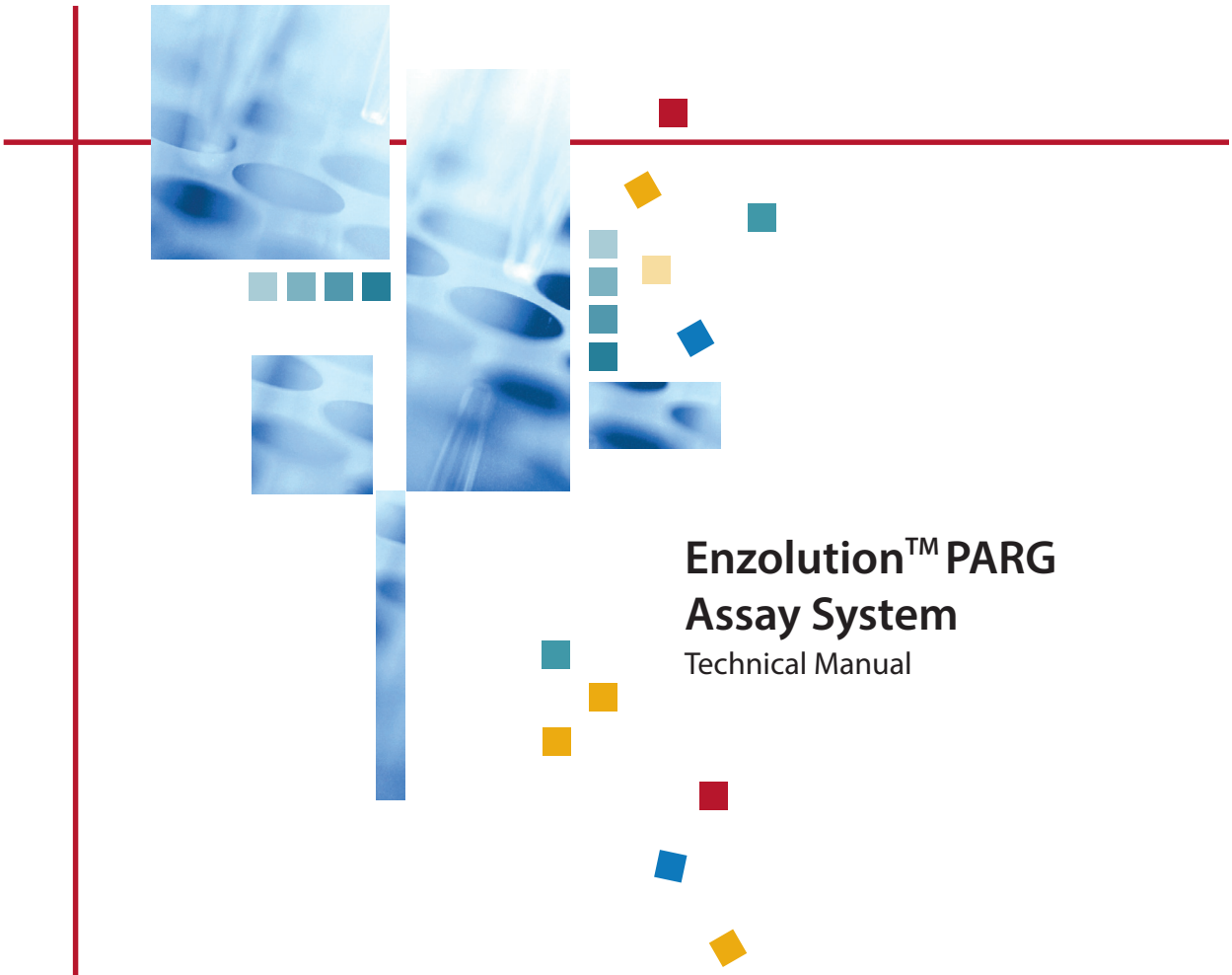
BellBrook Labs

PARG Assay System



# Enzolution™ PARG Assay System

## Technical Manual

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

Rev 2024-04

# Enzolution™ PARG Assay System 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 Enzolution™ 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 Enzolution™ 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

