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Enzolution™ SIRT3 Assay System Technical Manual

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We use this protocol and it's working

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Abstract

The Enzolution™ SIRT3 Assay System is intended for use with the Transcreener® OAADPr SIRT Assay Kits (Parts #3046 and #3047) to measure the enzymatic activity of SIRT3 (NAD-dependent protein deacetylase sirtuin 3). SIRT3 catalyzes transfer of acetyl groups from acetylated histones to NAD, yielding the unacetylated peptide, O-acetyl-ADP ribose (OAADPr), and nicotinamide as products (**Figure 1**). The assay relies on a Coupling Enzyme (CE) to convert OAADPr into AMP, which is then detected using a far-red, competitive 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 SIRT3 Assay System provides all reagents required to screen and profile SIRT3 inhibitors when used with the Transcreener OAADPr SIRT Assay Kits, including purified, human SIRT3 (aa 101-399) and acetylated peptide, H3K9Ac (1-21). 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.

Introduction

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Key Applications:

- Screening for SIRT3 inhibitors (or activators)
- Generating dose response curves and IC₅₀ values for SIRT3 inhibitors
- Kinetic and mechanistic analyses

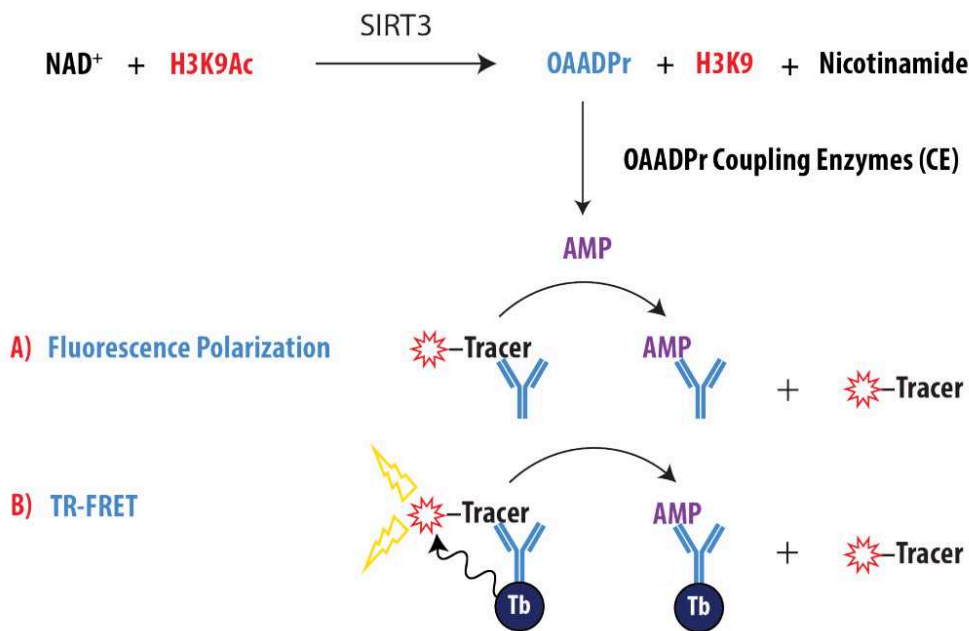


Figure 1. Schematic Overview of the Enzolution SIRT3 Assay System with the Transcreeper OAADPr SIRT Assays. OAADPr produced by the target SIRT enzyme is completely converted to AMP in real time by the OAADPr Coupling Enzyme. In the detection step, the CE is quenched by EDTA, and AMP displaces a fluorescent tracer from the $\text{AMP}^2/\text{GMP}^2$ Antibody, resulting in decreased fluorescence polarization (A) or TR-FRET (B).

Product Specifications

2

	Product	Quantity	Part#
	Enzolution SIRT3 Assay System	1,000 assays*	3052-1K
		10,000 assays*	3052-10K

***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.

Materials Provided

2.1

Component	Composition	Notes
SIRT3 Enzyme	0.095 mg/mL (2.81 μ M)* in 50mM HEPES pH 7.5, 200 mM NaCl, 1 mM TCEP, 1 mM EDTA, 10% glycerol,	Truncated protein (amino acids 101-399), N-Terminal His tag, 33.7 kDa. Sufficient enzyme is included in the kit to complete at least 1,000 assays (Part# 3052-1K) or 10,000 assays (Part# 3052-10K).
H3K9Ac (1-21)	10 mM in water	An acetylated peptide from the N-terminus of Histone H3, stored in RNase Free Water; to be used at 100 μ M in the SIRT3 reaction.
Enzyme Assay Buffer G, 10X	500 mM Tris (pH 7.5), 50 mM $MgCl_2$, and 0.1% Triton X-10	Enzyme Assay Buffer G, along with $MnCl_2$, has been optimized to support SIRT activity as well as CE activity. Changes to the assay buffer may affect SIRT enzyme activity and/or conversion of OAADPr to AMP.
$MnCl_2$, 1 M	1 M in water	Add to 1X Enzyme Assay Buffer G to a final concentration of 0.5 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.

