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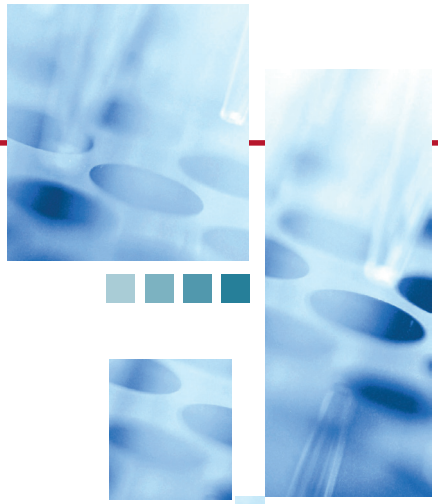
RED FI



TRANSCREENER®

ADP² FI Assay

Technical Manual

Instructions for Part Numbers 3013-A, 3013-1K, and 3013-10K

TM#133 • Rev 2021-03

Transcreener® ADP² FI Assay Technical Manual

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U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500, 11/958,515 and 11/958,965, U.S. Divisional Application 12/029,932, and International Patent Application Nos. PCT/US07/088111, European Application Nos. 04706975.2 and 05785285.7, Canadian Application 2,514,877, and Japanese Application 2006-503179 applied. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes other than use of the product or its components to provide a service, information, or data. Commercial Purposes means any activity by a party for consideration other than use of the product or its components to provide a service, information, or data and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (3) resale of the product or its components, whether or not such product or its components are resold for use in research. BellBrook Labs LLC will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitati1ns of this limited use statement, BellBrook Labs LLC is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, BellBrook Labs LLC, 5500 Nobel Drive, Suite 230, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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1.0 Introduction

The Transcreener® assay platform 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 FI Assay extends the Transcreener® platform for ADP detection by utilizing a simple fluorescent intensity (FI) output. It can be used on fluorescence readers typically found in academic and therapeutic research laboratories, as well as more complex multimode plate readers more commonly used in core facilities and HTS facilities.

The assay is a red, competitive FI method (**Figure 1**). 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® ADP² FI Assay provides the following benefits:

- Accommodates ATP concentrations ranging from 0.1 µM to 100 µM.
- Excellent data quality (Z′ ≥ 0.7) and signal at low substrate conversion (typically 2.5% or less) using 1 µM ATP.
- 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.
- Red tracer further minimizes interference from fluorescent compounds and light scattering.

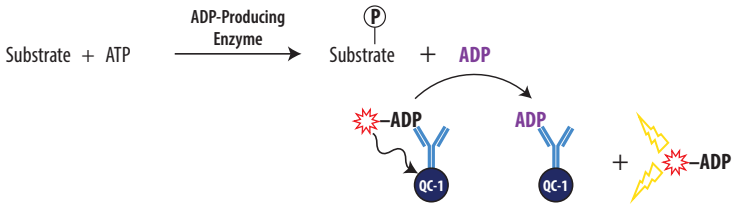


Figure 1. Schematic overview of the Transcreener® ADP² FI Assay. The Transcreener® ADP Detection Mixture contains a quenched ADP Alexa Fluor® 594 tracer bound to an ADP² antibody conjugated to an IRDye® QC-1 quencher. ADP produced by the target enzyme displaces the tracer, which is no longer quenched and causes a positive increase in FI.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® ADP² FI Assay	200 assays*	3013-A
	1,000 assays*	3013-1K
	10,000 assays*	3013-10K

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 96-well plates using 50 µL reaction volumes (3013-A), or 384-well plates (3013-1K and 3013-10K) using 20 µL reaction volumes.

Storage

Store all reagents at –20°C upon receipt.

2.1 Materials Provided

*Note: The exact antibody concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration..

Component	Composition	Notes
ADP² Antibody–IRDye® QC-1	1.4 mg/mL* solution in 100 mM KH_2PO_4 (pH 8.5)	The concentration of antibody needed for an enzyme target is dependent upon the ATP concentration and buffer conditions in the enzyme reaction (see **Section 4.2**). Sufficient antibody is included in the kit to complete 200 assays (Part # 3013-A), 1,000 assays (Part # 3013-1K), or 10,000 assays (Part # 3013-10K) at an ATP concentration up to 100 µM. **Note: The Antibody IR-Dye QC-1 can have precipitate upon thawing. If so,spin down the precipitate and continue using the supernatant in the assay.The antibody will perform as directed.**
ADP Alexa Fluor® 594 Tracer	800 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The final tracer concentration in the reaction is 4 nM.
Stop & Detect Buffer B, 10X	200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer B 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 B at the time of FI 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

