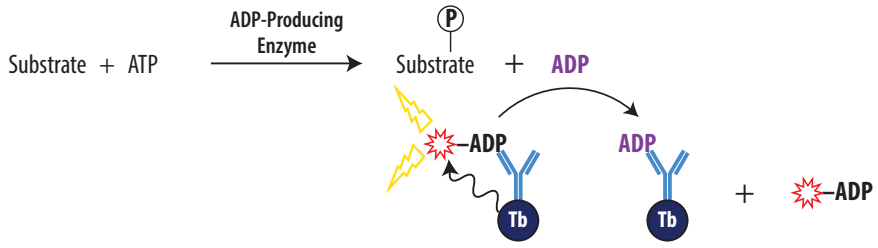


Figure 1. Schematic overview of the Transcreener® ADP² TR-FRET Red Assay. The Transcreener® ADP Detection Mixture contains an ADP HiLyte647 tracer bound to an ADP² antibody conjugated to terbium (Tb). Excitation of the Tb complex in the UV range (~330 nm) results in energy transfer to the tracer and emission at a higher wavelength (665nm) after a time delay. ADP produced by the target enzyme displaces the tracer, which causes a decrease in TR-FRET.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® ADP² TR-FRET Red Assay	1,000 assays*	3011-1K
	10,000 assays*	3011-10K

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using 20 µL reaction volumes.

Storage

Store all reagents at –80°C upon receipt.

2.1 Materials Provided

Component	Composition	Notes
ADP HiLyte647 Tracer	10 µM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The concentration of ADP HiLyte647 Tracer needed for an enzyme target depends upon the ATP concentration and buffer conditions in the enzyme reaction (see Section 4.2). Sufficient tracer is included in the kit to complete 1,000 assays (Part # 3011-1K) or 10,000 assays (Part # 3011-10K) at an ATP concentration up to 100 µM ATP.
ADP² Antibody-Terbium Conjugate	800 nM solution in HEPES-buffered saline	The final antibody concentration in the reaction is 4 nM in a 20 µL final reaction volume.
Stop & Detect Buffer C, 10X	500 mM HEPES (pH 7.5), 200 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer C components will stop enzyme reactions that require Mg^{2+}. To ensure that the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the reaction. The final concentration of Stop & Detect Buffer C at the time of FRET measurement is 0.5X.
ATP	5 mM	The ATP supplied in this kit can be used for the enzyme reaction and to create an ADP/ATP standard curve, if desired.
ADP	5 mM	ADP is used to create the ADP/ATP standard curve.

Caution: ATP is a common reagent in many laboratories; however, it is imperative that a highly purified preparation be used for the Transcreener® assay. If the ATP stock contains impurities, such as ADP, the assay window will be compromised.

2.2 Materials Required but Not Provided

- ***Ultrapure Water***—Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.
- ***Enzyme***—Transcreener® ADP² assays are designed for use with purified enzyme preparations. Contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
- ***Enzyme Buffer Components***—User-supplied enzyme buffer components include enzyme, buffer, acceptor substrate, $MgCl_2$ or $MnCl_2$, EGTA, Brij-35, and test compounds.
- ***Plate Reader***—A microplate reader configured to measure TR-FRET of the Tb:HiLyte647 donor:acceptor pair is required. This assay has been designed to provide high-quality data on any HTS-qualified instrument configured to measure TR-FRET using standard europium or terbium complexes with emission wavelengths at 615 nm and 665 nm. Validation was completed using PHERAstar Plus $Ex_{337}/Em_{620}/Em_{665}$ (BMG LABTECH) and Envision $Ex_{320}/ Em_{615}/Em_{665}$ (Perkin Elmer).
- ***Assay Plates***—It is important to use assay plates that are entirely white with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4513).
- ***Liquid Handling Devices***—Use liquid handling devices that can accurately dispense a minimum volume of 2.5 µL into 384-well plates.

Note: Contact BellBrook Labs Technical Service for suppliers and catalog numbers for buffer components, and additional information regarding setup of TR-FRET instruments.

