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Enzolution™ PARP1 Assay System Technical Manual

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Abstract

The Enzolution™ PARP1 Assay System is intended for use with the Transcreener® pADPr PARP Assay Kits (Parts #3043 and #3044) to measure the enzymatic activity of PARP1 (poly(ADP-ribose) polymerase 1). PARP1 forms poly(ADP-ribose) (pADPr) chains on target proteins, including itself, using NAD^+ as a donor substrate (**Figure 1**). The Transcreener pADPr PARP Assay Kits use pADPr Coupling Enzymes (CE) to convert pADPr formed by PARP1 to AMP, which is then detected using far-red, competitive fluorescence assays that enable single addition, mix-and-read detection in continuous or endpoint formats. The assay has been optimized and extensively validated for high throughput screening (HTS) and inhibitor dose response measurements using most multimode plate readers.

Introduction

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The Enzolution PARP1 Assay System provides all reagents required to screen and profile PARP1 inhibitors when used with the Transcreener pADPr PARP Assay Kits, including purified, full length human PARP1 (aa 1-1014) and Sheared Salmon Sperm DNA. Note that PARP1 can ADP-ribosylate multiple substrates, including itself. The assay was optimized to detect the auto-ADP-ribosylation reaction, so it is not necessary to add any other protein substrate. The protocol is configured for 384-well plates; use of different multi-well plate formats will require adjustment of reagents concentrations utilized in the assay.

Key Applications:

- Screening for PARP1 inhibitors (or activators)
- Generating dose response curves and IC_{50} values for PARP1 inhibitors
- Kinetic and mechanistic analyses

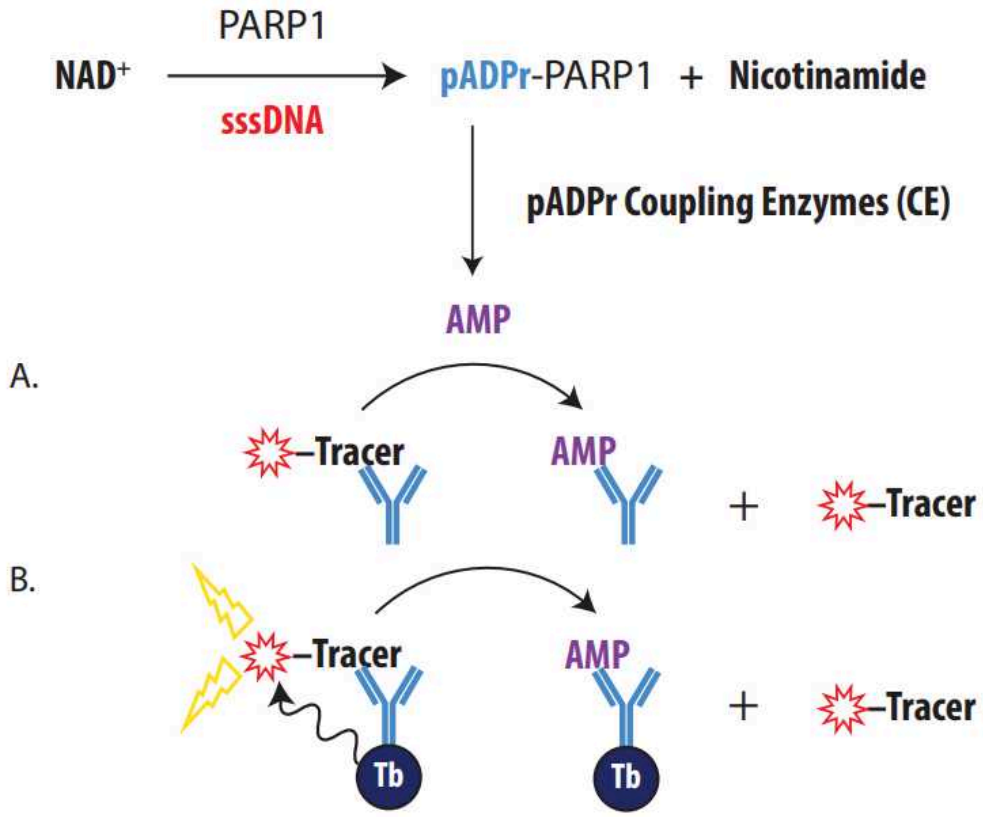


Figure 1. Schematic Overview of the Enzolution PARP1 Assay System with the Transcreener pADPr PARP Assays.