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

- ***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 $MnCl_2$, Brij-35, and test compounds.
- ***Plate Reader***—A multidetection microplate reader configured to measure FI of the Alexa Fluor® 594 tracer is required. The Transcreener ADP² FI Assay has been successfully used on the following instruments: Perkin Elmer EnVision®; Molecular Devices Spectramax M2; and Tecan Infinite® M200 and Safire2™ (see **Table 1**).
- ***Assay Plates***—It is important to use assay plates that are entirely black with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4514) and Corning 96-well, half-area plates (Cat. # 3686).
- ***Liquid Handling Devices***—Use liquid handling devices that can accurately dispense a minimum volume of 2.5 µL into 384-well plates.

3.0 Before You Begin

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

4.0 Protocol

The Transcreener® ADP² FI 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; increase each volume to 25 µL (final volume 50 µL) if performing the assay in 96 well half-volume plates. The use of different densities or reaction volumes will require changes in reagent quantities.

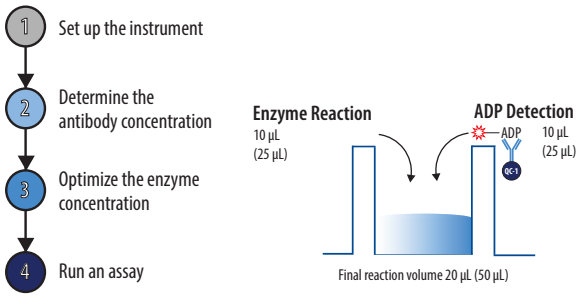


Figure 2. An outline of the procedure. The assay consists of 4 main steps with a mix-and-read format. Volumes shown are for 384-well plates (and 96-well plates).

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FI is essential to the success of the Transcreener® ADP² FI Assay. **Table 1** shows common instrument parameters.

Note that use of narrow bandwidth filters is critical for assay performance because the Stoke's shift (separation between excitation and emission maxima) for the Alexa Fluor® 594 is very narrow. It is possible to use wider bandwidth filters for some instruments, but it requires the use excitation and emission wavelengths different from those shown above in order to avoid spectral overlap.

Plate Reader	Excitation Filter/ Bandwidth	Emission Filter/ Bandwidth	Mirror Module	Other Parameters
Envision (Perkin Elmer)	545 nm/7 nm (Cat. # 2100-5070)	635 nm/15 nm (Cat. # 2100-5590)	D595	Mirror: Texas Red FP single mirror Cat. # 2100-4190
PHERAstar Plus (BMG Labtech)	580 nm/10 nm	620 nm/10 nm	NA	
Safire2 (Tecan)	580 nm/10 nm	620 nm/10 nm	NA	Monochromator-based
SpectraMax M2 (Molecular Devices)	584 nm	612 nm	NA	Emission filter auto-cutoff at 610 nm

Table 1. Instrument filters and settings for commonly used multimode plate readers. Contact BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

4.1.1 Verify That the Instrument Measures FI

Ensure that the instrument is capable of measuring FI of Alexa Fluor® 594. The optimal excitation/emission settings for the ADP Alexa Fluor® 594 Tracer are excitation 590 nm (10 nm bandwidth) and emission 617 nm (10 nm bandwidth). The ADP Alexa Fluor® 594 Tracer has been successfully used at excitations of 580–590 nm and emissions of 610–620 nm with bandwidths of 10 nm (see **Table 1**).

4.1.2 Define the Maximum FI Window for the Instrument

Measuring low (tracer + antibody) and high (free tracer) relative fluorescence units (RFUs) will define the maximum assay window of your specific instrument. Prepare Low and High RFU Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use both tracer and antibody at 0.5X concentration in the final reaction volume. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction. As an example, if the calculated antibody concentration is 10 µg/mL, the concentration used here would be 5 µg/mL.

The examples shown below are for an initial ATP concentration of 10 µM.

Low RFU Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
ADP² Antibody–IRDye® QC-1	1.4 mg/mL	5 µg/mL	1.8 µL	
10X Stop & Detect Buffer B	10X	0.5X	25.0 µL	
ADP Alexa Fluor® 594 Tracer	800 nM	4 nM	2.5 µL	
Water			470.7 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.

