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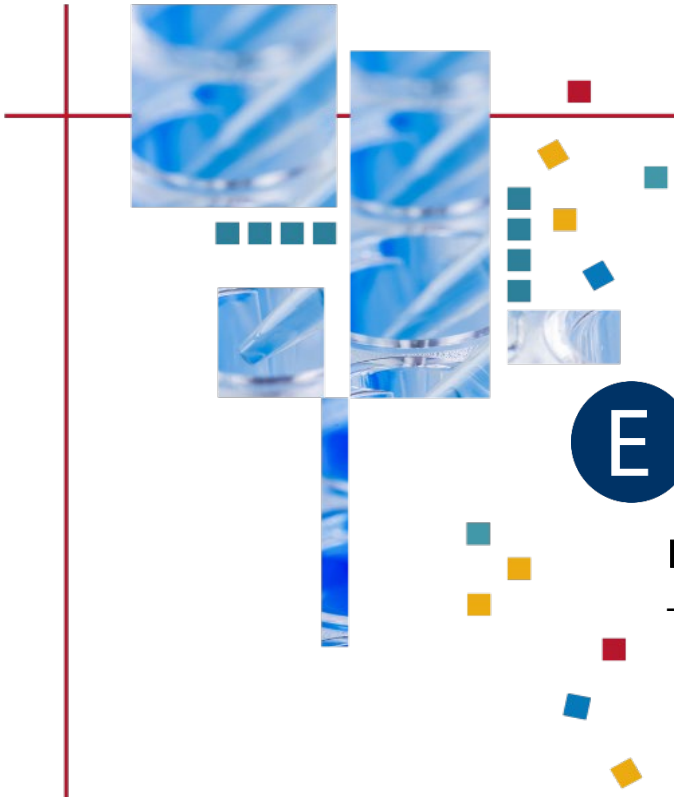

Enzolution™

PARP2 Assay System

Technical Manual

Instructions for Part Numbers 3050-1K and 3050-10K

Enzolution™ PARP2 Assay System Technical Manual

Contents

1.0 Introduction

The Enzolution™ PARP2 Assay System is intended for use with the Transcreener® pADPr PARP Assay Kits (Parts #3043 and #3044) to measure the enzymatic activity of PARP2 (poly(ADP-ribose) polymerase 2). Upon binding to DNA, PARP2 forms poly(ADP-ribose) (pADPr) chains on target proteins, including itself, using NAD^+ as a donor substrate (**Figure 1**). The assay relies on Coupling Enzymes (CE) to convert pADPr into AMP, which is detected using a far-red, fluorescence polarization (FP) or time-resolved Förster-resonance-energy-transfer (TR-FRET) assay. 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 PARP2 Assay System provides all reagents required to screen and profile PARP2 inhibitors when used with the Transcreener pADPr PARP Assay Kits, including purified, full length human PARP2 and sheared salmon sperm DNA ("sssDNA" in **Figure 1**). Since PARP2 can add pADPr to itself, this assay was optimized to detect the auto-ADP-ribosylation reaction and does not require the addition of other protein substrates. 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 PARP2 inhibitors (or activators)
- Generating dose response curves and IC_{50} values for PARP2 inhibitors
- Kinetic and mechanistic analyses