For FP readout (A): pADPr produced by PARP1 is converted to AMP in real time by the CE. AMP displaces an Alexa Fluor® 633 Tracer from the AMP^2/GMP^2 Antibody, resulting in decreased fluorescence polarization.

For TR-FRET readout (B): pADPr produced by PARP1 is converted to AMP in real time by the CE. AMP displaces a Hilyte647 Tracer from the AMP^2/GMP^2 Antibody conjugated to terbium (Tb), resulting in a decrease in TR-FRET.

Product Specifications

2

	Product	Quantity	Part #	Part #
	Enzolution PARP1 Assay System	1,000 assays*	FP	3045-1K-FP
			TR-FRET	3045-1K-TR
		10,000 assays*	FP	3045-10K-FP
			TR-FRET	3045-10K-TR

*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 should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that the PARP1 and Sheared Salmon Sperm DNA are stable and maintain greater than 80% activity 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.

Materials Provided

2.1

	Component	Composition	Notes
	PARP1 Enzyme	0.1 mg/mL (868 nM)* in 25 mM HEPES pH 7.5, 300 mM NaCl, 10% glycerol, 0.04% Triton X-100, 0.5 mM TCEP	Full-length (amino acids 1-1014), N-Terminal FLAG tag, 115.1 kDa. Sufficient enzyme is included in the kit to complete at least 1,000 assays (Part # 3045-1K) or 10,000 assays (Part # 3045-10K).
	Sheared Salmon Sperm DNA	10 mg/mL in H_2O	Salmon sperm DNA is resuspended in RNAse Free Water. Sufficient for the corresponding assay kit when 0.25 mg/mL is used in the 10 µL reaction.
	Enzyme Assay Buffer F, 10X	500 mM TRIS (pH 7.5), 100 mM $MgCl_2$, 0.1% Triton X-100	Use Enzyme Assay Buffer F in the Enzyme Reaction and for preincubation with inhibitors. Changes to the assay buffer could affect enzyme activity and/or detection of AMP.
	384-Well Low Volume Assay Plates	Corning #4514 - FP Only Corning #4513 - TR-FRET Only	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.

Materials Required But Not Provided

2.2

	Component	Notes
	Ultrapure Nuclease Free Water	Some deionized water systems are contaminated with nucleases 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 or 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 sub-microliter volumes into 384-well plates.
	Laboratory Incubator	An incubator model that is capable of maintaining temperature stability at 30°C is required.

Transcreener® pADPr PARP FP Assay - SOLD SEPARATELY

	Component	Composition	Notes
	AMP^2/GMP^2 Antibody	1.26 mg/mL solution in PBS with 10% glycerol*	1,000 assays (Part # 3043-1K) or 10,000 assays (Part # 3043-10K).
	AMP^2/GMP^2 Alexa Fluor® 633 Tracer	800 nM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	1,000 assays (Part # 3043-1K) or 10,000 assays (Part # 3043-10K).
	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 PARP1 Enzyme Reaction by chelating Mg^{2+}. The final concentrations of Mg^{2+} and EDTA in the Complete Assay are 10 mM and 20 mM, respectively.
	pADPr Coupling Enzyme (CE)	400X CE in 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM TCEP, 10% glycerol, 0.05% Triton X-100	1,000 assays (Part # 3043-1K) or 10,000 assays (Part # 3043-10K). pADPr Coupling Enzymes are present in excess to ensure pADPr is completely converted to AMP.
	NAD^+	5 mM NAD^+ in deionized water, pH 7.0	1,000 assays (Part # 3043-1K)or 10,000 assays (Part # 3043-10K).

	Component	Composition	Notes
	AMP	5 mM ADPR in deionized water, pH 7.0	Used for the AMP standard curve.