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the TR-FRET instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

4.0 Protocol

The Transcreener® ADP² TR-FRET Red Assay protocol consists of 4 steps (**Figure 2**). The protocol was developed for a 384-well format, using a 10 µL enzyme reaction and 20 µL final volume at the time that the plates are read. The use of different densities or reaction volumes will require changes in reagent quantities.

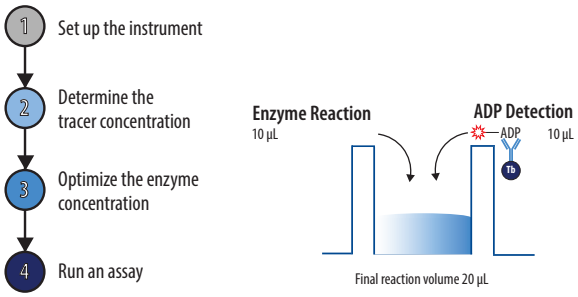


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for TR-FRET is essential to the success of the Transcreener® ADP² TR-FRET Red Assay.

4.1.1 Verify That the Instrument Measures TR-FRET

Ensure that the instrument is capable of measuring TR-FRET (not simply fluorescence intensity) of the terbium:HiLyte647 TR-FRET pair ($Ex_{320}/Em_{615}/Em_{665}$).

Note: A complete list of instruments and instrument-specific application notes can be found online at: https://www.bellbrooklabs.com/technical-resources/instrument-compatibility Contact BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

4.1.2 Define the Maximum TR-FRET Window for the Instrument

Measuring high (0% ATP conversion) and low (100% ATP conversion) FRET will define the maximum assay window of your specific instrument. Prepare High and Low FRET Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use ATP and ADP HiLyte647 Tracer at 0.5X concentration in a 20 µL final reaction volume. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction. As an example, the 1X detection mixture may contain 10 µM ATP. After adding this to the enzyme reaction, the concentration in the final 20 µL reaction volume would be 5 µM.

High FRET Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
ADP² Antibody-Tb	800 nM	4 nM	2.5 µL	
10X Stop & Detect Buffer C	10X	0.5X	25.0 µL	
ADP HiLyte647 Tracer	10 µM	13.4 nM	0.7 µL	
ATP	5 mM	5 µM	0.5 µL	
Water			471.3 µL	
Total			**500.0 µL**	

The assay window will depend upon your initial ATP concentration. These volumes can be adjusted for fewer assays and different ATP concentrations.

Low FRET Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
ADP² Antibody-Tb	800 nM	4 nM	2.5 µL	
10X Stop & Detect Buffer C	10X	0.5X	25.0 µL	
ADP HiLyte647 Tracer	10 µM	13.4 nM	0.7 µL	
ADP	5 mM	5 µM	0.5 µL	
Water			471.3 µL	
Total			**500.0 µL**	

The assay window will depend upon your initial ADP concentration. These volumes can be adjusted for fewer assays and different ADP concentrations.

4.1.3 Measure the TR-FRET

Test the Z′ factor and assay window on your instrument by adding 20 µL of the Low FRET Mixture in 16 wells and 20 µL of High FRET Mixture in 16 wells. Calculate the Z′ factor using the equation below; values greater than 0.7 are acceptable.

Caution: Contact BellBrook Labs Technical Service for assistance if the calculated Z′ factor is less than 0.7.

$$Z' = 1 - \frac{[(3 \times SD_{\text{High FRET Mixture}}) + (3 \times SD_{\text{Low FRET Mixture}})]}{|(\text{mean of High FRET Mixture ratio 665:615}) - (\text{mean of Low FRET Mixture ratio 665:615})|}$$

4.2 Determine the ADP HiLyte647 Tracer Concentration

The Transcreener® ADP² TR-FRET Red Assay requires 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. The concentration of ADP HiLyte647 tracer determines the total assay window and the ADP detection range; the amount needed primarily depends upon the ATP concentration in the enzyme reaction.