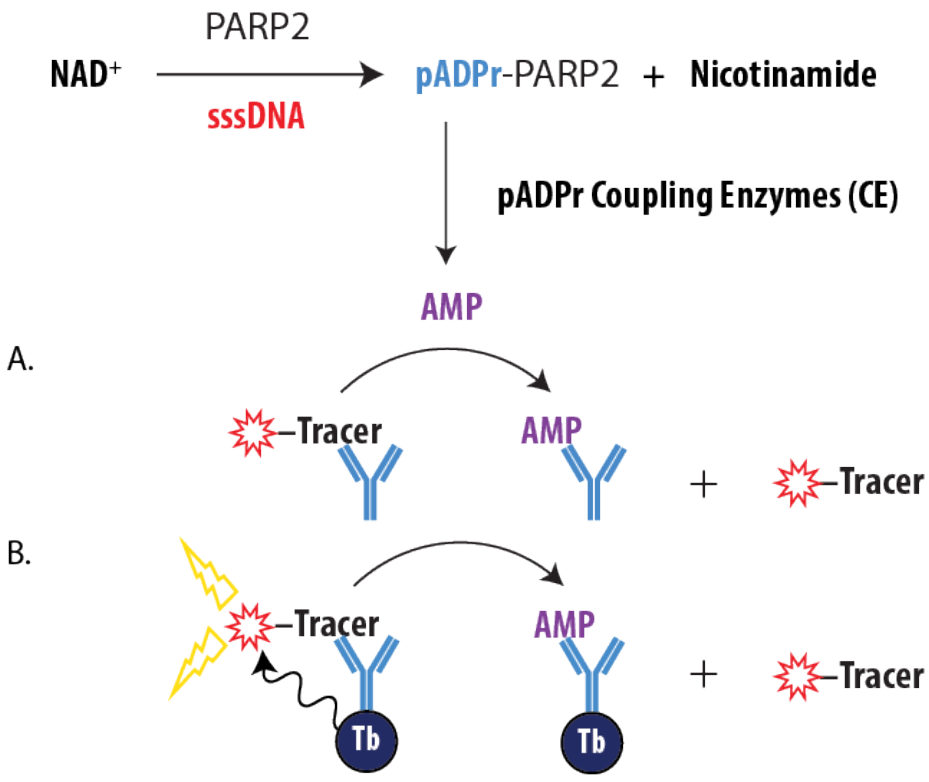

Figure 1. Schematic Overview of the Enzolution PARP2 Assay System with the Transcreener pADPr PARP Assays. pADPr produced by PARP2 is completely converted to AMP in real time by the pADPr Coupling Enzymes. In the detection step, the CE is quenched by EDTA, and AMP displaces a fluorescent tracer from the AMP^2/GMP^2 Antibody, resulting in decreased fluorescence polarization (A) or TR-FRET (B).

2.0 Product Specifications

Product	Quantity	Part#
Enzolution PARP2 Assay System	1,000 assays*	3050-1K
	10,000 assays*	3050-10K

***NOTE: The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using 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 enzyme reagents 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.

2.1 Materials Provided

Component	Composition	Notes
PARP2 Enzyme	0.5 mg/mL (7.46 µM)* in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol	Full-length protein (amino acids 1-583), N-Terminal His tag, 67 kDa. The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for and accurate concentration.
Sheared Salmon Sperm DNA	10 mg/mL in water	Salmon Sperm DNA stored in Nuclease Free Water; to be used at 0.25 mg/mL in the PARP2 reaction.
Enzyme Assay Buffer H, 10X	500 mM Tris (pH 8.0), 50 mM $MgCl_2$, 1 M NaCl, and 0.1% Triton	Enzyme Assay Buffer H, along with DTT, has been optimized to support PARP2 activity as well as CE activity. Changes to the assay buffer may affect PARP2 enzyme activity and/or conversion of pADPr to AMP.
DTT, 1 M	1 M in water	Add to 1x Enzyme Assay Buffer H to a final concentration of 1 mM.
384-Well Low Volume Assay Plates	Corning# 4514 - FP and FI 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.

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	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 TR-FRET Assay - SOLD SEPARATELY		
Component	**Composition**	**Notes**
AMP²/GMP² Antibody-Tb	800 nM solution in 25 mM HEPES buffered saline	1,000 assays (Part # 3044-1K) or 10,000 assays (Part # 3044-10K)
AMP²/GMP² 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 C, 10X	500 mM HEPES, 0.2% Brij, and 200 mM EDTA at a final pH 7.5	The Stop & Detect Buffer C components quench the CE reaction by chelating metals required for activity.
pADPr Coupling Enzymes (CE)	400X pADPr Coupling Enzyme	pADPr Coupling Enzymes are present in excess to ensure pADPr is completely converted to AMP.
NAD^+	5 mM in water	The use of NAD from different sources may require reoptimize the assay's dynamic range.
AMP	5 mM in water	Used for the AMP standard curve.

Transcreener® pADPr PARP FP Assay - SOLD SEPARATELY		
Component	**Composition**	**Notes**
AMP²/GMP² Antibody	1.26 mg/mL solution in PBS with 10% glycerol	1,000 assays (Part # 3043-1K) or 10,000 assays (Part # 3043-10K).
AMP²/GMP² 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 Stop & Detect Buffer B components quench the CE reaction by chelating metals required for activity.
pADPr Coupling Enzyme (CE)	400X pADPr Coupling Enzyme	pADPr Coupling Enzyme is present in excess to ensure pADPr is completely converted to AMP.
NAD^+	5 mM in water	The use of NAD from different sources may require reoptimize the assay's dynamic range.
AMP	5 mM in water	Used for the AMP standard curve.

