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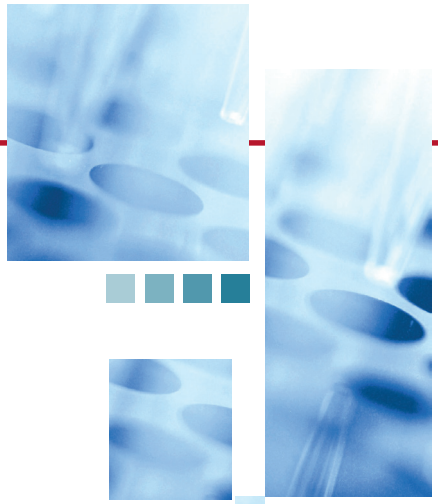
FAR RED TR-FRET



TRANSCREENER®

pADPr PARP TR-FRET Assay

Technical Manual

Instructions for Part Numbers 3044-1K and 3044-10K

Rev 2025-02

Transcreener® pADPr PARP TR-FRET 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 limitations 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, 1232 Fourier Drive, Suite 115, Madison, Wisconsin 53717. Phone (608)443-2400. Fax (608)441-2967.

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1.0 Introduction

The Transcreener® pADPr PARP TR-FRET Assay is a biochemical HTS assay for measuring the production of poly(ADP-ribose) (pADPr) in poly(ADP-ribose) polymerase (PARP) reactions **(Figure 1)**. The assay relies on pADPr Coupling Enzymes (CE) to convert pADPr into AMP, which is then detected using a far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) readout. As an example, the Transcreener pADPr PARP TR-FRET assay can be used to detect the activity of human PARP1, which uses NAD+ to form pADPr on itself, histones, and DNA repair proteins.

The Transcreener pADPr PARP TR-FRET assay is designed specifically for high throughput screening (HTS), with a single-addition, mix-and-read format. It is easy to integrate into automated HTS workflows, with outstanding reagent stability (deck stability > 16 hours, signal stability > 24 hours), robust detection of ADPR monomers released from pADPr over a range of down to 10 nM and as high as 100 µM, and compatibility with commonly used multimode plate readers. Data quality is excellent (Z′ ≥ 0.7), and the assay uses a far-red tracer to minimize interference from fluorescent compounds and light scattering.

Key Applications:

- Screening for enzyme inhibitors/activators
- Generating dose response curves and IC_{50} values for inhibitors.
- Kinetic and mechanistic analyses.

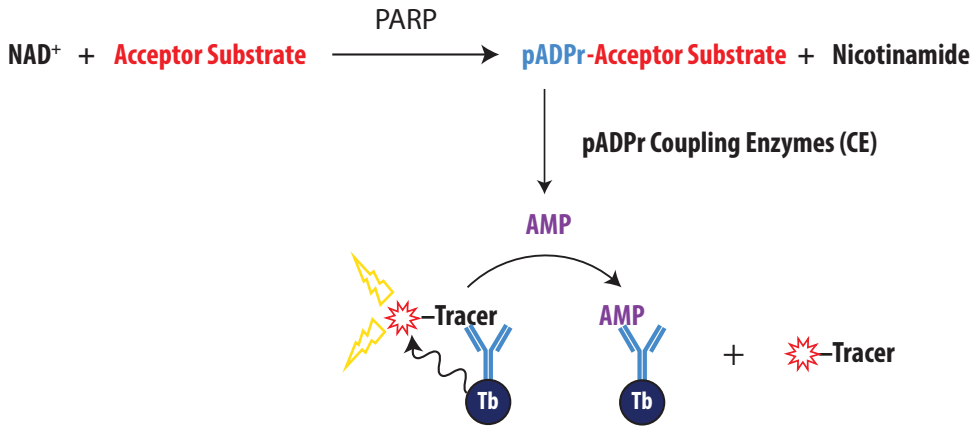


Figure 1. Schematic overview of the Transcreener pADPr PARP TR-FRET Assay. pADPr produced by the target PARP enzyme is converted to AMP in real time by the pADPr Coupling Enzymes. In the detection step, the PAPR Enzyme and CE are quenched by EDTA and AMP displaces a Hilyte 647 Tracer from an AMP²/GMP² Antibody conjugated to Terbium (Tb), resulting in a decrease in TR-FRET.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® pADPr PARP TR-FRET Assay	1,000 assays*	3044-1K
	10,000 assays*	3044-10K