***NOTE: The exact enzyme 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
Plate Reader	A multimode microplate reader configured to measure FP is required. Transcreener Assays have been validated on the



Component	Notes
	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.

Transcreeper OAADPr SIRT FP Assay - SOLD SEPERATELY

Component	Composition	Notes
AMP ² /GMP ² Antibody	1.26 mg/mL solution in PBS with 10% glycerol	1,000 assays (Part# 3046-1K) or 10,000 assays (Part # 3046-10K).
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# 3046-1K) or 10,000 assays (Part# 3046-10K).
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.
OAADPr Coupling Enzyme (CE)	200X OAADPr Coupling Enzyme in 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM TCEP, 10% glycerol, 0.05% Triton X-100	OAADPr Coupling Enzyme is present in excess to ensure OAADPr is completely converted to AMP.
NAD ⁺	5 mM in water	1,000 assays (Part# 3046-1K) or 10,000 assays (Part# 3046-10K).
AMP	5 mM in water	Used for the AMP standard curve.
Enzyme Assay Buffer G, 10X	500 mM Tris (pH 7.5), 50 mM MgCl ₂ , and 0.1% Triton X-100	Enzyme Assay Buffer G, along with MnCl ₂ , has been optimized to support SIRT activity as well as CE activity. Changes to the assay buffer could affect SIRT enzyme activity and/or conversion of OAADPr to AMP.
DTT, 1 M	1 M in water	Add to 1x Enzyme Assay Buffer G to a final concentration of 1 mM.
MnCl ₂ , 1 M	1 M in water	Add to 1X Enzyme Assay Buffer G to a final concentration of 0.5 mM.

**Transcreener OAADPr SIRT TR-FRET Assay - SOLD SEPERATELY**

Component	Composition	Notes
AMP ² /GMP ² Antibody-Tb	800 nM solution in 25 mM HEPES buffered saline	1,000 assays (Part# 3047-1K) or 10,000 assays (Part# 3047-10K)
AMP ² /GMP ² Hilyte 647 Tracer	10 μ M solution in 2 mM HEPES (pH 7.5) containing 0.01% Brij-35	1,000 assays (Part# 3047-1K) or 10,000 assays (Part# 3047-10K)
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.
OAADPr Coupling Enzyme (CE)	200X OAADPr Coupling Enzyme in 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM TCEP, 10% glycerol, 0.05% Triton X-100	OAADPr Coupling Enzyme is present in excess to ensure OAADPr is completely converted to AMP.
NAD ⁺	5 mM in water	1,000 assays (Part# 3047-1K) or 10,000 assays (Part# 3047-10K)
AMP	5 mM in water	Used for the AMP standard curve.
Enzyme Assay Buffer G, 10X	500 mM Tris (pH 7.5), 50 mM MgCl ₂ , and 0.1% Triton	Enzyme Assay Buffer G, along with MnCl ₂ and DTT, has been optimized to support SIRT activity as well as CE activity. Changes to the assay buffer could affect SIRT enzyme activity and/or conversion of OAADPr to AMP.
DTT, 1 M	1 M in water	Add to 1x Enzyme Assay Buffer G to a final concentration of 1 mM.
MnCl ₂ , 1 M	1 M in water	Add to 1X Enzyme Assay Buffer G to a final concentration of 0.5 mM.

Before You Begin

- 3
 - 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 OAADPr SIRT Assays **Full list of compatible plate readers and settings.**
 - Please read and understand the Transcreener OAADPr SIRT Assay Technical Manuals prior to use with this kit.

Protocol

- The methods described below are for endpoint detection of OAADPr formation by SIRT3 under initial velocity conditions ($\leq 10\%$ conversion of NAD^+ to OAADPr) with a sub-saturating concentration of NAD^+ ($50 \mu\text{M}$) and H3K9Ac peptide ($100 \mu\text{M}$). These conditions will ensure sensitive detection of inhibitors that compete with either substrate. Significant changes in the NAD^+ concentration may require adjustment of the dynamic range, as described in the **Transcreener OAADPr SIRT Assay** Tech Manuals (**Section 3.2**).