3.0 Before You Begin

- Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
- Check the plate reader and verify that it is compatible with the Transcreener® pADPr PARP Assays. Full List of Compatible Plate Reader Settings
- Please read and understand the Transcreener® pADPr PARP Assay Technical Manuals prior to use with this kit.

4.0 Protocol

The methods described below are for endpoint detection of pADPr formation by PARP2 under initial velocity conditions (≤ 20% conversion of NAD^+ to pADPr) with a sub-K_m 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^+. Significant changes in the NAD^+ concentration may require adjustment of the dynamic range, as described in the **Transcreener pADPr PARP Assay** Tech Manuals.

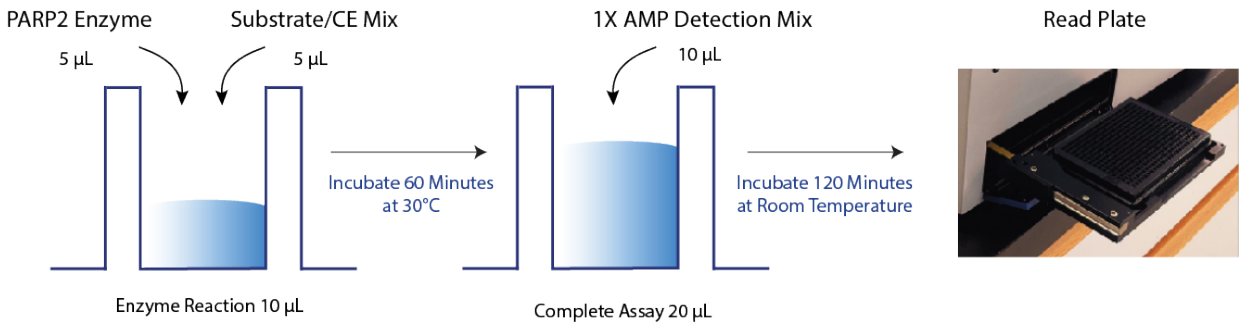

Figure 2. Simple Mix-and-Read Format. The PARP2 Enzyme Reaction is run in the presence of CE, so that pADPr is converted to AMP in real time. After the Enzyme Reaction incubation is complete, 1X AMP Detection Mix is added, which contains EDTA to quench the Enzyme Reaction. Plates are allowed to sit for 120 min at room temperature before reading to allow the detection reaction to reach equilibrium.

4.1 Performing an Enzyme Assay

The following assay protocol is for 384-well format, using a 10 µL Enzyme Reaction and 20 µL Complete Assay volume when the plates are read. The use of different formats will require changes in reagent quantities (see **Section 5.1**). All the reagent mixes can be prepared ahead of time and stored on ice for at least 2 hours before use, with the exception of the Substrate/CE mix, which should be used within 30 minutes of preparation.

1. **Prepare Working Stocks**
   a. Prepare Complete Assay Buffer by combining 1X Enzyme Assay Buffer H, and 1 mM DTT in Ultrapure Nuclease-Free Water.
   b. Dilute PARP2 enzyme to 2X the desired concentration* in Complete Assay Buffer.
   c. Prepare Substrate/CE Mix by combining 2X CE, 200 µM NAD^+, 0.5 mg/mL sheared salmon sperm DNA in Complete Assay Buffer.

Component	Stock	10 µL Enzyme Reaction Components	
		Working Stock	Final Concentration in 10 µL
Complete Assay Buffer	10X Enzyme Assay Buffer H, 1 M DTT	1X Enzyme Assay Buffer H, 1 mM DTT in Nuclease Free Water	50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 mM NaCl, 0.01% Triton X-100, 1 mM DTT
PARP2 Enzyme	0.5 mg/mL (7.46 µM)	2X in Complete Assay Buffer	3 nM – 4 nM (see **Section 4.1**)
Substrate/CE Mix	5 mM NAD^+, 10 mg/mL sheared salmon sperm DNA, 400X CE	200 µM NAD^+, 0.5 mg/mL sheared salmon sperm DNA, 2X CE in Complete Assay Buffer	100 µM NAD^+, 0.25 mg/mL salmon sheared sperm DNA, 1X CE

Table 1. PARP2 Enzyme Reaction Components.

