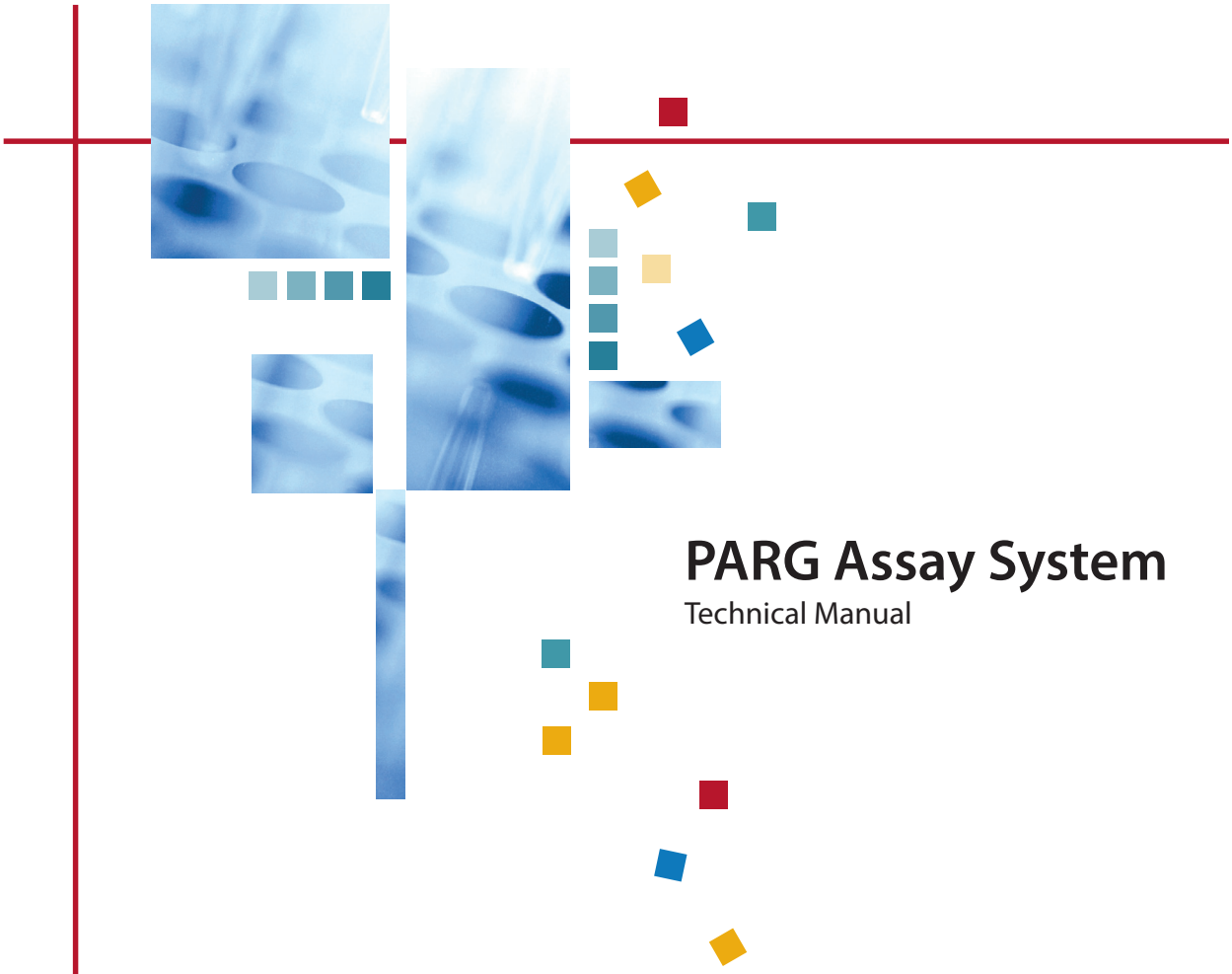
BellBrook Labs

PARG Assay System



PARG Assay System

Technical Manual

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Instructions for Part Numbers 3033-1K and 3033-10K