*The exact number of assays depends on the enzyme reaction conditions. The kits are designed for use with 384-well plates, using a 10 µL Enzyme Reaction and a 20 µL Complete Assay Volume.

IMPORTANT: Antibody centrifugation is required to remove aggregates that can disrupt data quality. Antibodies should be centrifuged at 10,000 x g for 10 minutes before use. Following centrifugation, pipet the solution needed from the top of the aliquot to ensure precipitate is not present in the detection reagents.

Storage

The pADPr Coupling Enzymes (CE) should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that the CE are stable up to 5 freeze-thaw cycles, we recommend aliquoting the reagents and snap-freezing for multiple uses to minimize loss of activity.

Use the reagents provided in this kit within 6 months from date of receipt.

2.1 Materials Provided

Component	Composition	Notes
AMP^2/GMP^2 Antibody-Tb	800 nM solution in 25 mM HEPES buffered saline	The final Antibody-Tb concentration 20 µL Complete Assay is 4 nM. Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3044-1K) or 10,000 assays (Part # 3044-10K).
AMP^2/GMP^2 Hilyte 647 Tracer	10 µM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	Sufficient tracer is included in the kit to complete 1,000 assays (Part # 3044-1K) or 10,000 assays (Part # 3044-10K).
pADPr Coupling Enzymes (CE)	400X CE in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM TCEP, 10% glycerol, 0.05% Triton X-100	Sufficient CE for 1,000 assays (Part # 3043-1K) or 10,000 assays (Part # 3043-10K) with coupling enzyme present in excess to ensure pADPr is completely converted to AMP.
Stop & Detect Buffer B, 10X	200mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer B components quench the PARP Enzyme and CE reactions by chelating Mg^{2+}. Therefore, it will work for any target enzyme, as long as the EDTA concentration is at least equimolar to the Mg^{2+}.
NAD^+, 5 mM	5 mM in H_2O	Sufficient NAD^+ is included in the kit to complete 1,000 assays (Part # 3043-1K) or 10,000 assays (Part # 3043-10K).
AMP, 5 mM	5 mM in H_2O	The AMP in this kit can be used to create a standard curve to convert mP values to AMP product formed.

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 TR-FRET instruments.

Component	Notes
Ultrapure Nuclease Free Water	Some deionized water systems are contaminated with enzymes that can degrade both nucleotide substrates and products, reducing assay performance. Use nuclease free water such as: Invitrogen Part # AM9930
Enzyme	The Transcreener pADPr PARP TR-FRET Assay is designed for use with purified PARP enzymes. It is not recommended for cell lysates or impure enzyme preparations as contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
Acceptor Substrate	PARP1 can ADP-ribosylate itself and does not require addition of an additional acceptor substrate. If a separate acceptor substrate is used, it should be highly purified to avoid degradation of AMP by contaminating enzymes.
Plate Reader	A multimode microplate reader configured to measure TR-FRET is required. Transcreener Assays have been validated on the following instruments: BioTek Synergy™2 and Synergy™4; BMG Labtech PHERAstar® Plus and CLARIOstar® Plus; Molecular Devices SpectraMax™ Paradigm; Perkin Elmer EnVision® and ViewLux; and Tecan Infinite® F500, Safire2™, and M1000. Full list of compatible plate readers and settings.
Liquid Handling Devices	Use liquid handling devices that can accurately dispense submicroliter volumes into 384-well plates.
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). The suggested plate has a square well top that enables easier robotic pipetting and a round bottom that allows good Z′ factors. It has a recommended working volume of 15–20 µL.

