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Transcreener® ADPR FP Assay Technical Manual

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Abstract

The Transcreener® ADPR FP Assay is a biochemical HTS assay for measuring the production of ADP-ribose (ADPR) in enzymatic reactions (**Figure 1**). The assay uses a Coupling Enzyme to convert ADPR into AMP, which is then detected using a far-red, competitive fluorescence polarization (FP) assay. As examples, the Transcreener® ADPR FP assay can be used to detect the activity of human CD38, which forms ADPR from the hydrolysis of NAD, or Poly (ADP-ribose) glycohydrolase (PARG), which produces ADPR from the breakdown of poly-ADP-ribose.

The Transcreener® ADPR FP 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), sensitive detection of ADPR down to 10 nM and as high as 50 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.

Introduction

1 The Transcreener® ADPR FP Assay is a biochemical HTS assay for measuring the production of ADP-ribose (ADPR) in enzymatic reactions (**Figure 1**). The assay uses a Coupling Enzyme to convert ADPR into AMP, which is then detected using a far-red, competitive fluorescence polarization (FP) assay. As examples, the Transcreener® ADPR FP assay can be used to detect the activity of human CD38, which forms ADPR from the hydrolysis of NAD, or Poly (ADP-ribose) glycohydrolase (PARG), which produces ADPR from the breakdown of poly-ADP-ribose.

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Key Applications:

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

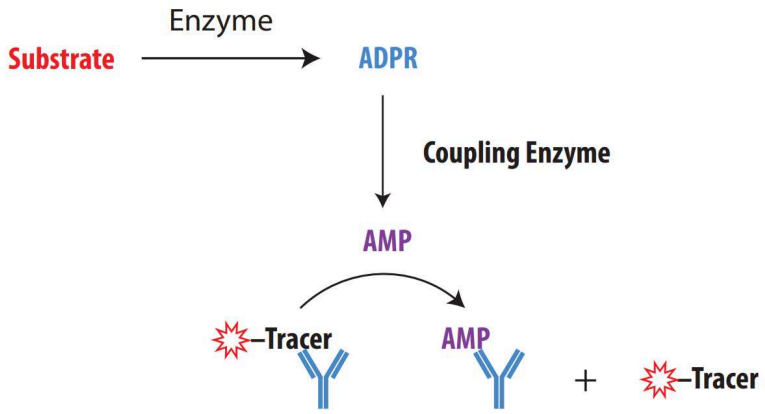

Figure 1. Schematic overview of the Transcreener ADPR FP Assay. ADPR produced by the target enzyme is converted to AMP by the Coupling Enzyme in real time. In the detection step, the Coupling Enzyme is quenched by EDTA and AMP displaces an Alexa Fluor® 633 tracer from the AMP^2/GMP^2 antibody, resulting in decreased fluorescence polarization.

Product Specifications

2

	Product	Quantity	Part #
	Transcreener® ADPR FP Assay	1,000 assays*	3030-1K
		10,000 assays*	3030-10K

*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
ADPR-AMP Coupling Enzyme should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that the ADPR-AMP Coupling Enzyme is stable up to 3 freeze-thaw cycles, we recommend aliquoting the enzyme 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.

Materials Provided

2.1

	Component	Composition	Notes
	AMP^2/GMP^2 Antibody	1.26 mg/mL solution in PBS with 10% glycerol*	Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3030-1K) or 10,000 assays (Part # 3030-10K).
	AMP^2/GMP^2 Alexa Fluor® 633 Tracer	800 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	The final tracer concentration in the 20 µL Complete Assay is 4 nM. Sufficient tracer is included in the kit to complete 1,000 assays (Part # 3030-1K) or 10,000 assays (Part # 3030-10K).
	ADPR-AMP Coupling Enzyme	400X ADPR-AMP Coupling Enzyme in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 10% glycerol	Sufficient for 1,000 assays (Part # 3030-1K) or 10,000 assays (Part # 3030-10K) with Coupling Enzyme present in excess to ensure ADPR 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 Coupling Enzyme Reaction 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+}.
	ADPR	5 mM ADPR in deionized water, pH 7.0	The ADPR in this kit can be used to create a standard curve to convert mP values to ADPR product formed.