High RFU Mixture

Prepare the following solution:

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
10X Stop & Detect Buffer B	10X	0.5X	25.0 µL	
ADP Alexa Fluor® 594 Tracer	800 nM	4 nM	2.5 µL	
Water			472.5 µL	
Total			**500.0 µL**	

Caution: Contact BellBrook Labs Technical Service for assistance if the ratio is <5.0.

4.1.3 Measure the FI

Subtract the Low RFU Mixture readings from the corresponding High RFU Mixture readings. The difference between the low and high RFU values will give the maximum assay window. The values will differ, depending on the units from the plate reader, but the ratio (High RFU Mixture):(Low RFU Mixture) should be >5.0.

4.2 Determine the Optimal ADP² Antibody–IRDye® QC-1 Concentration

The Transcreener® ADP² FI 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 ADP² Antibody–IRDye® QC-1 determines the total assay window and the ADP detection range; the amount needed primarily depends upon the ATP concentration in the enzyme reaction. To produce the most sensitive and robust assay signal, it is necessary to titrate the ADP² Antibody–IRDye® QC-1 in the buffer system ideal for your enzyme or drug target.

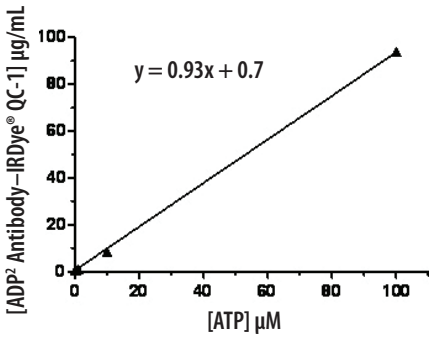


Figure 3. Linear relationship between [ATP] and [ADP² Antibody–IRDye® QC-1]. The antibody concentration can be calculated using the equation: y = 0.93x + 0.7

4.2.1 Calculate the Antibody Concentration

As shown in **Figure 3**, the relationship between ATP and ADP² Antibody–IRDye® QC-1 concentrations is linear. (Though shown for 0.1–100 µM ATP, the relationship is valid to 1,000 µM ATP.) Therefore, the quantity of ADP² Antibody–IRDye® QC-1 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 enzyme reaction, **y** = [ADP² Antibody–IRDye® QC-1] (µg/mL) in the 1X ADP Detection Mixture, **m** (slope) = 0.93, and **b** (y-intercept) = 0.7. We recommend a final reaction volume of 20 µL (384-well plate) or 50 µL (96-well plate).

For example, if you are using 3 µM ATP in a 10 µL enzyme reaction, the optimal ADP² Antibody–IRDye® QC-1 concentration in the 1X ADP Detection Mixture (assuming 10 µL of ADP Detection Mixture was added to each 10 µL enzyme reaction) would be (0.93 × 3) + 0.7 = 3.49 µg/mL.

4.2.2 Optimize the Antibody Concentration

Using the ADP² Antibody–IRDye® QC-1 concentration calculated using the equation in **Figure 3** will produce excellent results for most users. If it does not produce the results you require, refer to **Section 7.1** for instructions on titrating the antibody in the buffer system ideal for your enzyme target.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener® ADP² FI Assay. Use enzyme buffer conditions, substrate, and ATP concentrations that are optimal for your target enzyme. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 1% DMSO (test compound solvent), 0.01% Brij-35, and ATP. Run your enzymatic reaction at its requisite temperature and time period. Refer to **Section 5.2.3** 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 FI 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:

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

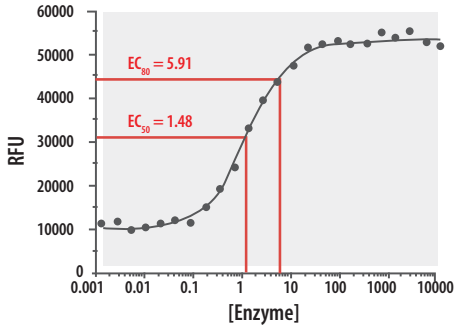


Figure 4. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

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 7.2** for a description of how to run the standard curve.
Background Control	Use only 0.5X enzyme reaction conditions and Stop & Detect Buffer B.