2. **Run the PARP2 Enzyme Reaction**

   a. Add 5 µL of 2x PARP2 enzyme to each well.

   b. Add 5 µL of Substrate/CE Mix to initiate the reaction. Mix gently on a plate shaker and incubate at 30°C for 60 minutes.

3. **Prepare and Dispense AMP Detection Mix**

   a. Centrifuge AMP^2/GMP^2 Antibody at 10,000 x g for 10 minutes to remove any aggregates. It is normal for the antibody to form aggregates over time or after freeze/thaw cycles. Removing these aggregates will not affect assay performance.

   b. Prepare 1X AMP Detection Mix by diluting Stop & Detect Buffer, AMP^2/GMP^2 Tracer, and AMP^2/GMP^2 Antibody in Ultrapure Nuclease-Free Water to the concentrations described in **Table 2**, according to the appropriate readout mode.

   c. Add 10 µL of the 1X AMP Detection Mix to each well and mix gently on a plate shaker. Incubate at room temperature for 120 minutes to allow the detection reaction to reach equilibrium.

Component	1X AMP Detection Mix – Add 10 µL Per Well			
	FP		TR-FRET	
	Detection Mix Conc.	Complete Assay	Detection Mix Conc.	Complete Assay
Stop & Detect Buffer	1X	0.5X	1X	0.5X
AMP^2/GMP^2 Tracer	8 nM	4 nM	280 nM	140 nM
AMP^2/GMP^2 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.

*Using the EC_{80} concentration suggested in the PARP2 Enzyme Certificate of Analysis should provide a robust signal that is within the linear range for AMP formation. However, for best results, it may be useful to perform an enzyme titration to identify the optimal enzyme concentration (EC_{50} to EC_{80}) (see **Figure 3a**). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

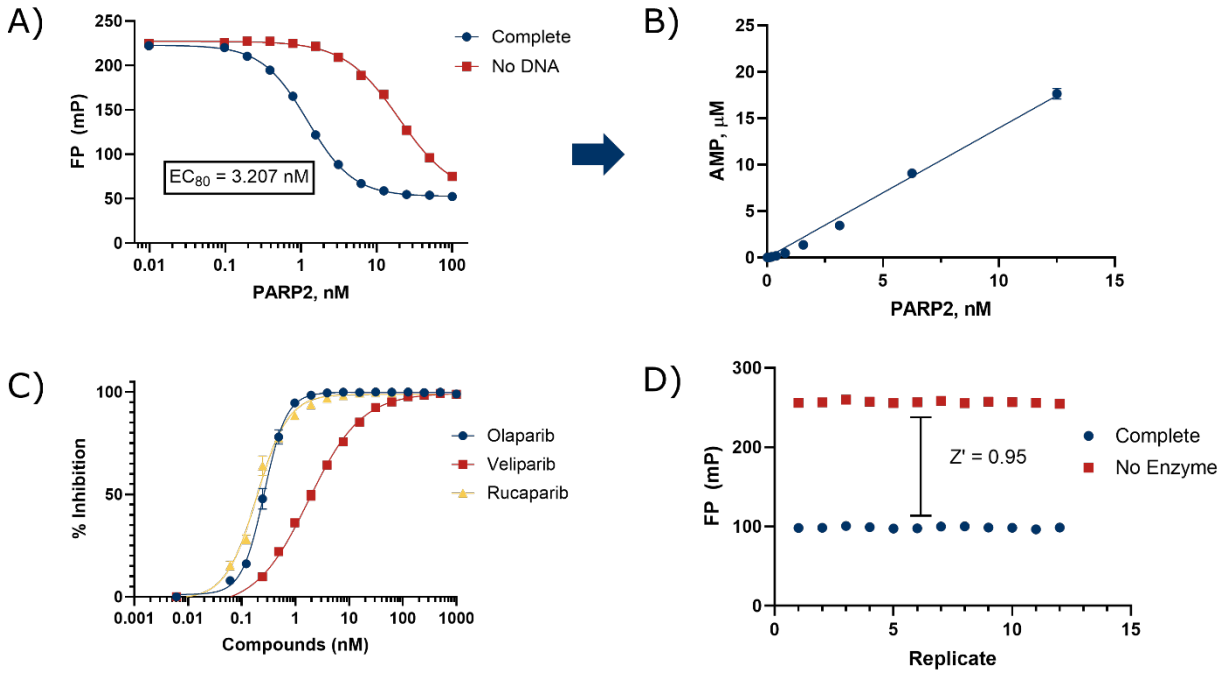

Figure 3. Sample Data for FP Readout Mode. (a) Enzyme titration curve. (b) mP values from A) are converted to AMP formed using the standard curve in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism (see **Section 5.2** for guidance). (c) Dose-Response Curves of probe inhibitors (see **Section 4.2**). (d) Z' Measurement (n=12) (see **Section 4.4**).