Rev 2023-08

PARG Assay System Technical Manual

Contents

1.0 Introduction

The PARG Assay System is intended for use with the Transcreener® ADPR FP Assay Kit (Part #3030) to measure enzymatic activity for PARG (poly(ADP-ribose) glycohydrolase). PARG produces ADPR from the breakdown of poly(ADP-ribose) (polyADPR). It plays an important role in DNA damage repair and has been seen as a potential target for anticancer therapy. The Transcreener® ADPR FP assay uses a Coupling Enzyme to convert ADPR to AMP, which is then detected using a far-red, competitive fluorescence polarization (FP) assay. It is in a single addition, endpoint mix-and-read format in which the Coupling Enzyme is quenched by addition of the detection reagents. The assay has been optimized and extensively validated for high throughput screening (HTS) and inhibitor dose response measurements using most multimode plate readers.

The PARG Assay System provides all reagents required to screen and profile PARG inhibitors when used with the Transcreener® ADPR FP Assay Kit, including purified human PARG (amino acids 1-976, C-terminal 6xHis) and polyADPR Substrate. Note that the assay has been optimized to minimize interference of test compounds with the Coupling Enzyme (excess Coupling Enzyme is present), however, we recommend counter screening against the detection reagents to triage false positives. Additionally, the protocol is configured for 384-well plates; use of different multi-well plate formats will require adjustment of reagent concentrations utilized in the assay.

Key Applications:

- Screening for PARG inhibitors
- Generating dose response curves and IC_{50} values for PARG inhibitors
- Kinetic and mechanistic analyses

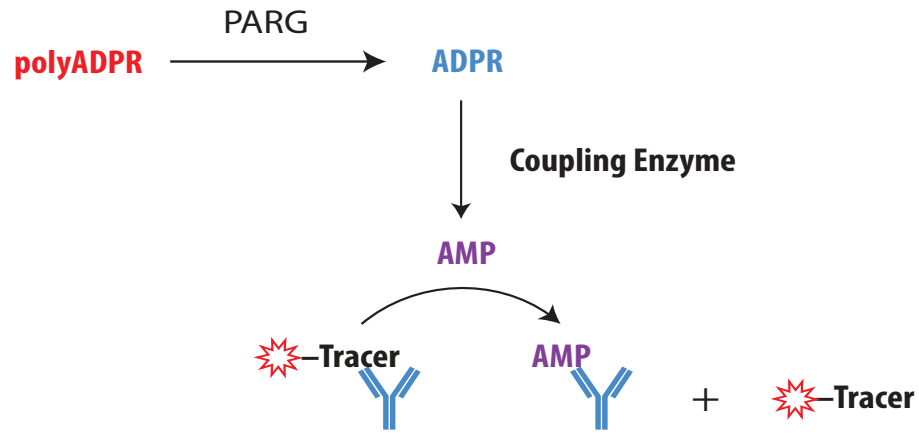


Figure 1. Schematic Overview of the PARG Assay System with the Transcreener® ADPR FP Assay. ADPR produced by PARG is converted to AMP by the Coupling Enzyme in real time. In the detection step, the Coupling Enzyme is quenched by EDTA, and AMP displaces an Alexa Fluor® 633 tracer from the AMP2/GMP2 antibody, resulting in decreased fluorescence polarization.

2.0 Product Specifications

Product	Quantity	Part #
PARG Assay System	1,000 assays*	3033-1K
	10,000 assays*	3033-10K

*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.