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the plate reader and verify that it is compatible with the Transcreener AMP²/ GMP² Assays. Full list of compatible plate readers and settings.

4.0 Protocol

The Transcreener pADPr PARP TR-FRET Assay kit optimization follows a simple protocol. First, the instrument parameters are configured to ensure optimal signal detection **(Section 4.1)**. Then, the Transcreener AMP²/GMP² Hilyte 647 Tracer concentration is selected for the desired dynamic range **(Section 4.2)**. Next, an optimal enzyme concentration is determined to provide a good assay window while meeting initial velocity requirements **(Section 4.3)**. Once the instrument parameters, tracer, and enzyme optimization are complete, the assay itself consists of a simple mix-and-read protocol **(Section 4.4)**.

The assay procedure was developed for a 384-well format, using a 10 µL Enzyme Reaction and 20 µL Complete Assay volume when the plates are read **(Figure 2)**. The use of different densities or reaction volumes will require changes in reagent quantities (see **Section 7.3** for example reaction volumes).

Note: The Antibody and CE concentrations remain constant in the 20 µL Complete Assay regardless of changes to other reaction conditions.

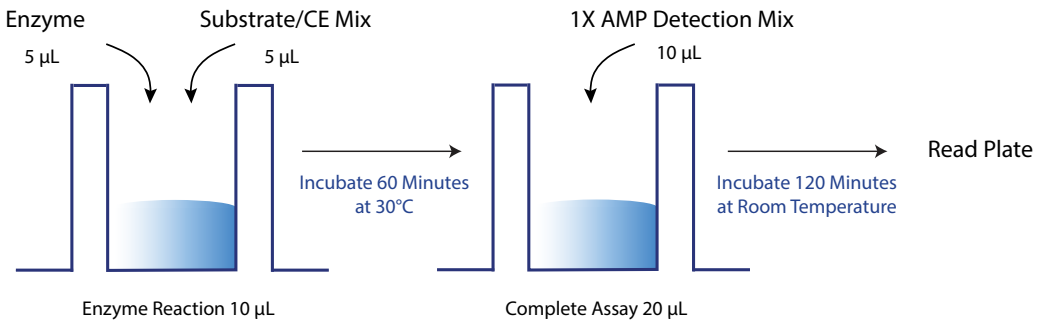


Figure 2. An outline of the Assay Procedure. The target Enzyme Reaction is run in the presence of CE, so that pADPr is converted to AMP in real time. After the Enzyme Reaction incubation is complete, AMP detecting reagents (Transcreener AMP²/GMP² Antibody-Tb and Tracer) are added along with EDTA to quench the Enzyme Reaction.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for TR-FRET is essential to the success of the Transcreener® pADPr PARP TR-FRET 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:Hilyte 647 TR-FRET pair (Ex320/Em615/Em665).

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 Window for the Instrument

Measuring High (Tracer + Antibody-Tb) and Low (Tracer + Antibody-Tb + AMP) signal will define the maximum assay window of your specific instrument. Prepare High and Low TR-FRET Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use AMP²/GMP² Antibody-Tb at 4 nM in a 20 µL Complete Assay. This mimics the 2-fold dilution when adding an equal volume of detection mix to an enzyme reaction.

High FRET Mixture

Prepare the following solution:

Component	As Provided	Final Concentration in 20 µL Complete Assay	Example: 25 Assays	Your Numbers
AMP²/GMP² Antibody-Tb	800 nM	4 nM	3 µL*	
AMP²/GMP² Hilyte 647 Tracer	10 µM	200 nM	12 µL	
Stop & Detect Buffer B	10X	0.5X	30 µL	
Water			555 µL	
Total			**600.0 µL**	

*Pipetting small sample volumes accurately requires the correct equipment and proper technique. An extra dilution step may be required to ensure accuracy.