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

Materials Required but Not Provided

2.2

	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® ADPR FP Assay is designed for use with purified enzymes capable of producing ADPR from a

	Component	Notes
		substrate. Contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
	Substrate	The Transcreener® ADPR FP Assay is designed for use with purified enzymes capable of producing ADPR from a substrate. Substrate impurities and degradation may produce background signal and reduce the assay window.
	Plate Reader	A multimode microplate reader configured to measure FP of the AMP^2/GMP^2 AlexaFluor® 633 Tracer is required. Transcreener® FP Assays have been successfully used 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 readersand 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 black with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4514). 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.

Note

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

Before You Begin

3 **1.** Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).

2. Check the FP instrument and verify that it is compatible with the assay being performed (see **Section 4.1**).

Protocol

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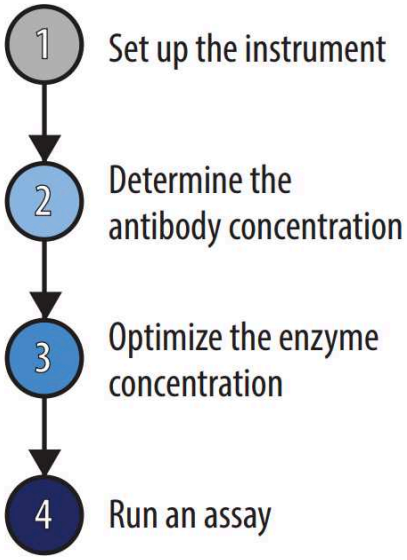


The Transcreener® ADPR FP Assay kit optimization follows a simple format. First, the instrument is configured with proper parameters to ensure a successful data readout (**Section 4.1**). This is followed by an optimization of the antibody concentration to obtain the desired dynamic range (**Section 4.2**). Next, an optimal enzyme concentration is determined to provide a good assay window while meeting initial velocity requirement (**Section 4.3**). Once the instrument parameters, antibody, and enzyme optimization are complete, the assay itself consists of a simple mix-and-read protocol (**Section 4.4**).

The assay protocol in **Section 4.4** 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 10 µL Enzyme Reaction is composed of two components: 5 µL of the target enzyme diluted to the desired concentration and 5 µL of a preparation containing both the Coupling Enzyme and the required substrate. The Coupling Enzyme working concentration is 1X in 10 µL Enzyme Reaction, which is present in at least 5x excess over what is required for complete conversion of ADPR to AMP in real time. Once the Enzyme Reaction is complete, simply add 10 µL of 1X AMP Detection Mix to your enzyme reaction and read the plate. The use of different densities or reaction volumes will require changes in reagent quantities (see **Section 7.3** for example reaction volumes).

Note: Tracer concentration remains constant at 4 nM in the 20 µL Complete Assay regardless of changes to other reaction conditions. It is not recommended that this parameter be changed.

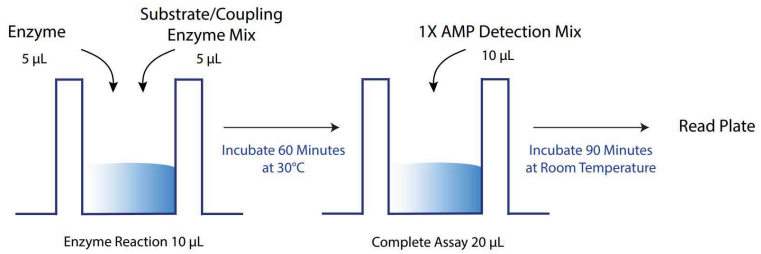


Figure 2. An outline of the Assay Protocol. The target enzyme reaction is run in the presence of Coupling Enzyme, so that ADPR is converted to AMP in real time. After the Enzyme Reaction incubation is complete, AMP detection reagents are added (Transcreener® AMP^2/GMP^2 Antibody and Tracer) along with EDTA to quench the Coupling Enzyme.

Set Up the Instrument

4.1 Becoming familiar with ideal instrument settings for FP is essential to the success of the Transcreener® ADPR FP Assay.

4.1.1 Verify That the Instrument Measures FP

Ensure that the instrument is capable of measuring FP (not simply fluorescence intensity) of the AMP^2/GMP^2 AlexaFluor 633 Tracer.