Storage

Enzymes and polyADPR should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that PARG and polyADPR are stable up to 3 freeze-thaw cycles, we recommend aliquoting and snap-freezing them 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
PARG Enzyme	0.1 mg/mL (894 nM)* in 50 mM Tris (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM TCEP	Amino acids 1-976, C-terminal 6xHis, 111.9 kDa. Sufficient enzyme is included in the kit to complete 1,000 assays (Part # 3033-1K) or 10,000 assays (Part # 3033-10K).
polyADPR, 200 µM	200 µM in TE buffer (10 mM Tris (pH 8), 1 mM EDTA)	polyADPR is produced by chemo-enzymatic synthesis; polymer size distribution can vary slightly from lot to lot. Refer to Certificate of Analysis for K_m of a specific lot.
Enzyme Assay Buffer B, 10X	500 mM Tris (pH 7.5), 100 mM $MgCl_2$, 0.01% BSA, and 0.1% Brij-35	Use the Enzyme Assay Buffer in the Enzyme Reaction and for preincubation with inhibitors. Changes to the assay buffer could affect enzyme activity and/or detection of ADPR.
384-Well Low Volume Black Assay Plates	Corning #4514	Black polystyrene non-binding surface assay plates in either a 3-pack (1,000+ Assays) or a 30-pack (10,000+ Assays). We strongly recommend the use of these plates as inconsistent results have been observed with other plates.

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

2.2 Materials Required But Not Provided

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
Plate Reader	A multimode microplate reader configured to measure FP 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 a submicroliter volumes into 384-well plates.
Laboratory Incubator	An incubator model that is capable of maintaining temperature stability at 30°C is required.

Transcreener® ADPR FP Assay - SOLD SEPARATELY		
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	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 (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	200 mM HEPES (pH 7.5), 400 mM EDTA, and 0.2% Brij-35	The EDTA in the Stop & Detect Buffer B quenches the Coupling Enzyme Reaction by chelating Mg^{2+}. Therefore, the assay should work as an end-point assay for any target enzymes, as long as the EDTA is at least equimolar to the Mg^{2+}. In the case of the PARG Assay System, the final concentrations of Mg^{2+} and EDTA in the Complete Assay are 5 mM and 20 mM, respectively.
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.

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 assay being performed (see Full list of compatible plate readers and settings)
3. Please read and understand the Transcreener® ADPR Assay Technical Manual prior to using this kit.

4.0 Protocol

The methods described below are for single-addition, endpoint detection: the Coupling Enzyme is quenched by the addition of EDTA along with the detection reagents (see **Figure 2**). The methods were designed for 384-well plates using a 10 µL PARG Enzyme Reaction and 10 µL of detection/quench reagents (final volume 20 µL when the plates are read). The use of different plate densities or reaction volumes will require changes in reagent quantities (see **Section 5.1** for example reaction volumes).

The methods were optimized for initial velocity detection of ADPR formation by PARG over a range of 0.1 to 1.0 µM ADPR with polyADPR at approximately 5X K_m concentration. Running the assay at this saturated concentration allows better dynamic range and reduces data variation. If it is desired to run the assay at substrate K_m, a reduction of the AMP^2/GMP^2 Antibody concentration by half would be necessary to adjust the dynamic range toward detection of lower amount of ADPR.

Note: Tracer concentrations remain constant at 4 nM in the 20 µL Complete Assay regardless of changes to other reaction conditions. Additionally, the Coupling Enzyme is present in at least 5X excess over what is required for complete conversion of ADPR to AMP in real time over a range of initial polyADPR concentrations; it is not recommended that this parameter is changed.

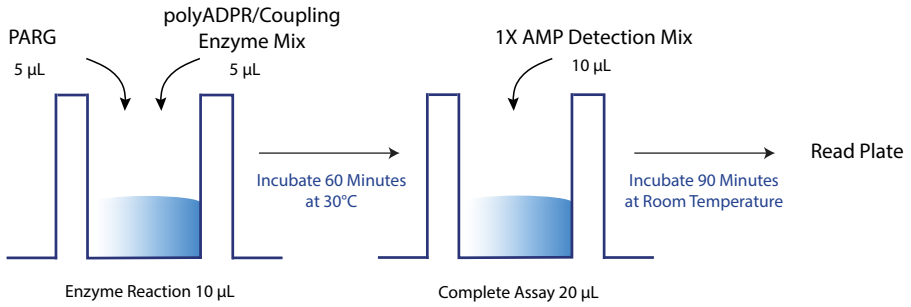


Figure 2. An Outline of the Procedure. The PARG Enzyme Reaction is initiated by the addition of polyADPR/Coupling Enzyme Mix. After the Enzyme Reaction incubation is completed, AMP detection reagents are added (Transcreener® AMP^2/GMP^2 Antibody and Tracer) along with EDTA to quench the Coupling Enzyme.