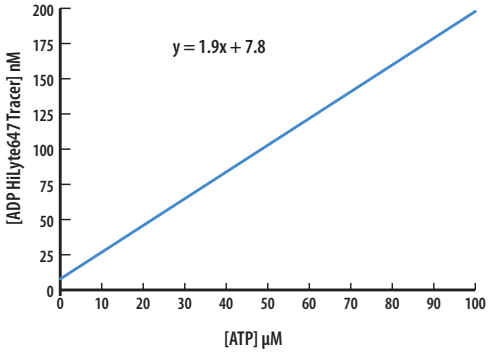


Figure 3. Linear relationship between [ATP] and [ADP Tracer]. The tracer concentration can be calculated using the equation: **y = 1.9x + 7.8**

4.2.1 Calculate the Tracer Amount

As shown in **Figure 3**, the relationship between ATP and ADP HiLyte647 Tracer concentrations is linear. (Though shown for 0.1–100 µM ATP, the relationship is valid to 1,000 µM ATP.) Therefore, the quantity of ADP HiLyte647 Tracer for enzyme reactions that use between 0.1 µM and 1,000 µM ATP can be determined using the equation **y = mx + b**, where **x** = [ATP] (µM) in the 10 µL enzyme reaction, **y** = [ADP HiLyte647 Tracer] (nM) in the 10 µL of 1X ADP Detection Mixture, **m** (slope) = 1.9, and **b** (y-intercept) = 7.8. We recommend a final reaction volume of 20 µL.

For example, if you are using 3 µM ATP in a 10 µL enzyme reaction, the optimal ADP HiLyte647 Tracer concentration in the 1X ADP Detection Mixture (assuming 10 µL of ADP Detection Mixture was added to each 10 µL enzyme reaction) would be (1.9 × 3) + 7.8 = 13.6 nM.

4.2.2 Optimize the Tracer Concentration

Using the [ADP HiLyte647 Tracer] calculated using the equation in **Figure 3** will produce excellent results for most users. If it does not produce the results you require, simply optimize the tracer concentration in a stepwise fashion using the [ADP HiLyte647 Tracer] (X) from the line as a starting point. Try performing a standard curve (see **Section 7.1**) at 0.5 × [Y], [Y], and 1.5 × [Y] tracer concentrations to find an assay window that suits your needs. See **Section 6** for troubleshooting suggestions.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener® ADP² TR-FRET Red Assay. Use enzyme buffer conditions, substrate, and ATP concentrations that are optimal for your target enzyme and ADP HiLyte647 Tracer concentration calculated as described in **Section 4.2**. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 35 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, 1% DMSO (test compound solvent), 0.015% Brij-35, and ATP. Run your enzymatic reaction at its requisite temperature and time period. Refer to **Section 7.2** for the tolerance of different components for your buffer conditions.

4.3.1 Enzyme Titration Steps

To achieve the most robust assay and a high signal, the quantity of enzyme required to produce a 50–80% change in FRET signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose-response curves (see **Figure 4**). To determine the EC_{80} enzyme concentration, use the following equation:

$$EC_{80} = (80 \div (100 - 80))^{(1 \div \text{hillslope})} \times EC_{50}$$

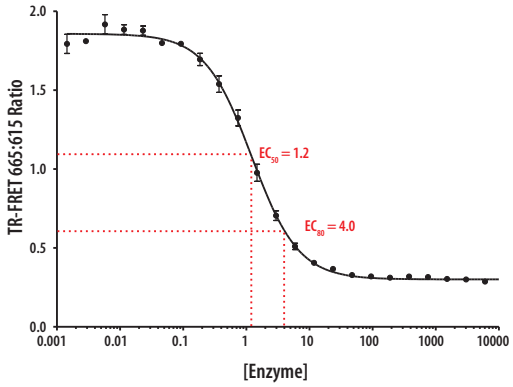


Figure 4. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

4.3.2 Enzyme Assay Controls

The enzyme reaction controls define the limits of the enzyme assay.