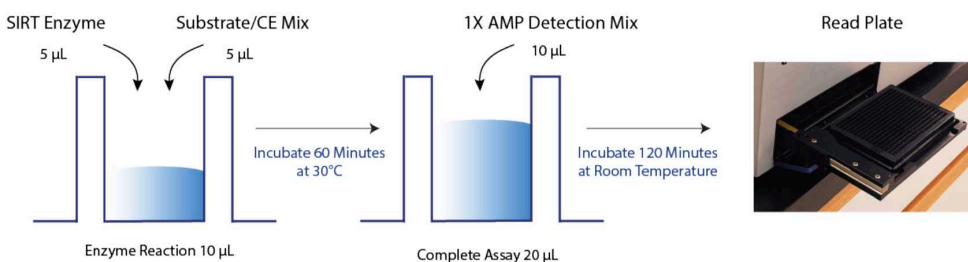


Figure 2. Simple Mix-and-Read Format. The SIRT Enzyme Reaction is run in the presence of CE, so that OAADPr is converted to AMP in real time. After the Enzyme Reaction incubation is complete, 1X AMP Detection Mix is added, which contains EDTA to quench the Enzyme Reaction. Plates are allowed to sit for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

10 µL Enzyme Reaction Components

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	150 nM	75 nM
AMP ² /GMP ² Antibody	20 µg/mL	10 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.

Performing an Enzyme Assay

- 4.1 The following assay protocol is for a 384-well format, using a 10 μ L Enzyme Reaction and 20 μ L 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 G, and 0.5 mM MnCl_2 in Ultrapure Nuclease-Free Water.
- b. Dilute SIRT3 enzyme to 2X the desired concentration* in Complete Assay Buffer.
- c. Prepare Substrate/CE Mix by combining 2X CE, 100 μ M NAD^+ , 200 μ M H3K9Ac peptide in Complete Assay Buffer.

2. Run the SIRT3 Enzyme Reaction

- a. Add 5 μ L of 2X SIRT3 enzyme to each well, followed by 5 μ L 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 $\text{AMP}^2/\text{GMP}^2$ Antibody at 10,000 x g for 10 minutes. This step is critical to ensure optimal assay performance.
- b. Prepare 1X AMP Detection Mix by diluting Stop & Detect Buffer, $\text{AMP}^2/\text{GMP}^2$ Tracer, and $\text{AMP}^2/\text{GMP}^2$ Antibody in Ultrapure Nuclease-Free Water to the concentrations described in **Table 2**, according to the appropriate readout mode.
- c. Add 10 μ L 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.

***Note: Each time an assay is performed with a new batch of enzyme, an enzyme titration is required to determine the working concentration.**

*Using the EC_{80} concentration suggested in the SIRT3 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:

$$\text{EC}_X = (X \div (100 - X))^{(1 \div |\text{hillslope}|)} \times \text{EC}_{50}$$

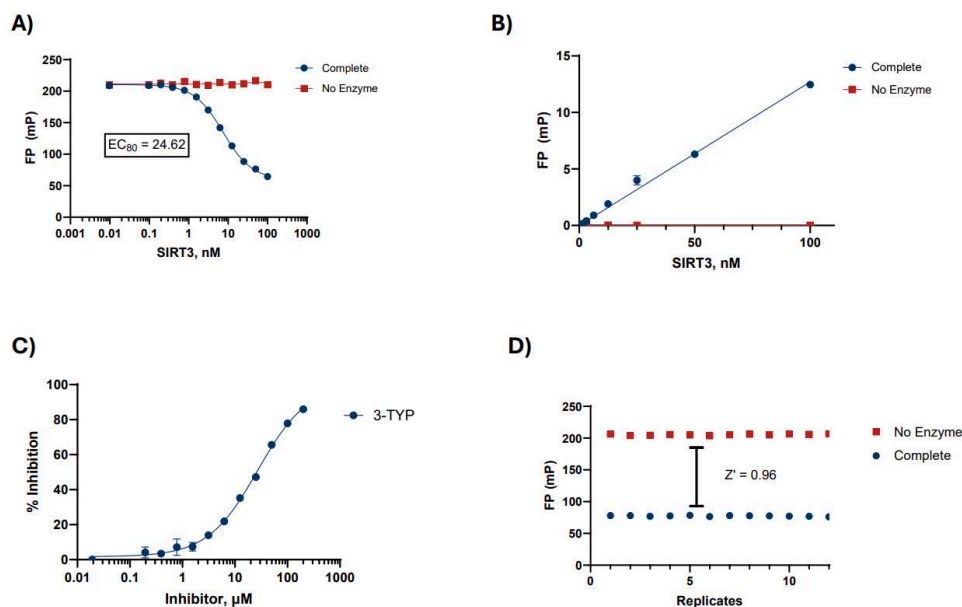


Figure 3. Sample Data for FP Readout Mode. (a) Enzyme titration curve. (b) Raw polarization in enzyme titration curve is converted to AMP formed using the standard curve in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism (see **Section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

Performing Single Compound Screening and Dose-Response Assays

- 4.2 Single Compound Screening and Dose-Response Assays follow the protocol listed in **Section 4.1**. The target SIRT Enzyme is added to the test compounds pre-dispensed in wells; the total mixture volume should be 5 μ L 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 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.