Component	10 µL Enzyme Reaction Components	
	Working Stock	Final Concentration in 10 µL
Enzyme Assay Buffer B, 10X	1X in Nuclease Free Water	1X (50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.001% BSA, and 0.01% Brij-35)
PARG, 0.1 mg/mL (894 nM)	2X in 1X Enzyme Assay Buffer B	5 pM - 100 pM*
polyADPR, 200 µM	10X K_m concentration in 1X Enzyme Assay Buffer B (with 2X Coupling Enzyme)	5X K_m concentration**
Coupling Enzyme, 400x	2X in 1X Enzyme Assay Buffer B (with 10X K_m polyADPR)	1X

Table 1. PARG Enzyme Reaction Components. Concentrations are provided for the standard protocol using 5 µL of PARG Enzyme Mix and 5 µL of polyADPR/Coupling Enzyme Mix for the Enzyme Reaction.

*See Section 4.1 for Determining the Optimal Enzyme Concentration.

**polyADPR is produced by chemo-enzymatic synthesis; polymer size distribution can vary slightly from lot to lot, which can affect the K_m. Please refer to Certificate of Analysis for K_m of a specific lot.

Table 2. 1X AMP Detection Mix Components. Volumes provided in the table are based on preparation of a 10 mL solution; adjust these appropriately for the desired volume, including 10% extra for pipetting dead volume.

	1X AMP Detection Mix - Add 10 µL Per Well			
Component	As Provided	Detection Mix Concentration	Final Concentration in 20 µL Complete Assay	Example
AMP²/GMP² Antibody*	1.26 mg/mL	2 µg/mL	1 µg/mL	15.9 µL
AMP²/GMP² Alexa Fluor ® 633 Tracer	800 nM	8 nM	4 nM	100.0 µL
Stop & Detect Buffer B, 10X	10X	1X	0.5X	1,000.0 µL
Nuclease Free Water	-	-	-	8,884.1 µL
Total Volume	-	-	-	10,000 µL

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

4.1 Determining the Optimal Enzyme Concentration

Using the enzyme concentration suggested in the PARG Enzyme Certificate of Analysis should provide a robust signal that is within the linear range for ADPR formation. However, for best results, we suggest performing an enzyme titration to identify the optimal enzyme concentration (EC_{50} to EC_{80}), especially when running the assay in a different buffer system or with a different polyADPR concentration. This example uses a 2X serial dilution; it should be performed at least in duplicate. If a compound screen is planned, you should include the solvent (e.g., DMSO) at its final assay concentration.

4.1.1 Enzyme Titration Steps

1. Prepare 1500 µL 1X Enzyme Assay Buffer B: dilute 150 µL of 10X Enzyme Assay Buffer B in 1350 µL Ultrapure Nuclease Free Water.
2. Prepare 223 µL of 4 nM PARG Enzyme: dilute 1 µL of 894 nM PARG Enzyme in 222 µL 1X Enzyme Assay Buffer B.
3. Add 10 µL of the PARG Enzyme to well 1 (including replicates).
4. Add 5 µL of 1X Enzyme Assay Buffer B to wells 2-16, DO NOT add the 1X Enzyme Assay Buffer B 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 15 has received PARG Enzyme. Well 16 is to be used as a blank and should not include enzyme.
   IMPORTANT: After mixing the last well (15) 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 250 µL of polyADPR/Coupling Enzyme Mix: dilute 1.25 µL 400X Coupling Enzyme and 10X K_m concentration of polyADPR in 1X Enzyme Assay Buffer B.
   Note: Prepare the polyADPR/Coupling Enzyme Mix right before use to avoid degradation of the substrate and possible reduction of the assay window. As for polyADPR, please refer to Certificate of Analysis for K_m of a specific lot.
7. Start the Enzyme Reaction by adding 5 µL of the polyADPR/Coupling Enzyme Mix to every well (1-16). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
8. Prepare 650 µL 1X AMP Detection Mix based on the concentrations provided in **Table 2**: 65 µL 10X Stop & Detect Buffer B, 6.5 µL AMP²/GMP² Alexa Fluor® 633 Tracer and 2 µg/mL AMP²/GMP² Antibody in Ultrapure Nuclease Free Water.
9. Add 10 µL of 1X AMP Detection Mix to every well (1-16), in replicate.
10. 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.
    Note: The reagent volumes indicated above are sufficient for running the enzyme titration in duplicate plus excess for pipetting dead volume. Scaling of volumes can be performed if necessary.