Component	Notes
0% ATP Conversion Control	This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme), and 100% ATP (0% ADP). It defines the upper limit of the assay window.
100% ATP Conversion Control	This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme), and 100% ADP (0% ATP). It defines the lower limit of the assay window.
Minus-Nucleotide Control and Minus-Substrate Control	To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of nucleotide (i.e., ATP) or acceptor substrate.
ADP/ATP Standard Curve	Although optional, an ADP/ATP standard curve can be useful to ensure day-to-day reproducibility and that the assay conditions were performed using initial rates. It can also be used to calculate product formed and inhibitor IC_{50} values. See **Section 7.1** for a description of how to run the standard curve.
Background Control	Use only 0.5X enzyme reaction conditions and Stop & Detect Buffer C.

4.4 Run an Assay

1. Add the enzyme reaction mixture to test compounds and mix on a plate shaker.
2. Start the reaction by adding ATP and acceptor substrate, then mix. The final volume of the enzyme reaction mixture should be 10 µL. Incubate at a temperature and time ideal for the enzyme target before adding the ADP Detection Mixture.
3. Prepare 1X ADP Detection Mixture as follows:

	ATP Concentration: Examples			
Component	**1 µM**	**10 µM**	**100 µM**	**Your Numbers**
ADP² Antibody-Tb	100 µL	100 µL	100 µL	
ADP HiLyte647 Tracer	9.7 µL	26.8 µL	197.8 µL	
10X Stop & Detect Buffer C	1,000 µL	1,000 µL	1,000 µL	
Water	8,890.3 µL	8,873.2 µL	8,702.2 µL	
Total	**10,000 µL**	**10,000 µL**	**10,000 µL**	

Final concentrations in the detection mixture should be 8 nM ADP² Antibody-Tb, 1X Stop & Detect Buffer C, and the tracer concentration calculated using the equation in **Figure 3**. An example is shown below:

	y = 1.9x + 7.8		
ATP	1 µM	10 µM	100 µM
ADP HiLyte647 Tracer	9.7 nM	26.8 nM	197.8 nM

4. Add 10 µL of 1X ADP Detection Mixture to 10 µL of the enzyme reaction. Mix using a plate shaker.
5. Incubate at room temperature (20–25°C) for 1 hour and measure TR-FRET.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® ADP² TR-FRET Red Assay is designed for endpoint readout. The Stop & Detect Buffer C contains EDTA to stop Mg^{2+}-dependent enzyme reactions by chelating available Mg^{2+}. Contact BellBrook Labs regarding stop buffers for non-Mg^{2+}-dependent enzymes.

5.1.2 Real-Time Assay

You can perform real-time experiments by adding the ADP Detection Mixture, without the Stop & Detect Buffer C, directly to an enzyme reaction at initiation of the reaction. ADP detection equilibration time is not instantaneous, making it difficult to quantify ADP production; however, this method can provide insight into optimal enzyme concentration and incubation time. If Mn^{2+} or heavy metal ions, such as Cr^{3+}, Co^{2+}, $Fe^{2+/3+}$, or Cu^{2+} are present, they can negatively quench the terbium chelate at high enough concentrations, so this method may not be possible for all enzymes. As an alternative, the Transcreener® ADP² FP Assay is recommended to perform real-time assays. Note that the optimal ADP HiLyte647 Tracer concentration may change when EDTA is omitted from the reaction.

5.2 Reagent and Signal Stability

The Transcreener® technology provides a robust and stable assay method to detect ADP.

5.2.1 Signal Stability

The stability of the TR-FRET ratio assay window at 10% substrate conversion was determined after the addition of the ADP Detection Mixture to the standard samples. The ratio assay window at 10% substrate conversion (10 µM) remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read TR-FRET on the following day, seal the plates to prevent evaporation.

5.2.2 ADP Detection Mixture Stability

The ADP Detection Mixture is stable for at least 8 hours at room temperature (20–25°C) before addition to the enzyme reaction (i.e., when stored on the liquid handling deck).