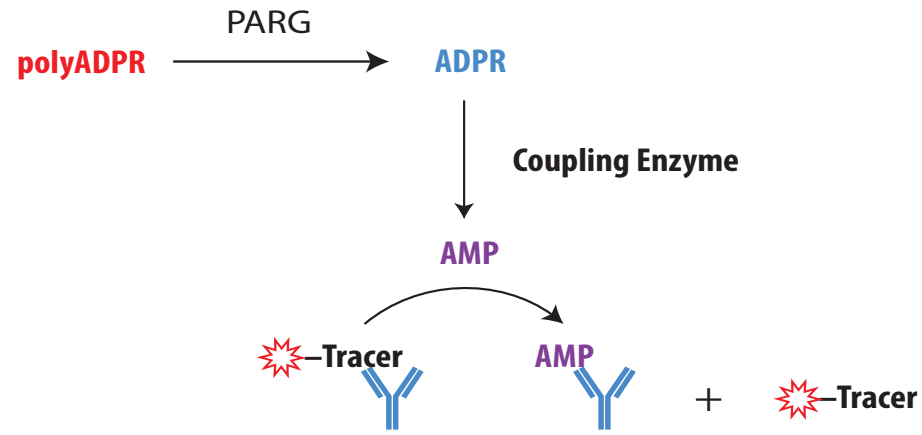



**Figure 1. Schematic Overview of the Enzolution™ PARG Assay System with the Transcreener® ADPR FP Assay.** ADPR produced by PARG is converted to AMP by the Coupling Enzyme in real time. In the detection step, the Coupling Enzyme is quenched by EDTA, and AMP displaces an Alexa Fluor® 633 tracer from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

## 2.0 Product Specifications

| Product | Quantity | Part # |
|---|---|---|
| Enzolution™ 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.**

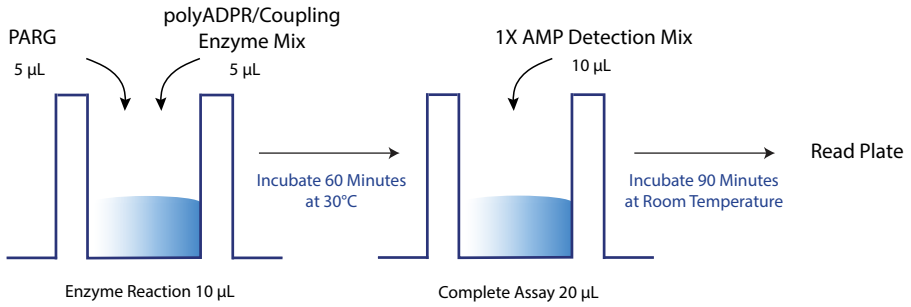



**Figure 2. An Outline of the Procedure.** The PARG Enzyme Reaction is initiated by the addition of polyADPR/Coupling Enzyme Mix. After the Enzyme Reaction incubation is completed, AMP detection reagents are added (Transcreener® AMP$^2$/GMP$^2$ Antibody and Tracer) along with EDTA to quench the Coupling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

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

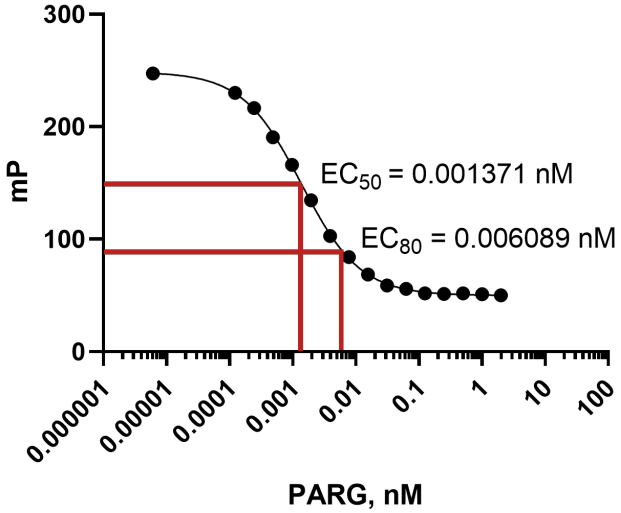
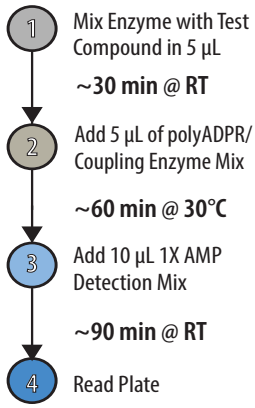



**Figure 3. Enzyme Titration Curve.** Example PARG Enzyme titration. The ideal range of enzyme concentrations is between $EC_{50}$ and $EC_{80}$; the specific concentration may vary depending on the enzyme lot.