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 formation (see **Figure 7**). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

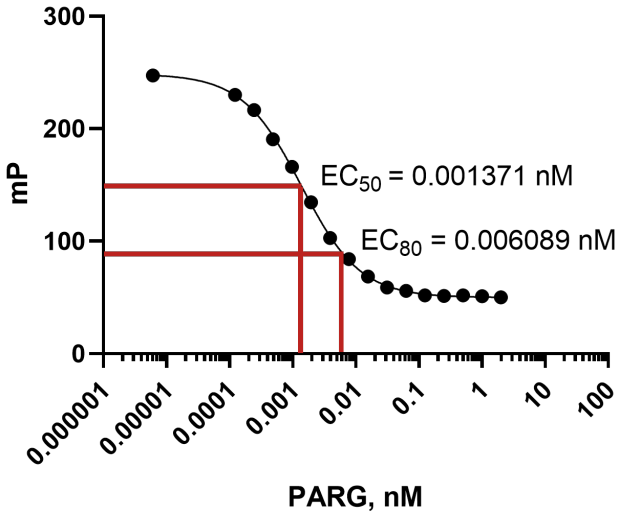
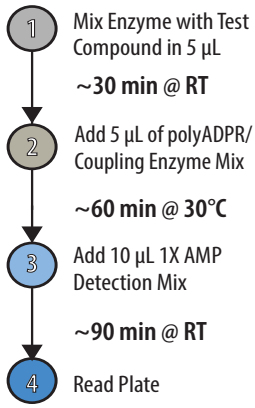


Figure 3. Enzyme Titration Curve. Example PARG Enzyme titration. The ideal range of enzyme concentrations is between EC_{50} and EC_{80}; the specific concentration may vary depending on the enzyme lot.

4.2 Performing Single Compound Screening and Dose-Response Assays

4.2.1 Experimental Samples

1. Perform a serial dilution of test compounds with your method of choice. Add PARG 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: Final concentration of test compounds should be based on the volume of the Enzyme Reaction.
2. Start the Enzyme Reaction by adding 5 µL of the polyADPR/Coupling Enzyme Mix. It is recommended to incubate the Enzyme Reaction at 30°C for 60 minutes.
   Note: The final volume of the Enzyme Reaction mixture should be 10 µL for 384 well plates. See Section 5.1 for a list of other plate formats.
3. After the incubation, add 10 µL of 1X AMP Detection Mix to the 10 µL Enzyme Reaction and mix the 20 µL Complete Assay using a plate shaker for 40 to 60 seconds.
4. Incubate at room temperature for 90 minutes and measure FP.



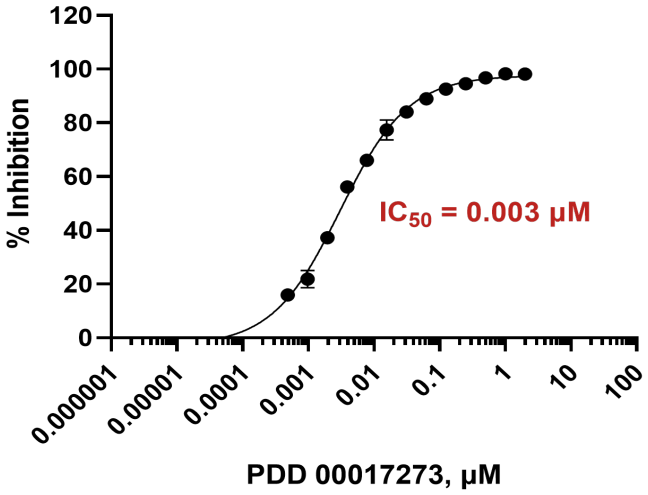


Figure 4. Dose-Response Curve. Example dose response curve with probe inhibitor PDD 00017273.