Transcreener® pADPr PARP TR-FRET Assay - SOLD SEPARATELY

	Component	Composition	Notes
	AMP^2/GMP^2 Antibody-Terbium Conjugate	800 nM solution in 25 mM HEPES buffered saline	1,000 assays (Part # 3044-1K) or 10,000 assays (Part # 3044-10K).
	AMP/GMP HiLyte 647 Tracer	10 µM solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	1,000 assays (Part # 3044-1K) or 10,000 assays (Part # 3044-10K).
	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 PARP1 Enzyme and the CE by chelating Mg^{2+}. The final concentrations of Mg^{2+} and EDTA in the Complete Assay are 10 mM and 20 mM, respectively.
	pADPr Coupling Enzyme (CE)	400X CE in 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM TCEP, 10% glycerol, 0.05% Triton X-100	1000 assays (Part # 3044-1K) or 10,000 assays (Part # 3044-10K). pADPr Coupling Enzymes are present in excess to ensure pADPr is completely converted to AMP.
	NAD^+	5 mM NAD^+ in deionized water, pH 7.0	1,000 assays (Part # 3044-1K) or 10,000 assays (Part # 3044-10K)
	AMP	5 mM ADPR in deionized water, pH 7.0	Used for the AMP standard curve.

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

Before You Begin

3 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 pADPr PARP Assays. **Full list of compatible plate readers and settings.**

3. Please read and understand the Transcreener pADPr PARP Assay Technical Manuals prior to use with this kit.

Protocol

4 The methods described below are for single-addition, endpoint detection: the PARP1 Enzyme Reaction and the CE are 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 PARP1 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 AMP formation (≤ 20% conversion of NAD^+ to AMP) by PARP1 with a sub-Km concentration of NAD^+ (100 µM) and a saturating concentration of Sheared Salmon Sperm DNA (0.25 mg/mL). These conditions will ensure sensitive detection of inhibitors that compete with NAD^+ while minimizing the effects of compounds that inhibit PARP1 via non-specific interactions with DNA. Significant changes in the NAD^+ concentration may require optimization of the AMP^2/GMP^2 Antibody concentration (in the case of the FP readout mode) or the AMP^2/GMP^2 Hilyte 647 Tracer (in the case of the TR-FRET readout mode) to adjust the dynamic range as described in the Transcreener pADPr PARP Assay Manuals.

Note: Antibody (TR-FRET) and Tracer (FP) concentrations remain constant in the 20 µL Complete Assay regardless of changes to other reaction conditions. Additionally, the CE is present in at least 5X excess over what is required for complete conversion of pADPr to AMP in real time over a range of initial NAD^+ concentrations; it is not recommended that this parameter be changed.

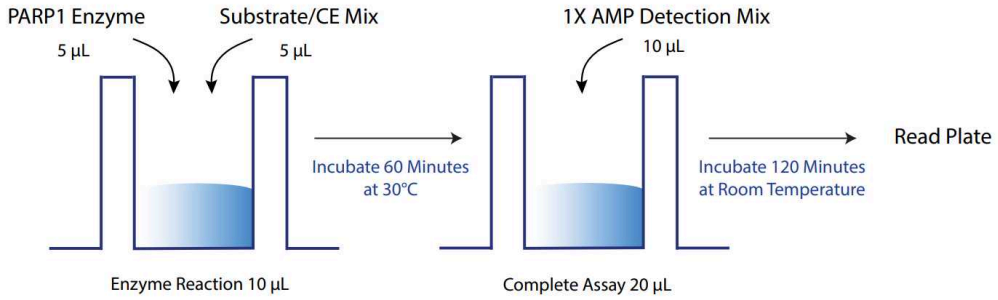


Figure 2. An Outline of the Procedure. The PARP1 Enzyme Reaction is initiated by the addition of Sheared Salmon Sperm DNA, NAD^+ and CE. After the Enzyme Reaction incubation is completed, AMP detection reagents are added (Transcreener AMP^2/GMP^2 Antibody and Tracer) along with EDTA to quench the enzyme reactions.