Low FRET Mixture

Prepare the following solution:

Component	As Provided	Final Concentration in 20 µL Complete Assay	Example: 25 Assays	Your Numbers
AMP²/GMP² Antibody-Tb	800 nM	4 nM	3 µL	
AMP²/GMP² Hilyte 647 Tracer	10 µM	200 nM	12 µL	
AMP	5 mM	100 nM	12 µL	
Stop & Detect Buffer B	10X	0.5X	30 µL	
Water			543 µL	
Total			**600.0 µL**	

4.1.3 Measure the Ratio of High and Low Signal

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

Calculate the Z′-Factor using the equation below; values greater than 0.7 acceptable.

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

4.2 Calculate the Optimal AMP²/GMP² Hilyte 647 Tracer Concentration

The sensitivity of the Transcreener pADPr PARP TR-FRET Assay, and thus the dynamic range, is determined by the concentration of the Transcreener AMP²/GMP² Hilyte 647 Tracer: a lower concentration of tracer increases the sensitivity, higher tracer results in lower sensitivity. Assuming that PARP reactions will be run under initial velocity conditions (≤ 10% consumption of NAD^+), as is typical for almost all applications, the concentration of NAD+ used establishes the desired dynamic range. For example, if 100 µM NAD^+ is used in the PARP reaction, then the desired dynamic range is 1-10 µM AMP. The optimal Transcreener AMP²/GMP² Hilyte 647 Tracer concentration for the 1X AMP Detection Mix can be calculated based on the concentration of NAD^+ in the Enzyme Reaction:

[Tracer] (nM) = 3.78 x [NAD+] (µM) + 22.3

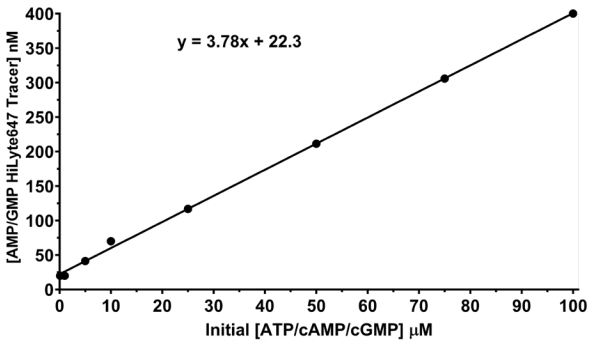


Figure 3. Relationship between NAD⁺ concentration in the Enzyme Reaction and the Optimal Transcreener AMP²/GMP² Hilyte 647 Tracer Concentration for detection under initial velocity conditions.

This equation, though not shown, is valid for NAD⁺ concentrations as high as 1000 µM, provided that the Enzyme Reaction and the 1X AMP Detection Mix are equivalent in volume. For example, if you are using 10 µM NAD+ in a 10 µL Enzyme Reaction, the optimal AMP²/GMP² Hilyte 647 Tracer concentration in the 10 µL 1X AMP Detection Mix would be (3.78 × 10) + 22.3 = 67.7 nM. In the 20 µL Complete Assay, the optimal AMP²/GMP² Hilyte 647 Tracer concentration would be half the concentration in the 1X AMP Detection Mix, or 33.83 nM for this example.

Please see **Section 7.2** for more information about optimizing the AMP²/GMP² Hilyte 647 Tracer concentration for cases in which the concentration calculated is not yielding the results that you require.

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener pADPr PARP TR-FRET Assay. Use enzyme buffer conditions and substrate concentrations that are optimal for your enzyme and experimental goals. If a compound screen is planned, you should include the library solvent at its final assay concentration. Run your enzymatic reaction at its requisite temperature and time period.