4.3 Setting Up a Standard Curve

Use of a standard curve for conversion of mP values to amount of ADPR formed allows quantitative measurement of PARG activity and accurate IC_{50} determinations; it is not typically done for screening at single concentrations. Here, we describe preparation of a standard curve using 2X serial dilution from 25 µM ADPR, which encompasses the appropriate range for this assay using 5X K_m concentration of polyADPR.

Note: The reagent volumes indicated below are sufficient for running the standard curve in duplicate plus excess for pipetting dead volume.

1. Prepare 600 µL 1X Enzyme Assay Buffer B: dilute 60 µL 10X Enzyme Assay Buffer B in 540 µL Ultrapure Nuclease Free Water.
2. Prepare 100 µL of 50 µM ADPR: dilute 1 µL 5 mM ADPR stock in 99 µL 1X Enzyme Assay Buffer B.
3. Prepare 250 µL of polyADPR/Coupling Enzyme Mix: dilute 1.25 µL 400X Coupling Enzyme and 10X K_m concentration of polyADPR in 1X Enzyme Assay Buffer B.
   Note: Prepare the polyADPR/Coupling Enzyme Mix right before use to avoid degradation of the substrate and possible reduction of the assay window. As for polyADPR, please refer to Certificate of Analysis for K_m of a specific lot.

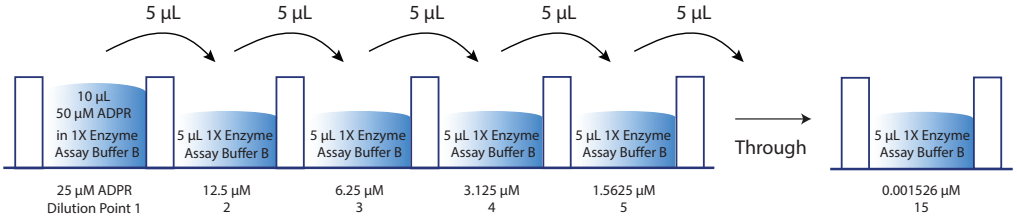


Figure 5. Performing a Serial Dilution. Example 2-fold serial dilution of ADPR to generate a standard curve.

4. Add 10 µL of the 50 µM ADPR to well 1 (including replicates).
5. Add 5 µL of 1X Enzyme Assay Buffer B to wells 2-16, **DO NOT** add the 1X Enzyme Assay Buffer B to well 1.
6. 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 15 has received ADPR. Well 16 is to be used as a blank and should correspond to 0 µM of ADPR on the standard curve.
   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.
7. Add 5 µL of the polyADPR/Coupling Enzyme Mix to every well (1-16). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
8. Prepare 650 µL 1X AMP Detection Mix based on the concentrations provided in **Table 2**: 65 µL 10X Stop & Detect Buffer B, 6.5 µL AMP²/GMP² Alexa Fluor® 633 Tracer and 2 µg/mL AMP²/GMP² Antibody in Ultrapure Nuclease Free Water.
9. Add 10 µL of 1X AMP Detection Mix to every well (1-16).
10. 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.