## 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.



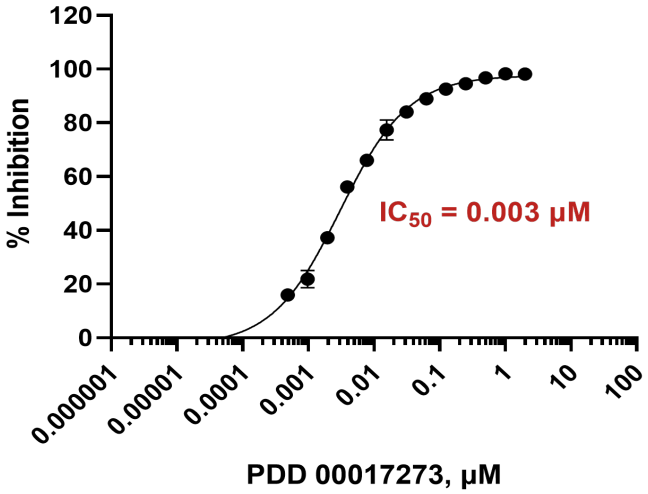




**Figure 4. Dose-Response Curve.** Example dose response curve with probe inhibitor PDD 00017273.

## 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.**

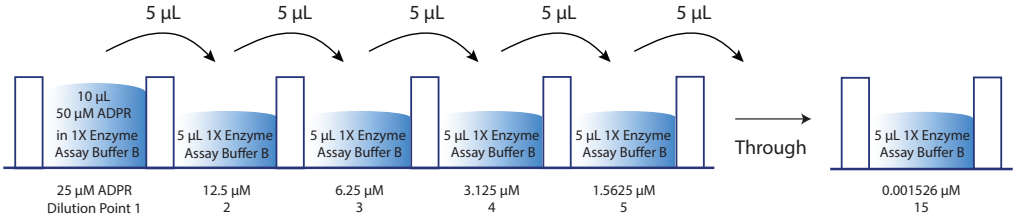



**Figure 5. Performing a Serial Dilution.** Example 2-fold serial dilution of ADPR to generate a standard curve.

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.

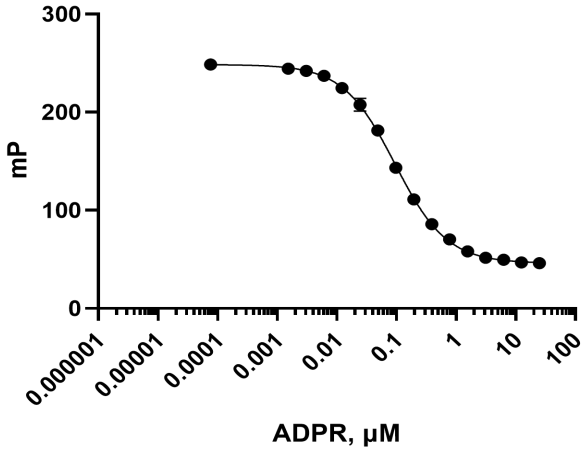



**Figure 6. ADPR Standard Curve.** Standard curve using 1X AMP Detection Mix as shown in **Table 2**.

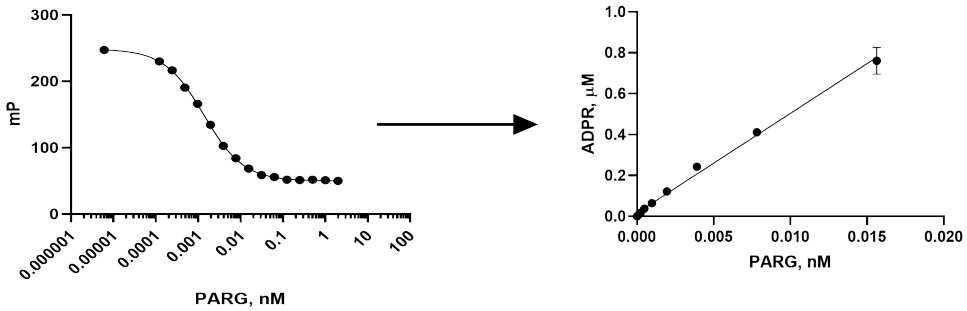



**Figure 7. Enzyme titration curve converted to ADPR formed.** Raw polarization signal (mP) is converted to ADPR formed using a standard curve as described in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism.

## 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'.

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

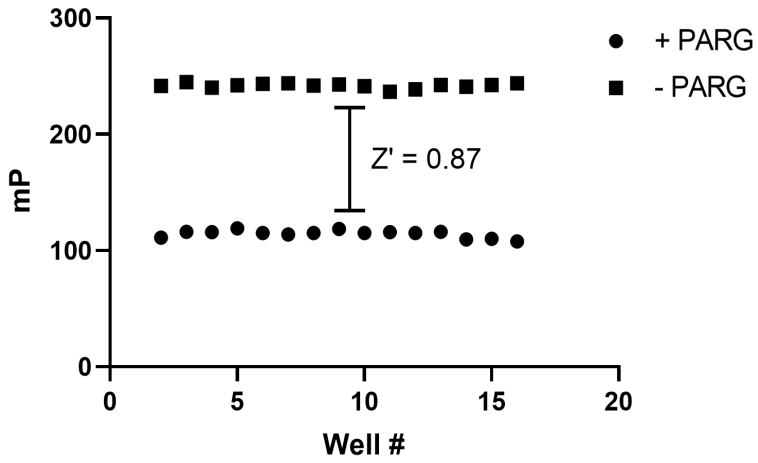



**Figure 8. Z' Measurement.** Complete Assay is performed with and without PARG enzyme (n=16). Z' is then calculated based on the formula shown in **Section 4.4**.

# 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

1232 Fourier Drive, Suite 115
Madison, Wisconsin 53717 USA
Email: TechSupport@bellbrooklabs.com
Phone: 608.443.2400
Toll-Free: 866.313.7881
FAX: 608.441.2967