Note

Note: A complete list of instruments and instrument-specific application notes can be found online at:
https://www.bellbrooklabs.com/technicalresources/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 mP Window for the Instrument

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

Use AMP^2/GMP^2 Alexa Fluor 633 Tracer at 4 nM in a 20 µL Complete Assay. This mimics the 2-fold dilution when adding an equal volume of 1X AMP Detection Mix to an Enzyme Reaction.

High FP Mixture

	Component	As Provided	Final Concentration in 20 µL Complete Assay	Example: 25 Assays	Your Numbers
	AMP^2/GMP^2 Antibody	1.26 mg/mL*	5 µg/mL	2.4 µL**	
	AMP^2/GMP^2 Alexa Fluor 633 Tracer	800 nM	4 nM	3 µL	
	Stop & Detect Buffer B	10X	0.5X	30 µL	

	Component	As Provided	Final Concentration in 20 µL Complete Assay	Example: 25 Assays	Your Numbers
	Water			564.6 µL	
	Total			**600.0 µL**	

*Please note AMP^2/GMP^2 Antibody concentration varies by lot number. This is an example and should be adjusted based on stock concentration accordingly.
**Pipetting small sample volumes accurately requires the correct equipment and proper technique. An extra dilution step may be required to ensure accuracy.

Low FP Mixture
Prepare the following solution.

	Component	As Provided	Final Concentration in 20 µL Complete Assay	Example: 25 Assays	Your Numbers
	AMP^2/GMP^2 Alexa Fluor 633 Tracer	800 nM	4 nM	3 µL	
	Stop & Detect Buffer B	10X	0.5X	30 µL	
	Water			567 µL	
	Total			**600.0 µL**	

4.1.3 Measure the FP
Subtract the Low FP Mixture readings from the corresponding High FP Mixture readings. The difference between the low and high FP values should be >100 mP.

Note

Caution: Contact BellBrook Labs Technical Service for assistance if the assay window is <100 mP.

Determine Optimal AMP^2/GMP^2 Antibody Concentration

4.2 The antibody is the only assay component that requires adjustment for different reaction conditions. Its concentration will define the dynamic range of the assay, and it should be adjusted based on the ADPR concentration produced in the Enzyme Reaction. We have determined optimal AMP^2/GMP^2 Antibody concentrations for up to 50 µM ADPR.

Using the AMP^2/GMP^2 Antibody concentration calculated in the chart below will produce excellent results for most users. If it does not produce satisfactory results, an AMP^2/GMP^2 antibody titration in your specific conditions is recommended. Please refer to **Section 7.2** for instructions on preparing the AMP^2/GMP^2 Antibody titration in the buffer system ideal for your target enzyme.

	ADPR Concentration in 10 µL Enzyme Reaction	AMP2/GMP2 Antibody Concentration in 10 µL 1X AMP Detect Mix
	0.2 - 0.7 µM	2 µg/mL
	0.7 - 3 µM	10 µg/mL

	ADPR Concentration in 10 µL Enzyme Reaction	AMP2/GMP2 Antibody Concentration in 10 µL 1X AMP Detect Mix
	3 - 10 µM	50 µg/mL
	>10 µM	100 µg/mL

Optimize the Enzyme Concentration

4.3 Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener ADPR FP 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 FP signal (EC_{50} to EC_{80}) (see **Figure 3**) and an assay window of at least 100 mP. This will result in initial velocity conditions, which correspond to the linear phase of the reaction after conversion of mP values to ADPR formed. The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