6.0 Troubleshooting

Problem	Possible Causes and Solutions
Low selectivity	*Suboptimal tracer concentration* • Under the reaction conditions used in the Transcreener® ADP² TR-FRET Red Assay, the ADP antibody is >140-fold selective for ADP over ATP. To achieve maximum sensitivity and assay window, the ADP tracer concentration must be optimized for each starting ATP concentration. *ATP concentration out of range* • Ensure that the starting ATP concentration is in the range of 1–1,000 µM.
No change in TR-FRET observed	*Low antibody/tracer activity* • The tracer and antibody are stable for up to 6 freeze-thaw cycles. For frequent use, aliquot the antibody and tracer and store the aliquots at –20°C. Use a minimum of 20 µL aliquots. *Interference from metal ions* • Mn^{2+} or heavy metals like Cu^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+}, or Co^{2+} can quench terbium at higher concentrations. This effect can be relieved by increasing EDTA concentration or adding additional quantities of EDTA-containing Stop & Detect Buffer C. Use a minimum molar ratio of at least 4X EDTA to metal ions.
High background signal	*Nonproductive ATP hydrolysis* • Certain kinases catalyze some level of nonproductive ATP hydrolysis, to the extent that water is able to get into the active site. However, the rates are generally low even in the absence of acceptor substrate and are even further reduced when acceptor substrate is present. If you are using the assay to screen for potential acceptor substrates, then background from ATP hydrolysis has to be taken into account on a case-by-case basis. We recommend a "no substrate" control to detect nonproductive ATP hydrolysis. *Interference from impurities* • Since the assay measures ADP production from any source, impurities that cause ADP production—such as a contaminating kinase, phosphatase, or ATPase—will interfere with accurate measurement of the desired kinase activity. Care should be taken to minimize these potential contaminants in both kinase and protein substrate preparations.

7.0 Appendix

7.1 ADP/ATP Standard Curve

The standard curve mimics an enzyme reaction (as ATP concentration decreases, ADP concentration increases); the adenine nucleotide concentration remains constant. The ADP/ATP standard curve allows calculation of the concentration of ADP produced in the enzyme reaction and, therefore, the % ATP consumed (% ATP conversion). In this example, a 16-point standard curve was prepared using concentrations of ADP and ATP corresponding to 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 2.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100% ATP conversion (see **Table 1**). Commonly, 8- to 12-point standard curves are used.

Table 1. Concentrations of ATP/ADP to prepare a 16-point standard curve.

% Conv.	ATP (µM)	ADP (µM)
100	0	100
50	50	50
25	75	25
10	90	10
5	95	5
2.5	97.5	2.5
1	99	1
0.8	99.2	0.8
0.6	99.4	0.6
0.5	99.5	0.5
0.4	99.6	0.4
0.3	99.7	0.3
0.2	99.8	0.2
0.1	99.9	0.1
0	100	0