4.3.1 Enzyme Titration Considerations

For detection of inhibitors at single concentration or in dose response mode, we recommend selecting an enzyme concentration that produces a 50–80% change in TR-FRET signal (EC_{50} to EC_{80}) **(see Figure 4)** and an assay Z' factor of at least 0.7. This will result in initial velocity conditions, which correspond to the linear phase of the reaction (after conversion of TR-FRET values to AMP formed). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

4.3.2 Performing an Enzyme Assay

1. Prepare Assay Buffer with the required components for the Enzyme Reaction.
2. Dilute the target PARP Enzyme to 2X the desired starting concentration.
3. Add 10 µL of the PARP Enzyme to well 1 (including replicates).
4. Add 5 µL of Assay Buffer to wells 2-11, DO NOT add the Assay Buffer to well 1.
5. Transfer 5 µL from well 1 to well 2 and mix by pipetting, then transfer 5 µL from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 11 has received PARP Enzyme. Well 12 is to be used as a blank and should not include enzyme.
   IMPORTANT: After mixing the last well (11) in the dilution series, remove 5 µL from that well only and discard, so that all the wells contain 5 µL final volume.
6. Prepare a Substrate/CE Mix composed of the CE, NAD⁺ and any other components necessary for enzymatic activity.
   Note: Prepare the Substrate/CE Mix right before use to avoid substrate degradation. All components should be added at 2X the desired concentration in the 10 µL Enzyme Reaction. For example, CE concentration is 2X in the Substrate/CE Mix and 1X in the 10 µL Enzyme Reaction.
7. Start the enzyme reaction by adding 5 µL of the Substrate/CE mix in each well. It is recommended to incubate the Enzyme Reaction at 30°C for 60 minutes.
8. Prepare 1X AMP Detection Mix with an appropriate AMP²/GMP² Hilyte 647 Tracer concentration based on the NAD⁺ concentration in the Enzyme Reaction. The following table contains an example preparation based on 100 µM NAD⁺:

Note: This is an example of running an assay for HTS or to obtain a dose response. Your volumes and concentrations may vary. It is important to have a 1:1 ratio of Enzyme Mix and Detection Mix for the final assay readout.

Component	1X AMP Detection Mix - Add 10 µL Per Well			
	As Provided	Detection Mix Concentration	Final Concetration in 20 µL Complete Assay	Final Volume in AMP Detect Mix
AMP²/GMP² Antibody-Tb	800 nM	8 nM	4 nM	100 µL
AMP²/GMP² Hilyte 647 Tracer	10 µM	400 nM	200 nM	400 µL
Stop & Detect Buffer B	10X	1X	0.5X	1000 µL
Water				8,500 µL
Total				**10,000 µL**

9. Following the incubation, add 10 µL of 1X AMP Detection Mix and mix gently on a plate shaker for 40 to 60 seconds.
   Note: After 1X AMP Detection Mix is added to the Enzyme Reaction the final concentration of components in the 20 µL Complete Assay will be 0.5X AMP Detection Mix (200 nM AMP²/GMP² Hilyte 647 Tracer, 4 nM AMP²/GMP² Antibody-Tb, and 0.5X Stop & Detect Buffer B).
10. Incubate at room temperature (20–25°C) for 120 minutes to allow equilibration of assay components and measure TR-FRET.

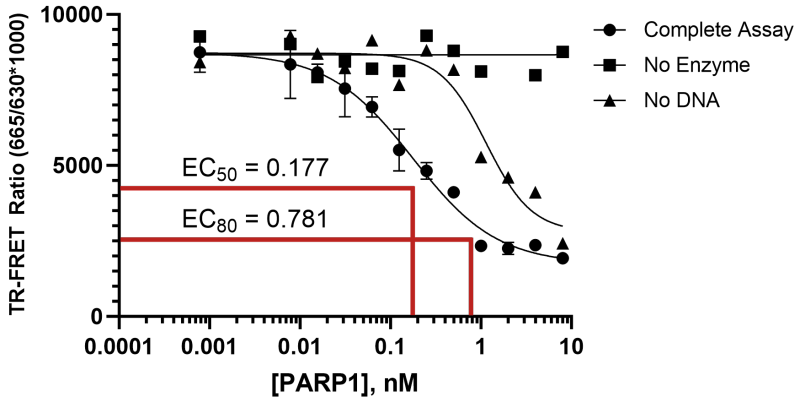


Figure 4. Enzyme titration curve. Example PARP1 Enzyme titration. The ideal range of enzyme concentrations is between EC_{50} and EC_{80}; the specific concentration may vary depending on the enzyme lot.