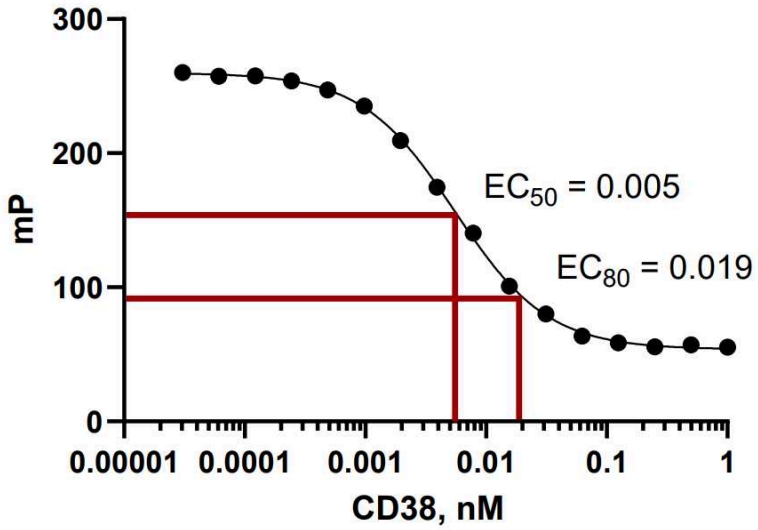


Figure 3. Enzyme titration curve. Example enzyme titration with CD38. The ideal range of enzyme concentrations is between EC_{50} and EC_{80}; the specific concentration may vary depending on the enzyme lot.

4.3.2 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 FP 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 FP 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.

Performing Single Compound Screening and Dose-Response Assays

4.4 **4.4.1 Experimental Samples**

1. Perform a serial dilution of test compound with your method of choice. Add the enzyme to the test compounds at the desired concentration so that the total volume of this mixture is 5 µL. Mix gently on a plate shaker for 40 to 60 seconds. Preincubate the Enzyme Inhibitor Mix for the desired time (typically at least 30 minutes) at room temperature to allow equilibration of the E-I complex.

Note

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.

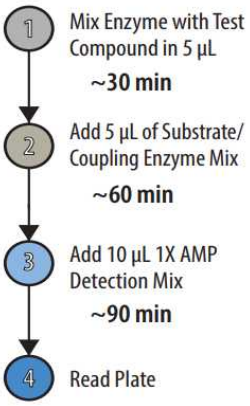
2. Start the enzyme reaction by adding 5 µL of the Substrate/Coupling Enzyme Mix then mix gently on a plate shaker for 40 to 60 seconds. It is recommended to incubate the enzyme reaction at 30°C for 60 minutes.

Note: Prepare the Substrate/Coupling Enzyme Mix right before use to avoid substrate degradation. Coupling Enzyme concentration is 2X in the Substrate/Coupling Enzyme Mix and 1X in the 10 µL Enzyme Reaction.

3. Prepare 1X AMP Detection Mix as follows:

1X AMP Detection Mix - Add 10 µL Per Well

	Component	As Provided	Detection Mix Concentration	Final Concentration in 20 µL Complete Assay	Final Volume in AMP Detect Mix
	AMP^2/GMP^2 Antibody	1.26 mg/mL	10 µg/mL	5 µg/mL	79.4 µL
	AMP^2/GMP^2 Alexa Fluor 633 Tracer	800 nM	8 nM	4 nM	100 µL
	Stop & Detect Buffer B	10X	1X	0.5X	1000 µL
	Water				8,820.6 µL
	Total				**10,000 µL**



4. Following the 60 minute incubation, add 10 µL of 1X AMP Detection Mix and mix gently on a plate shaker for 40 to 60 seconds.

Note: After Detection Mix is added to Enzyme Reaction the final concentration of components in the 20 µL Complete Assay will be 0.5X the Detection Mix (4 nM tracer, 5 µg/mL AMP^2/GMP^2 Antibody, and 0.5X Stop & Detect Buffer B).

5. Incubate at room temperature (20–25°C) for 90 minutes to allow equilibration of assay components and measure FP.

4.4.2 AMP Detection Controls

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

	Component	Notes
	Minus Antibody (Free Tracer) Control	This control contains 4 nM AMP^2/GMP^2 Tracer in the 20 µL Complete Assay without the AMP^2/GMP^2 Antibody and is set to low mP, typically between 20-50 mP depending on the instrument.
	Minus Tracer Control	This control contains the AMP^2/GMP^2 Antibody without the AMP^2/GMP^2 Tracer and is used as a sample blank for all wells. It contains the same AMP^2/GMP^2 Antibody concentration that will be used in the assay.

General Considerations

5

Endpoint Assay

5.1 The Transcreener® ADPR FP Assay is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA, which quenches the coupling enzyme by chelating Mg^{2+}.

Reagent and Signal Stability

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

5.2.1 Signal Stability

The stability of the FP assay window at 10% substrate conversion was determined after the addition of the 1X AMP Detection Mix to the standard samples. The FP 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 FP on the following day, seal the plates to prevent evaporation.