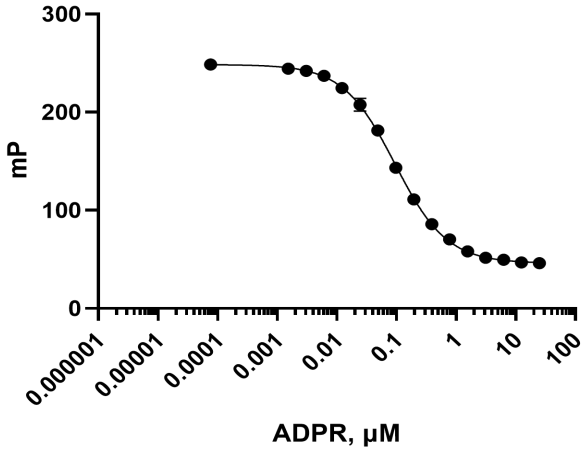


Figure 6. ADPR Standard Curve. Standard curve using 1X AMP Detection Mix as shown in **Table 2**.

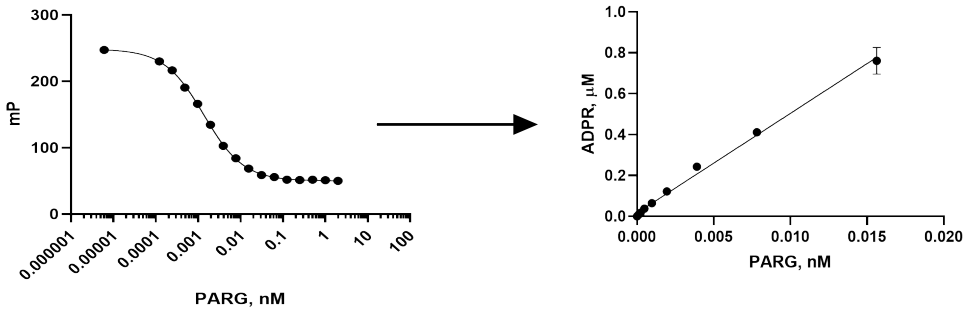


Figure 7. Enzyme titration curve converted to ADPR formed. Raw polarization signal (mP) is converted to ADPR formed using a standard curve as described in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism.

4.4 Measuring Assay Robustness with Z'

By taking into account both dynamic range and data variability at the high and low ranges of the assay, the Z' statistic provides a measure of what is of most interest when considering the suitability of an assay for HTS: the usable screening or "assay window." It is a dimensionless coefficient for the quality of the screening window that is relevant for any assay, regardless of detection method or readout, without the intervention of test compounds. As a guideline, a Z' value of 0.5 or greater is generally considered to be indicative of a very good screening window for a biochemical assay, thus the assay is an excellent assay. When running the PARG assay, run the controls with and without enzyme (no test compound) to achieve final results. Use the following formula to determine Z'.

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

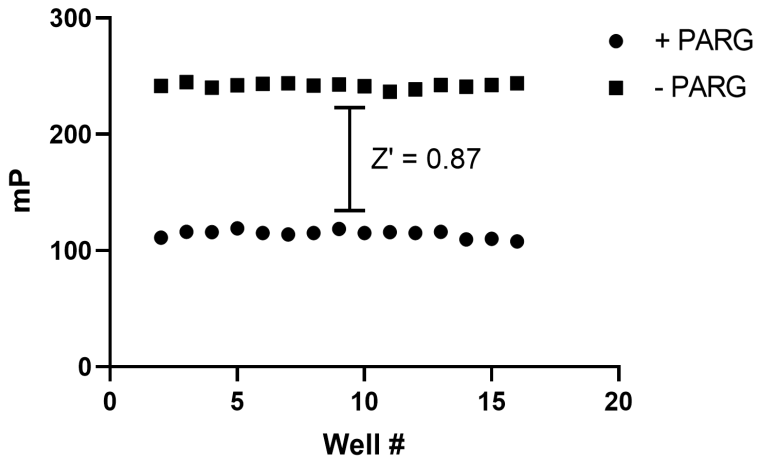


Figure 8. Z' Measurement. Complete Assay is performed with and without PARG enzyme (n=16). Z' is then calculated based on the formula shown in **Section 4.4**.

5.0 Appendix

5.1 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.

5.2 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
- Using GraphPad Prism to Interpolate Data from a Standard Curve to Generate a Dose-Response
- List of Commonly Used Plate Readers and Settings

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