10 µL Enzyme Reaction Components

	Component	Working Stock	Final Concentration in 10 µL
	Enzyme Assay Buffer F, 10X	1X in Nuclease Free Water	1X (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.01% Triton X-100)
	PARP1 Enzyme, 0.1 mg/mL (894 nM)	2X in 1X Enzyme Assay Buffer F	0.6 nM – 0.8 nM*
	NAD^+, 5 mM	200 µM in 1X Enzyme Assay Buffer F (with 2X CE and 0.5 mg/mL Sheared Salmon Sperm DNA)	50 µM
	Sheared Salmon Sperm DNA, 10 mg/mL	0.5 mg/mL in 1X Enzyme Assay Buffer F (with 2X CE and 200 µM NAD^+)	0.25 mg/mL
	pADPr Coupling Enzymes, 400X	2X in 1X Enzyme Assay Buffer F (with 200 µM NAD^+ and 0.5 mg/mL Sheared Salmon Sperm DNA)	1X

*See Section 4.1 for Determining the Optimal Enzyme Concentration.

	1X AMP Detection Mix - Add 10 µL Per Well			
	FP		TR-FRET	
Component	Detection Mix Concentration	Complete Assay	Detection Mix Concentration	Complete Assay
Stop & Detect Buffer	1X	0.5X	1X	0.5X
AMP²/GMP² Tracer	8 nM	4 nM	200 nM	100 nM
AMP²/GMP² Antibody	36 µg/mL	18 µg/mL	8 nM	4 nM

Table 2. 1X AMP Detection Mix Components. The optimal concentrations for each of the detection reagents based on the preferred readout mode are shown. Changes to the concentrations may require re-optimization of the assay.

Determining the Optimal Enzyme Concentration

4.1 Using the enzyme concentration suggested in the PARP1 Enzyme Certificate of Analysis should provide a robust signal that is within the linear range for AMP 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 NAD^+ or Sheared Salmon Sperm DNA 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 F: Dilute 150 µL of 10X Enzyme Assay Buffer F in 1350 µL Ultrapure Nuclease Free Water.
2. Prepare 54.25 µL of 16 nM PARP1 Enzyme: dilute 1 µL of 868 nM PARP1 Enzyme in 53.25 µL 1X Enzyme Assay Buffer F.
3. Add 10 µL of the PARP1 Enzyme to well 1 (including replicates).
4. Add 5 µL of 1X Enzyme Assay Buffer F 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 PARP1 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 250 µL of Substrate/CE Mix: mix 1.25 µL of 400X CE, 12.5 µL of 10 mg/mL Sheared Salmon
Sperm DNA and 10 µL of 5 mM NAD^+ with 25 µL of 10X Enzyme Assay Buffer F in 201.25 µL of Ultrapure Nuclease Free Water.
Note: Prepare the Substrate/CE Mix right before use to avoid degradation of the substrate and possible reduction of the assay window.
7. Start the Enzyme Reaction by adding 5 µL of the Substrate/CE Mix to every well (1-12). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
8. Prepare 600 µL 1X AMP Detection Mix based on the concentrations provided in Table 2: 60 µL 10X
Stop & Detect Buffer B, 6 µL AMP^2/GMP^2 Alexa Fluor 633 Tracer and 36 µg/mL AMP^2/GMP^2 Antibody in Ultrapure Nuclease Free Water.
Note: The 1X AMP Detection Mix varies for the readout mode used. Table 2 lists the 1X AMP Detection Mix for each readout.
9. Add 10 µL of 1X AMP Detection mix to every well (1-12), in replicate.
10. Gently mix on a plate shaker for 40 to 60 seconds and then allow it to incubate at room temperature for 120 minutes before measuring signals.

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 signal (EC_{50} to EC_{80}) (see **Figure 3**). This will result in initial velocity conditions, which

correspond to the linear phase of the reaction after conversion of values to AMP formation (see **Figure 6**). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