5.2.2 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.

Frequently Asked Questions

6

	Question	Possible Solutions
	No change in FP observed	Low antibody/tracer activity or Δ mP 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 ADPR standard curve if you want to convert raw mP 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 FP. 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.

Appendix

7

Standard Curve

7.1 The standard curve mimics any enzyme reaction where ADPR is formed and then converted to AMP. In this example a 16-point standard curve was prepared using the concentration of ADPR shown in **Table 1**. Commonly, 12-16 point standard curves are used. You may choose to not run a standard curve depending on your experiment.

	Data Point	ADPR (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	0.049
	13	0.024
	14	0.012
	15	0.006
	16	0.003

Table 1. ADPR Standard Curve. Standard curve to help convert raw mP values to product formed.

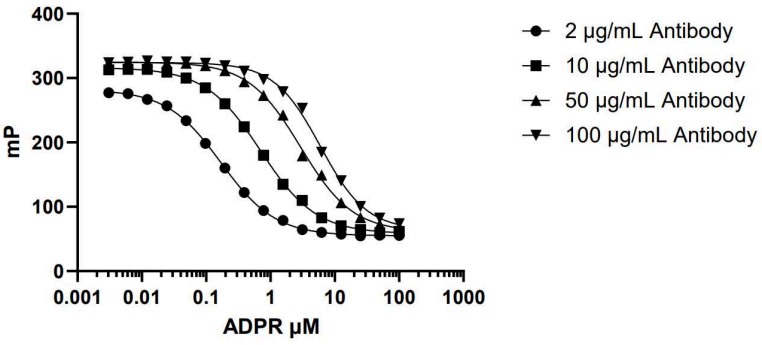


Figure 4. ADPR Standard Curve. Standard curve with varying AMP^2/GMP^2 Antibody concentrations, in 10 µL 1X AMP Detection Mix, to obtain ideal sensitivity.

Standard curves illustrate tuning the assay to the appropriate levels for the sensitivity desired. In this example, 100 µM ADPR standard curves were completed using 2, 10, 50,

and 100 µg/mL final concentrations of AMP^2/GMP^2 antibody in the 10 µL 1X AMP Detection Mix. Using the correct antibody concentration for the amount of ADPR produced in the assay is imperative to achieving quality results. Use the following equations to calculate the Z′ factor:

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

Optimizing the AMP^2/GMP^2 Antibody Concentration

7.2 Using an antibody concentration from the chart 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 AMP^2/GMP^2 Antibody titration in the buffer system ideal for your enzyme target. This titration will determine the optimal antibody concentration for your assay conditions. The substrate concentration in the enzyme reaction generally determines the appropriate concentration of AMP^2/GMP^2 Antibody. We recommend using a concentration of antibody equivalent to the EC_{70} to EC_{85} acquired from the titration.

7.2.1. Titrate the AMP^2/GMP^2 Antibody

1. Prepare the reaction buffer. Example: 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.001% BSA and 0.01% Brij-35. Include desired substrate but omit the enzyme.
2. Add 10 µL of the reaction buffer to wells 2–16 (including replicates). Do not add reaction buffer to well 1.
3. Add 20 µL of AMP^2/GMP^2 Antibody (at 0.2 mg/mL concentration in the same reaction buffer) to well 1 of each replicate.
4. Transfer 10 µL from well 1 to well 2 and mix by pipetting, then transfer 10 µL from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 16 has received AMP^2/GMP^2 Antibody.

IMPORTANT: After mixing the last well in the dilution series, remove 10 µL from that well only and discard, so that all the wells contain 10 µL final volume.

5. Add 10 µL of 8 nM AMP^2/GMP^2 Alexa Fluor® 633 Tracer in 1X Stop & Detect Buffer B to each well.
6. Gently mix on a plate shaker for 40 to 60 seconds and then allow it to incubate at room temperature for 90 minutes before reading.

7.2.2 Calculate the Optimal AMP^2/GMP^2 Antibody Concentration

To determine the AMP^2/GMP^2 antibody concentration for EC_X, input the EC_{50} and hillslope values from a sigmoidal dose-response curve fit into the equation below.

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

Using the Assay with Different Volumes and Plate Formats

7.3

	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.

Contact Information

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