4.3.3 Enzyme Assay Controls

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

Component	Notes
No Inhibitor Control	This is a complete Enzyme Reaction with all detection components. It provides the maximum signal (minimal TR-FRET value) for an uninhibited enzyme reaction.
No Enzyme Control	This contains all Enzyme Reaction components in the absence of the target enzyme. It provides the minimal signal (maximal TR-FRET value), mimicking 100% Inhibition.
Minus-Substrate Control	This is an alternative to a No Enzyme Control; it may be more appropriate for enzymes that produce some background signal that is not substrate-dependent.

1. Mix Enzyme with Test Compound in 5 µL
   ~30 min
2. Add 5 µL of Substrate/ Coupling Enzyme Mix
   ~60 min
3. Add 10 µL 1X AMP Detection Mix
   ~120 min
4. Read Plate

4.4 Performing Single Compound Screening and Dose-Response Assays

Single Compound Screening and Dose-Response Assays follow the protocol listed in **Section 4.3.2**, with a slight change to the initial steps. Rather than performing a serial dilution of the target PARP Enzyme, a serial dilution of the test compound is performed. The target PARP Enzyme is then added to the test compounds; the total mixture volume should be 5 µL. The enzyme is added at a total concentration which yields the concentration calculated in **Section 4.3.1** after addition of the remaining components that make up the 10 µL Enzyme Reaction. We recommend mixing gently on a plate shaker for 40 to 60 seconds and preincubating the Enzyme Inhibitor Mix for the desired time (typically at least 15 minutes) at room temperature to allow equilibration of the E-I complex.

Note: Final concentration of test compounds should be based on the volume of the Enzyme Reaction. Compound volume added should be minimized to avoid signal interference caused by the compound or its solvent.

5.0 General Considerations

5.1 Endpoint Assay

The Transcreener pADPr PARP TR-FRET Assay is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA, which quenches the PARP enzymes and the CE by chelating Mg^{2+}.

5.2 Real-Time Assay

This assay can be performed in real time by eliminating stop reagents and including the 1X AMP Detection Mix components (antibody and tracer) in the Enzyme Reaction. However, this mode should only be used for relative activity comparisons, because the extended signal equilibration time precludes accurate quantitation of AMP. A standard curve run under similar conditions (continuous mode) will help in extrapolating the FRET ratios to amount of AMP product formed.

5.3 Reagent and Signal Stability

5.3.1 Signal Stability

The stability of the TR-FRET assay window at 10% substrate conversion was determined after the addition of the 1X AMP Detection Mix to the standard samples. The TR-FRET assay window at 10% substrate conversion 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.

5.3.2 AMP Detection Mix Stability

The AMP Detection Mix is stable for at least 16 hours at room temperature (20–25°C). If you prepare the AMP Detection Mix more than 30 minutes before addition, store it on ice or at 4°C until needed to help decrease the equilibration time.