4.4 Run an Assay

4.4.1 Experimental Samples

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 (384-well plates) or 25 µL (96-well plates). 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–IRDye® QC-1	11.6 µL	71.4 µL	669.3 µL	
ADP Alexa Fluor® 594 Tracer	100 µL	100 µL	100 µL	
10X Stop & Detect Buffer B	1,000 µL	1,000 µL	1,000 µL	
Water	8,888.4 µL	8,828.6 µL	8,230.7 µL	
Total	**10,000 µL**	**10,000 µL**	**10,000 µL**	

Final concentrations in the detection mixture should be 8 nM tracer, 1X Stop & Detect Buffer B, and the antibody concentration calculated in **Figure 3 (Section 4.2)**. An example is shown below:

	$y = 0.93x + 0.7$		
ATP	1 µM	10 µM	100 µM
ADP² Antibody–IRDye® QC-1	1.63 µg/mL	10 µg/mL	93.7 µg/mL

4. Add 10 µL (384-well plates) or 25 µL (96-well plates) of 1X ADP Detection Mixture to the enzyme reaction. Mix using a plate shaker.
5. Incubate at room temperature (20–25°C) for 1 hour and measure FI.

4.4.2 ADP Detection Controls

These controls are used to calibrate the FI plate reader and are added to wells that **do not contain enzyme**.

Component	Notes
Minus Antibody (Free Tracer) Control	This control contains the ADP Alexa Fluor® 594 Tracer without the ADP² Antibody–IRDye® QC-1 and determines the maximum RFU value achievable.
Minus Tracer Control	This control contains the ADP² Antibody–IRDye® QC-1 without the ADP Alexa Fluor® 594 Tracer and is used as a sample blank. It contains the same antibody concentration in all wells.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener® ADP² FI Assay is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA to stop Mg^{2+}-dependent enzyme reactions by chelating available Mg^{2+}.

5.1.2 Real-Time Assay

You can perform real-time experiments by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA. However, the equilibration time for the tracer and ADP² Antibody–IRDye® QC-1 is greater than 15 minutes, making it difficult to quantitate ADP produced during short-term enzyme reactions. Note that the optimal antibody concentration may change when EDTA is omitted.

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 FI assay window at 10% substrate conversion was determined after the addition of the ADP Detection Mixture to the standard samples. The RFU value at 10% substrate conversion (10 µM ATP) remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read FI 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 24 hours at room temperature (20–25°C) before addition to the enzyme reaction (i.e., when stored on the liquid handling deck).

5.2.3. Solvent Compatibility

The RFU 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 for further reagent compatibility information.

6.0 Troubleshooting

Problem	Possible Causes and Solutions
Low selectivity	*Suboptimal antibody concentration* • Under the reaction conditions used in the Transcreener® ADP² FI Assay, the ADP² Antibody–IRDye® QC-1 is >140-fold selective for ADP over ATP. To achieve maximum sensitivity and assay window, the antibody 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 FI 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.
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 Optimizing the ADP² Antibody–IRDye® QC-1 Concentration

Using an antibody concentration calculated using the equation in **Figure 3 (Section 4.2)** will produce excellent results for most users. If it does not produce the results you require, we recommend that you titrate the ADP² Antibody–IRDye® QC-1 in the buffer system ideal for your enzyme target. This titration will determine the optimal antibody concentration for your assay conditions. The nucleotide substrate concentration in the enzyme reaction generally determines the appropriate concentration of antibody. We recommend using the EC_{20} concentration of antibody.

7.1.1 Titrate the ADP² Antibody–IRDye® QC-1

1. Prepare your reaction buffer. We have used the follow buffer, but we recommend using your buffer for the titration: 50 mM HEPES (pH 7.5), 2 mM $MgCl_2$, and 0.01% Brij-35. Include ATP and substrate but omit the enzyme.
2. Dispense 10 µL (384-well plates) or 25 µL (96-well plates) of the reaction buffer into each well of columns 2–24.
3. Dispense 20 µL (384-well plates) or 50 µL (96-well plates) of ADP Antibody–IRDye® QC-1 (at the starting highest concentration in the same reaction buffer) into each well of column 1.
4. Remove 10 µL (384-well plates) or 25 µL (96-well plates) from each well of column 1 and add it to the corresponding well of column 2.
5. Repeat step 4 for the remaining columns, thereby performing a 2-fold serial dilution across the plate to column 24.

6. Add 10 µL (384-well plates) or 25 µL (96-well plates) of ADP Alexa Fluor® 594 Tracer (to a final concentration of 4 nM) in 1X Stop & Detect Buffer B to each well.
7. Mix the plate, equilibrate at room temperature for 1 hour, and measure FI.