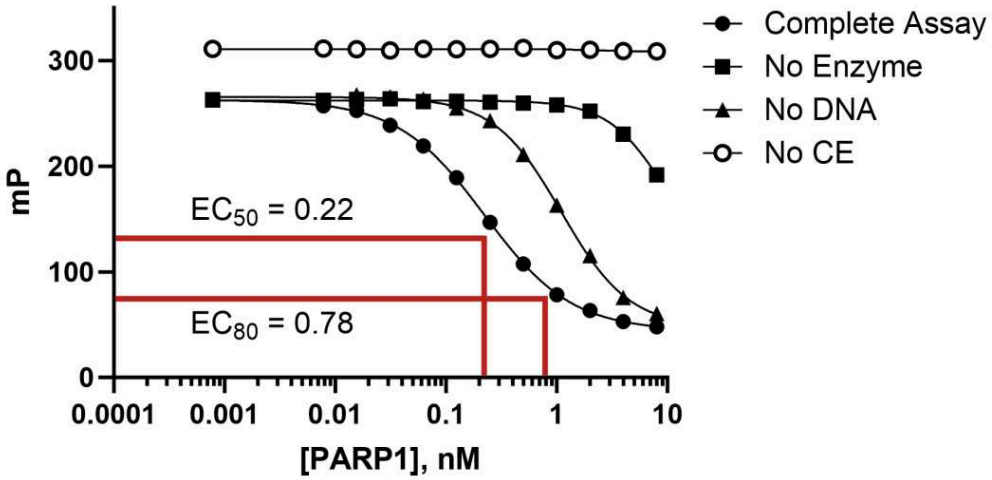


Figure 3. Enzyme Titration Curve. Example PARP1 Enzyme titration. The ideal range of enzyme concentrations is between EC_{50} and EC_{80}; the specific concentration may vary depending on the enzyme lot.

Performing Single Compound Screening and Dose-Response Assays

4.2 **4.2.1 Experimental Samples**

1. Perform a serial dilution of test compounds 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 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.

2. 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.

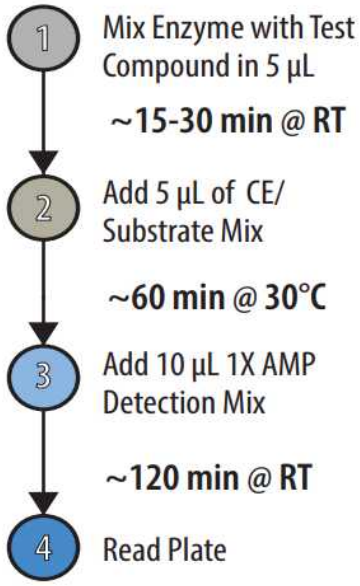
Note: The final volume of the Enzyme Reaction mixture should be 10 µL for 384 well plates. See Section 5.1 for a list of other plate formats.

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.

Note: The 1X AMP Detection Mix varies for the readout mode used. Table 2 lists the 1X

AMP Detection Mix for each readout.

4. Incubate at room temperature for 120 minutes before measuring signals.



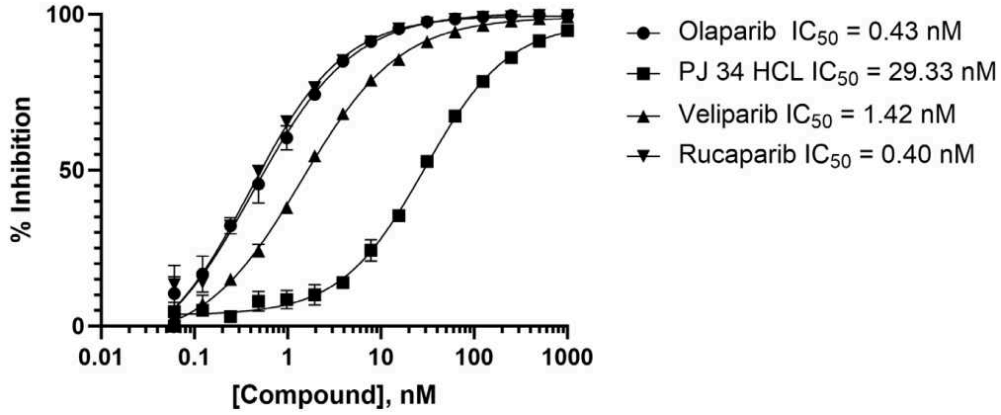


Figure 4. Dose-Response Curve. Example titration of probe inhibitors in the presence of PARP1 Enzyme.

Setting Up a Standard Curve

4.3 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. Here we describe preparation of a standard curve from 100 µM to 0.1 µM AMP, which encompasses the appropriate range for this assay.