6.0 Frequently Asked Questions

Question	Possible Solutions
Low Selectivity	Suboptimal tracer concentration • To achieve maximum sensitivity and assay window, the AMP²/GMP² Hilyte 647 Tracer concentration must be optimized for each starting NAD^+ concentration
No change in FRET observed	Low antibody/tracer activity or signal. • 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.
Is a standard curve required?	No, it is not required to run a standard curve. We recommend running the AMP standard curve if you want to convert raw signal ratio values to product formed. While designing a standard curve, make sure that most of the points are within the area of interest (initial velocity conditions). We do not recommend using a standard curve from previous experiments, rather generate a new curve with each experiment to achieve the most accurate result.
Can this assay be used with cell lysates?	The assay will only work with purified recombinant protein. The presence of nucleases in the lysates prohibits the use of Transcreener assays with lysates.
High background signal or change in signal after incubation with detection mixture.	Interference from impurities • Nuclease contamination in the buffer can cause the assay window to collapse, causing a change in signal. We recommend using nuclease-free water and freshly prepared buffer for each assay. • Some compounds may interfere with the detection mixture, causing a change in signal. • Bovine serum albumin (BSA) at concentrations >1% interferes with the detection reagents. Detergents, such as Brij-35, can be substituted for BSA in the enzyme reaction to prevent nonspecific binding of enzymes and substrates to the plate.

7.0 Appendix

7.1 Standard Curve

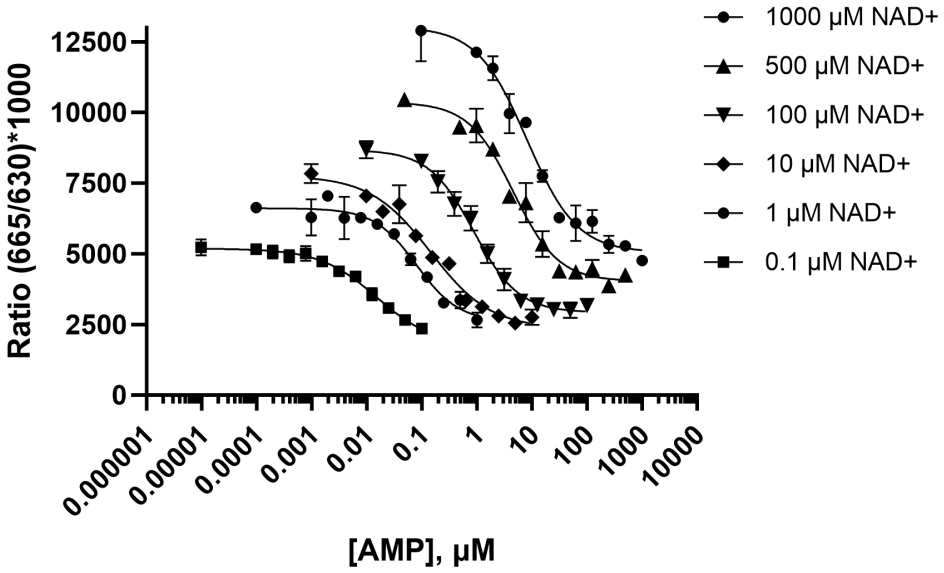
Use of a standard curve for conversion of values to amount of AMP formed allows quantitative measurement of the enzyme activity and accurate IC_{50} determinations; it is not typically done for screening at single concentrations. The standard curve mimics the Enzyme Reaction; the NAD^+ concentration remains constant to account for the background generated due to its degradation and reaction with the CE. The AMP standard curve allows calculation of the concentration of AMP produced in the Enzyme Reaction and, therefore, the percent AMP conversion.

In this example, a 12-point standard curve was prepared using the concentrations of AMP shown in the following Table. Commonly, 8- to 12-point standard curves are used.

Table 1. AMP Standard Curve. Concentrations of AMP to prepare a 12-point standard curve. Concentrations of NAD^+ are kept constant at a concentration equivalent to the highest concentration of AMP used (100 µM in this case).