4.2 Performing Single Compound Screening and Dose-Response Assays

For Single Compound Screening and Dose-Response Assays, follow the protocol listed in **Section 4.1**. Add PARP2 enzyme to the test compounds pre-dispensed in wells; the total mixture volume should be 5 µL, and DMSO concentration should not exceed 1-2%. We recommend mixing gently on a plate shaker for 40 to 60 seconds and preincubating for 30 minutes at RT to allow equilibration of the E-I complex.

NOTE: Final concentration of test compounds should be based on the volume of the Enzyme Reaction (10 µL).

4.3 Setting Up a Standard Curve

Use of a standard curve for conversion of raw FP or TR-FRET data 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 and follows the protocol outlined in **Section 4.1**, with the PARP2 enzyme replaced by a titration of AMP pADPr concentrations. The Substrate/CE Mix remains the same as in the PARP2 Enzyme Reaction to account for background generated by NAD^+ degradation.

Typically, an 8- to 12-point standard curve is used, with the starting concentration of AMP matching the NAD^+ concentration in the PARP2 Enzyme Reaction (see **Figure 4**). An example protocol for a 12-point standard curve is shown below:

1. **Prepare Working Stocks**

   a. Prepare Complete Assay Buffer and Substrate/CE Mix as described in **Section 4.1**.

   b. Prepare 200 µM AMP in Complete Assay Buffer.

2. **Perform AMP Titration**

   a. Add 5 µL of Complete Assay Buffer to well 2-12.

   b. Add 10 µL of 200 µM AMP to well 1. Perform a 2-fold serial dilution by pipetting 5 µL of the AMP solution from well 1 to well 2, and so on. Well 12 should be kept as a blank.

   c. Add 5 µl of Substrate/CE Mix, then mix gently on a plate shaker and incubate at 30°C for 60 minutes.

3. **Prepare and Dispense AMP Detection Mix** as described in **Section 4.1**

Data Point	AMP (µM)	NAD^+ (µM)
1	100	100
2	50	100
3	25	100
4	12.5	100
5	6.25	100
6	3.125	100
7	1.56	100
8	0.78	100
9	0.39	100
10	0.195	100
11	0.0976	100
12	0	100

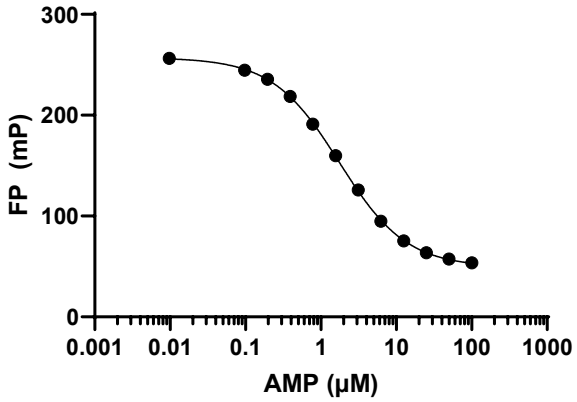

Figure 4. AMP Standard Curves. Example concentration and data for a standard curve corresponding to 100 µM NAD^+ in the PARP2 Enzyme Reaction. Note that NAD^+ would decrease proportionally in a PARP reaction, but it can be held constant to simplify the standard curve protocol.

4.4 Measuring Assay Robustness with Z'

The Z' value is a dimensionless coefficient that quantifies the separation between the positive and negative controls, which is key to determining the robustness and reliability of an assay. A Z' value of 0.5 or greater is typically considered indicative of a very good screening window for a biochemical assay, suggesting the assay is of excellent quality. To calculate the Z' value, run the controls both with and without the enzyme (without test compounds). Then, use the following formula for the calculation:

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

5.0 Appendix

5.1 Using the Assay with Different Volumes and Plate Formats

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

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

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

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