	% Conversion	AMP (µM)
	100	100
	50	50
	25	25
	12.5	12.5
	6.25	6.25
	3.125	3.125
	1.56	1.56
	0.78	0.78
	0.39	0.39
	0.195	0.195
	0.0976	0.0976
	0	0

Table 3. Concentration of AMP to prepare a 12-point standard curve.

4.3.1 Preparing the Standard Curve - Manual Preparation

1. Prepare 1X Enzyme Assay Buffer F.
2. Dilute 5 mM AMP to 200 µM in 1X Enzyme Assay Buffer F.
3. Add 10 µL of the 200 µM AMP to well 1 (including replicates).
4. Add 5 µL of 1X Enzyme Assay Buffer F 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 200 µM AMP. 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 Substrate/CE Mix containing 200 µM NAD^+, 0.5 mg/mL Sheared Salmon Sperm DNA, 2X CE and 1X Enzyme Assay Buffer F in Ultrapure Nuclease Free Water.
Note: Prepare the Substrate/CE Mix right before use to avoid degradation of the substrate and possible reduction of the assay window.

7. Add 5 µL of Substrate/CE Mix to every well (1-12). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.

8. Afterwards, add 10 µL of 1X AMP Detection Mix. The 1X AMP Detection Mix varies for the readout mode used. Table 2 lists the 1X AMP Detection Mix for each readout.

9. Finally, mix for 40 to 60 seconds. Incubate at room temperature for 120 minutes before measuring signals.

4.3.2 Preparing the Standard Curve - Auto Dispenser Preparation

1. Prepare 4.5 mM AMP with 0.1% Triton.

2. Prepare 1X Enzyme Assay Buffer F.

3. Add 5 µL of 1X Enzyme Assay Buffer F to wells 1-12. Dispense the concentrations of AMP shown in **Table 2** on the respective wells.

4. Prepare Substrate/CE Mix containing 200 µM NAD^+, 0.5 mg/mL Sheared Salmon Sperm DNA, 2X CE and 1X Enzyme Assay Buffer F in Ultrapure Nuclease Free Water.
Note: Prepare the Substrate/CE Mix right before use to avoid degradation of the substrate and possible reduction of the assay window.

5. Add 5 µL of Substrate/CE Mix to every well (1-12). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.

6. Afterwards, add 10 µL of 1X AMP Detection Mix. The 1X AMP Detection Mix varies for the readout mode used. **Table 2** lists the 1X AMP Detection Mix for each readout.