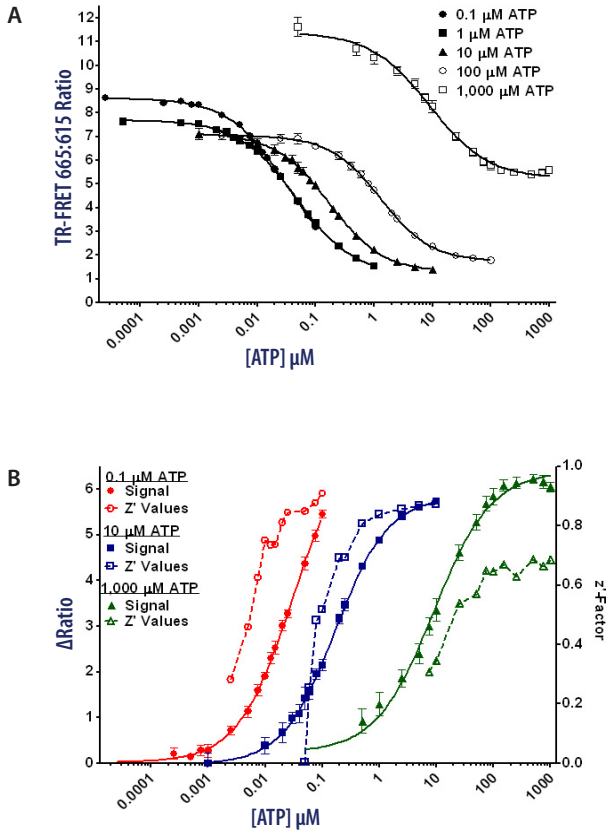


Figure 5. ATP/ADP standard curves. **A)** Sample data for 0.1 µM, 1 µM, 10 µM, 100 µM, and 1,000 µM ADP/ATP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the ADP Detection Mixture. Curves are obtained in a final 20 µL assay volume consisting of 35 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.015% Brij-35, 10 mM EDTA, 4 nM ADP² Antibody-Tb, ADP/ATP standards, and ADP HiLyte647 Tracer (concentration from equation in Figure 3) (n = 24). The data are plotted as FRET ratio and change in ratio vs. log [ADP] using 4-parameter nonlinear regression curve fitting. Alternatively, a 2-phase exponential decay and nonlinear regression can be used to present the data (GraphPad Prism). **B)** Excellent Z' values are obtained at <10% ATP conversion for the range of ATP concentrations used. Shown are 0.1 µM, 10 µM, and 1,000 µM ATP standard curves.

Use the following equations to calculate the Z' factor:

$$\Delta ratio = ratio_{initial\ [ATP]} - ratio_{sample}$$

$$Z' = 1 - \frac{[(3 \times SD_{initial\ [ATP]}) + (3 \times SD_{sample})]}{|(ratio_{initial\ [ATP]}) - (ratio_{sample})|}$$

7.2 Summary of Additive Effects on the Transcreener® ADP² TR-FRET Assay

The assay window at 10% substrate conversion (10 µM ATP) remains constant (<10% change) when up to 10% DMSO, DMF, ethanol, acetonitrile, ethanol, or methanol are used in the enzyme reaction. Contact BellBrook Labs Technical Service for further reagent compatibility information.

Component	5-Hour Tolerance (0–100% Conversion Signal)	5-Hour Tolerance (0–10% Conversion Signal)
Solvents		
Acetonitrile	>50%	12.5%
DMSO	3.13%	3.13%
Ethanol	>50%	12.50%
Methanol	>50%	25.00%
Glycerol	>50%	0.39%
Detergents		
Brij-35	0.94%	0.47%
CHAPS	1.25%	0.08%
NP40	0.16%	0.16%
SDS	0.04%	0.04%
Triton X-100	0.31%	0.31%
Sodium deoxycholate	0.00%	0.16%
N-lauroyl sarcosine	0.08%	0.04%
Metal chelates		
EDTA	125 mM	62.5 mM
EGTA	>250 mM	125 mM
Reductants		
Beta mercaptoethanol	6.25%	0.02%
Dithiothreitol	>500 mM	0.031 mM
Salts		
Ammonium acetate	125 mM	125 mM
Ammonium sulfate	3.91 mM	3.91 mM
Calcium chloride	15.62 mM	7.81 mM
Magnesium acetate	15.62 mM	3.91 mM
Magnesium chloride	15.62 mM	7.81 mM
Magnesium sulfate	1.95 mM	1.95 mM
Manganese chloride	15.62 mM	7.81 mM
Potassium chloride	500 mM	125 mM
Sodium azide	1.25%	1.25%
Sodium bromide	12.5 mM	3.12 mM
Sodium chloride	12.5 mM	6.25 mM

Component	5-Hour Tolerance (0–100% Conversion Signal)	5-Hour Tolerance (0–10% Conversion Signal)
Phosphatase Inhibitors		
Glycerol phosphate	15.62 mM	7.81 mM
Imidazole	62.5 mM	62.5 mM
Sodium fluoride	7.81 mM	3.91 mM
Sodium molybdate	31.25 mM	31.25 mM
Sodium tartrate	>400 mM	200 mM
Sodium orthovanadate	7.81 mM	7.81 mM
Sodium pyrophosphate	0.39 mM	0.78 mM
Carrier Proteins/Coactivators		
BSA	>1.0 mg/mL	>1.0 mg/mL
BGG	>2.5 mg/mL	>2.5 mg/mL

Not all combination of these components have been tested together. Results may vary depending on your assay conditions.

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