Data Point	AMP (µM)
1	100.000
2	50.000
3	25.000
4	12.500
5	6.250
6	3.125
7	1.563
8	0.781
9	0.391
10	0.195
11	0.098
12	0.000

Figure 4. AMP Standard Curve. Sample data for 0.1 µM, 1 µM, 10 µM, 100 µM, and 1,000 µM AMP standard curves. The nucleotide concentration reflects the amount in the enzyme reaction, prior to the addition of the 1X AMP Detection Mix. Curves are obtained from a 20 µL Complete Assay consisting of 4 nM AMP^2/ GMP^2 Antibody-Tb, 0.5X Stop and Detect Buffer B, 0.5X reaction buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 0.1% Triton 100-X), 0.5X Substrate/ CE Mix (1X CE, and NAD+), AMP standards, and AMP^2/GMP^2 Hilyte 647 Tracer at varying concentrations based on the initial amounts of NAD+, as described in **Section 4.2.1**.



Use the following equations to calculate the Z' factor:

$$Z' = 1 - \frac{[(3 \times SD_{No\ Enzyme}) + (3 \times SD_{Complete\ Reaction})]}{|(Mean_{No\ Enzyme}) - (Mean_{Complete\ Reaction})|}$$

7.2 Optimizing the AMP²/GMP² Hilyte 647 Concentration

Using a tracer concentration calculated in **Section 4.2** will produce excellent results for most users. If it does not produce the results you require, we recommend that you perform an AMP²/GMP² Hilyte 647 Tracer titration in the buffer system ideal for your enzyme target. This titration will determine the optimal tracer concentration for your assay conditions. The substrate concentration in the enzyme reaction generally determines the appropriate concentration of AMP²/GMP² Hilyte 647 Tracer. We recommend using a concentration of tracer equivalent to the EC_{70} to EC_{85} acquired from the titration.

7.2.1. Titrate the AMP²/GMP² Hilyte 647 Tracer

1. Prepare the reaction buffer. Example: 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 0.1% Triton 100-X.
2. Add 5 µL of the reaction buffer to wells 2–12 (including replicates). Do not add it to well 1.
3. Dilute the AMP²/GMP² Hilyte 647 Tracer to 4 µM in reaction buffer. Add 10 uL to well 1 of each replicate.
4. Transfer 5 µL from well 1 to well 2 and mix by pipetting, then transfer 5 µL from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 12 has received AMP²/GMP² Hilyte 647 Tracer.
   IMPORTANT: After mixing the last well in the dilution series, remove 5 µL from that well only and discard, so that all the wells contain 5 µL final volume.
5. Prepare Substrate/CE Mix in reaction buffer and add 5 µL into each well. Gently mix the plate for few seconds using a plate shaker.
   Note: Prepare the Substrate/CE Mix right before use to avoid substrate degradation. CE concentration is 2X in the Substrate/CE Mix.
6. Add 10 µL of 8 nM AMP²/GMP² Antibody-Tb in 1X Stop & Detect Buffer B to each well.
7. Gently mix on a plate shaker for 40 to 60 seconds and then allow it to incubate at room temperature for 120 minutes before reading.

7.2.2 Calculate the Optimal AMP²/GMP² Hilyte Tracer Concentration

To determine the AMP²/GMP² Hilyte 647 Tracer concentration for EC_x, input the EC_{50} and hillslope values from a sigmoidal dose-response curve fit into the equation below.

$$EC_X = (X \div (100 - X))^{(1 \div |hillslope|)} \times EC_{50}$$

7.3 Using the Assay with Different Volumes and Plate Formats

Component	Total Volume	Enzyme Reaction Volume	1X AMP Detection Mix Volume
96 Well Low Volume Plate	50 µL	25 µL	25 µL
384 Well Low Volume Plate	20 µL	10 µL	10 µL
1536 Well Low Volume Plate	8 µL	4 µL	4 µL

Please check the working plate volumes from the manufacturer to ensure they are within the suggest volumes ranges of your plate.

7.4 Links to Applicable Application Notes

- A Guide to Navigating Hit Prioritization After Screening Using Biochemical Assays
- A Guide to Measuring Drug-Target Residence Times with Biochemical Assays
- List of Commonly Used Plate Readers and Settings

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