7. Finally, mix for 40 to 60 seconds. Incubate at room temperature for 120 minutes before measuring signals.

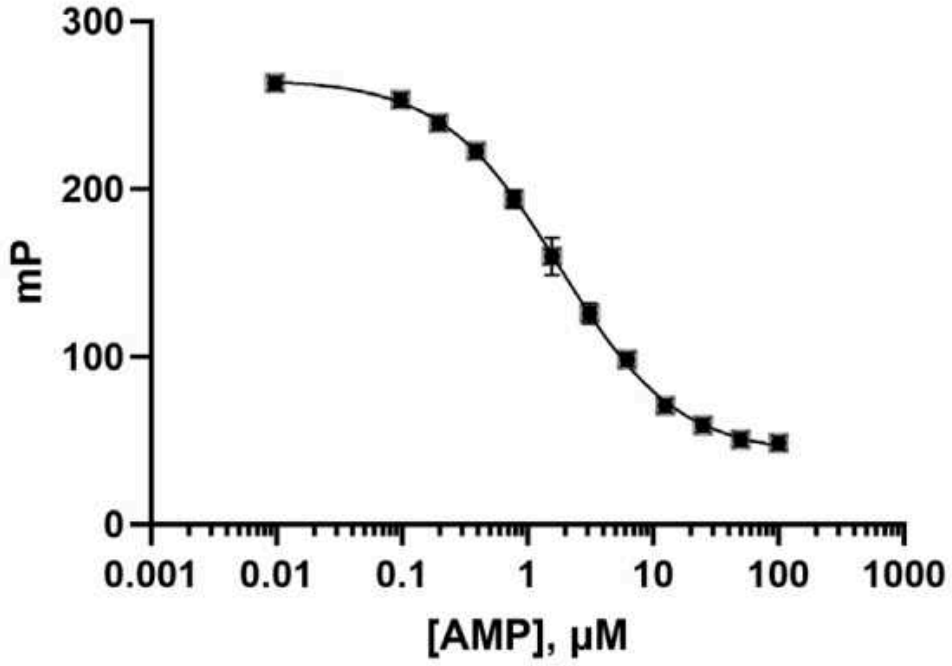


Figure 5. AMP Standard Curve. Standard curve using 1X AMP Detection Mix for FP readout as shown in **Table 2**. (36 µg/mL AMP^2/GMP^2 Antibody, 8 nM AMP^2/GMP^2 Tracer)

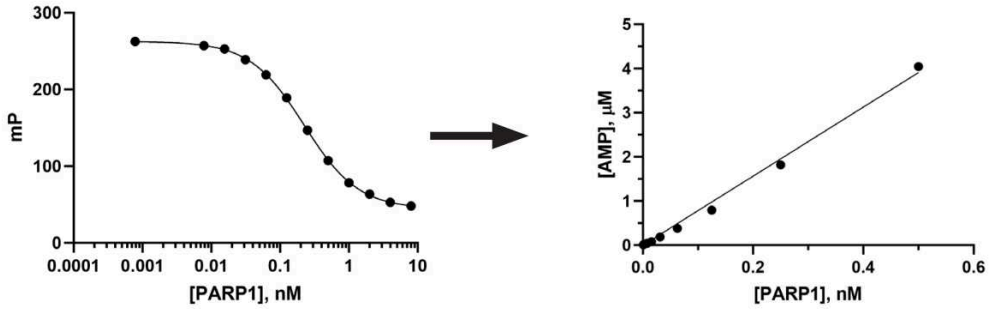


Figure 6. Enzyme Titration Curve Converted to AMP Formed. Raw polarization signal (mP) is converted to AMP formed using a standard curve as described in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism.

Measuring Assay Robustness with Z'

4.4 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 PARP1 Assay, run the controls with and without enzyme (no test compound) to achieve final results. Use the following formula to determine Z′.

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

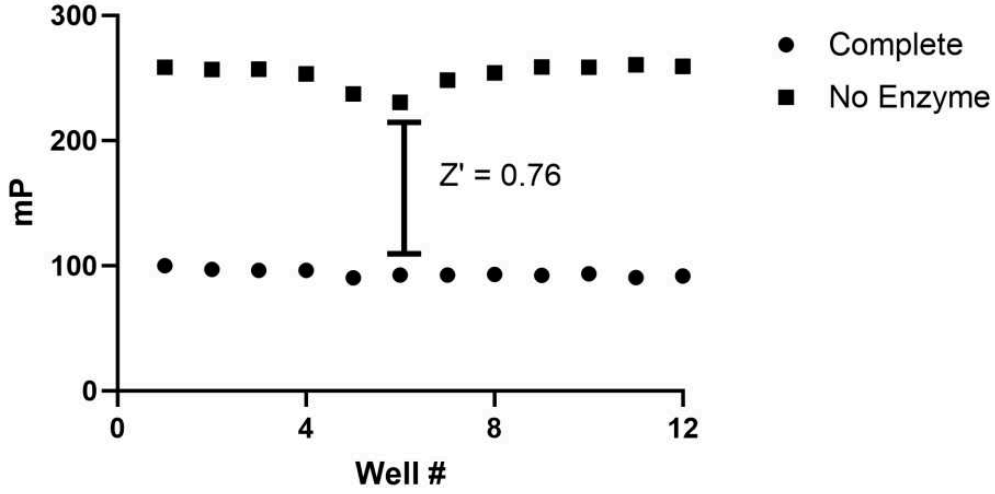


Figure 7. Z′ Measurement. Complete Assay is performed with and without PARP1 Enzyme (n=12). Z′ is then calculated based on the formula shown in **Section 4.4**.

Appendix

5

Using the Assay with Different Volumes and Plate Formats

5.1

	Component	Total Volume	Enzyme Reaction Volume	1X ADP Detection Mixture 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.

Links to Applicable Application Notes

5.2

- 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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6

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