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 and follows the protocol outlined in **Section 4.1**, with the SIRT enzyme

replaced by a titration of AMP concentrations. The Substrate/CE Mix remains the same as in the SIRT Enzyme Reaction to account for background generated by NAD⁺ degradation and reaction with the CE.

Typically, an 8- to 12-point standard curve is used, with the starting concentration of AMP matching the NAD⁺ concentration in the SIRT Enzyme Reaction (see **Figure 4**). The AMP standard curve allows the calculation of the AMP concentration produced in the Enzyme Reaction and, consequently, the percent AMP conversion. 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 100 μ M AMP in Complete Assay Buffer.

2. Perform AMP Titration

- a. Add 5 μ L of Complete Assay Buffer to well 2-12.
- b. Add 10 μ L of 100 μ M AMP to well 1. Perform a 2-fold serial dilution by pipetting 5 μ L of the AMP solution from well 1 to well 2, and so on. Well 12 should be kept as a blank.
- c. Add 5 μ L 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

- a. Prepare the AMP Detection Mix as described in **Section 4.1** and add 10 μ L to each well. Mix gently on a plate shaker and incubate at room temperature for 120 minutes.

	Data Point	AMP (μ M)	NAD ⁺ (μ M)
	1	50	50
	2	25	50
	3	12.5	50
	4	6.25	50
	5	3.125	50
	6	1.563	50
	7	0.781	50
	8	0.391	50

	Data Point	AMP (μM)	NAD ⁺ (μM)
	9	0.195	50
	10	0.098	50
	11	0.049	50
	12	0	50

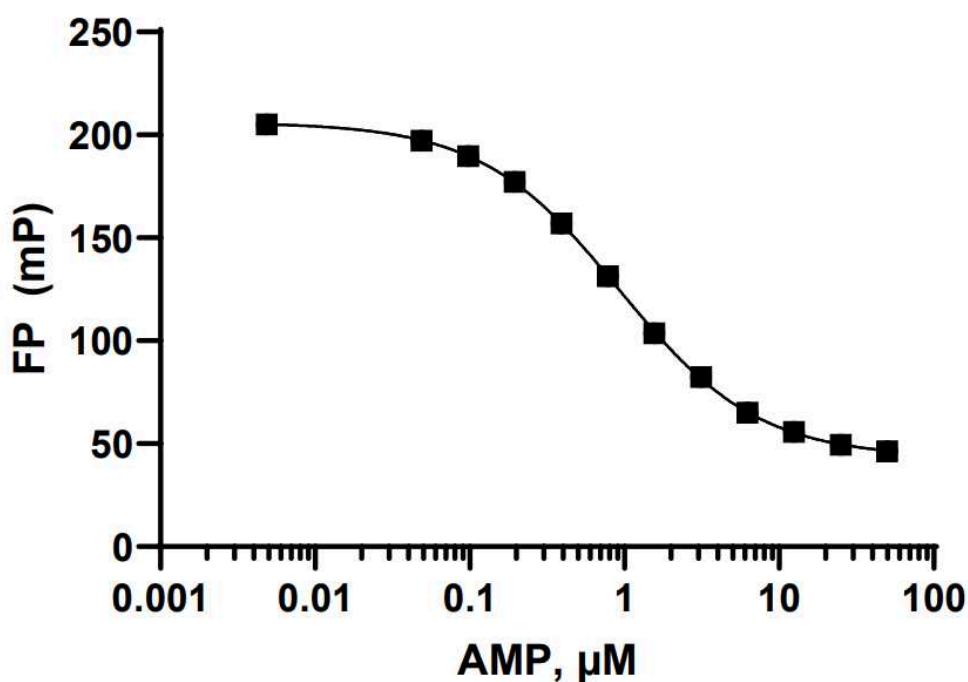


Figure 4. AMP Standard Curves. Example concentration and data for a standard curve corresponding to 50 μM NAD⁺ in SIRT Enzyme Reaction.

Measuring Assay Robustness with Z'

- 4.4 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:



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

Appendix

5

Using the Assay with Different volumes and Plate Formats

- 5.1 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

Links to Applicable Application Notes

- 5.2
- [A Guide to Navigating Hit Prioritization After Screening Using 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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