7.1.2 Calculate the Optimal ADP² Antibody–IRDye® QC-1 Concentration

The antibody concentration at the EC_{20} is often used as a good compromise between sensitivity and maximal assay window. The EC_{20} is determined by inputting the EC_{50} and hillslope values from a sigmoidal dose-response curve fit into the equation below.

$$EC_{20} = (20 \div (100 - 20))^{(1 \div \text{hillslope})} \times EC_{50}$$

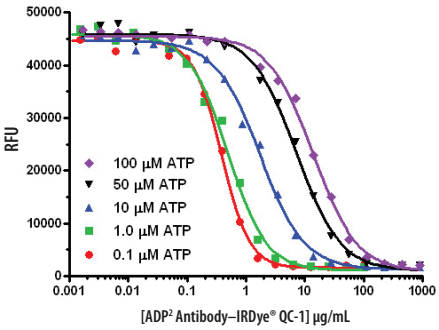


Figure 5. ADP² Antibody–IRDye® QC-1 titration at various ATP concentrations. The final 20 µL assay volume consisted of 4 nM ADP Alexa Fluor® 594 Tracer, 0.5X Stop & Detect Buffer B, 0.5X enzyme reaction mixture (50 mM HEPES [pH 7.5], 2 mM $MgCl_2$, 0.5% DMSO, 0.01% Brij-35, and ATP), and ADP² Antibody–IRDye® QC-1 (n=3).

7.2 ADP/ATP Standard Curve

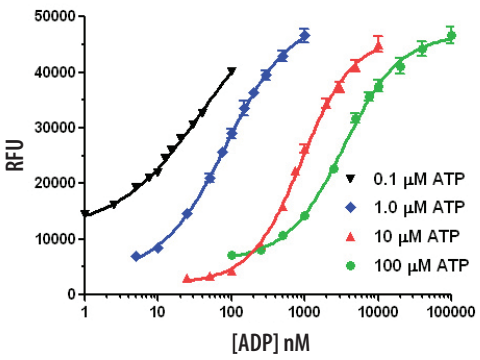
The standard curve mimics an enzyme reaction (as ATP concentration decreases, ADP concentration increases); the adenosine concentration remains constant. The ADP/ATP standard curve allows calculation of the concentration of ADP produced in the enzyme reaction and, therefore, the % ATP consumed (% ATP conversion). In this example, a 12-point standard curve was prepared using the concentrations of ADP and ATP shown in **Table 2**. Commonly, 8- to 12-point standard curves are used.

% Conv.	ATP (µM)	ADP (µM)
100	0	100
60	40	60
40	60	40
30	70	30
20	80	20
15	85	15
10	90	10
8.0	92	8.0
6.0	94.0	6.0
4.0	96.0	4.0
2.0	98.0	2.0
0	100	0

Table 2. Concentrations of ATP/ADP to prepare a 12-point standard curve.

Figure 6. ATP/ADP standard curves. A) Sample data for 0.1 µM, 1 µM, 10 µM, and 100 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 were obtained in a final 20 µL assay volume after adding 10 µL of reaction mix (50 mM HEPES [pH 7.5], 4 mM $MgCl_2$, 1% DMSO, 0.01% Brij, and ADP/ATP standards) to 10 µL detection mix (ADP² Antibody–IRDye® QC-1, 8 nM ADP Alexa Fluor® 594 Tracer, 1X Stop & Detect Buffer B) using the antibody concentration determined from Figure 3 (n = 24). The data are plotted as RFU vs. log [ADP] using 4-parameter nonlinear regression curve fitting. **B)** Excellent Z′ values are obtained for the range of ATP concentrations used.

A



B

% ATP Conversion	Z′ Values at Varying ATP Concentration			
	0.1 µM ATP	1 µM ATP	10 µM ATP	100 µM ATP
0.5	nd	0.4	0.7	0.6
1.0	nd	0.6	0.8	0.8
2.5	0.3	0.8	0.9	0.9
5.0	0.7	0.8	0.9	0.9
10	0.7	0.9	0.9	0.9
100	0.9	0.8	0.9	0.9

Use the following equation to calculate the Z′ factor:

$$Z' = 1 - \frac{[(3 \times SD_{x\% \text{ conversion}}) + (3 \times SD_{0\% \text{ conversion}})]}{|(RFU_{x\% \text{ conversion}}) - (RFU_{0\% \text{ conversion}})|}$$

8.0 Bibliography

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