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Enzolution™ SIRT2 Assay System Technical Manual

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We use this protocol and it's working

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Abstract

The Enzolution™ SIRT2 Assay System is intended for use with the Transcreener® OAADPr SIRT Assay Kits (Parts #3046 and #3047) to measure the enzymatic activity of SIRT2 (NAD-dependent protein deacetylase sirtuin 2). SIRT2 catalyzes transfer of acetyl groups from lysine residues 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 SIRT2 Assay System provides all reagents required to screen and profile SIRT2 inhibitors when used with the Transcreener OAADPr SIRT Assay Kits, including purified, full length human SIRT2 (aa 1-389) 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

1 The Enzolution™ SIRT2 Assay System is intended for use with the Transcreener® OAADPr SIRT Assay Kits (Parts #3046 and #3047) to measure the enzymatic activity of SIRT2 (NAD-dependent protein deacetylase sirtuin 2). SIRT2 catalyzes transfer of acetyl groups from lysine residues 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 SIRT2 Assay System provides all reagents required to screen and profile SIRT2 inhibitors when used with the Transcreener OAADPr SIRT Assay Kits, including purified, full length human SIRT2 (aa 1-389) 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.

Key Applications:

- Screening for SIRT2 inhibitors (or activators)
- Generating dose response curves and IC_{50} values for SIRT2 inhibitors
- Kinetic and mechanistic analyses

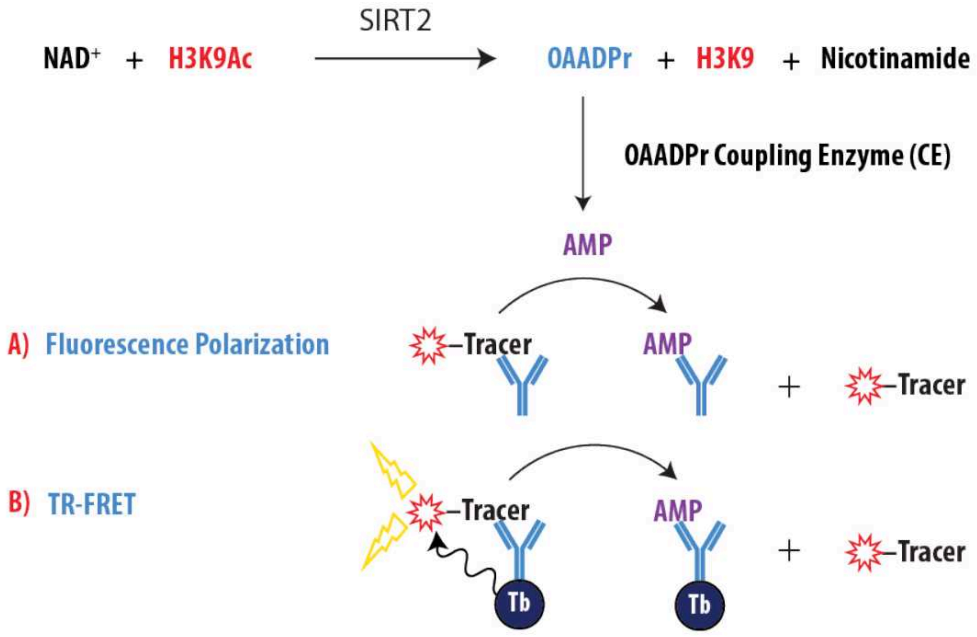

Figure 1. Schematic Overview of the Enzolution SIRT2 Assay System with the Transcreener OAADPr SIRT Assays. OAADPr produced by the target SIRT enzyme is completely converted to AMP in real time by the OAADPr Coupling Enzyme. In the detection step, the CE is quenched by EDTA, and AMP displaces a fluorescent tracer from the AMP[2]/GMP[2] Antibody, resulting in decreased fluorescence polarization (A) or TR-FRET (B).

Product Specifications

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	Product	Quantity	Part#
	Enzolution SIRT2 Assay System	1,000 assays*	3049-1K
		10,000 assays*	3049-10K

***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
	SIRT2 Enzyme	0.5 mg/mL (11.36 µM)* in 50mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol,	Full-length protein (amino acids 1-389), C-Terminal His tag, 44.01 kDa. Sufficient enzyme is included in the kit to complete at least 1,000 assays (Part# 3049-1K) or 10,000 assays (Part# 3049-10K).
	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 µM in the SIRT2 reaction.
	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 $MnCl_2$ and DTT, 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.
	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.
	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 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

	Component	Notes
	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 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 OAADPr SIRT 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# 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 $MnCl_2$ 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.

	Component	Composition	Notes
	$MnCl_2$, 1 M	1 M in water	Add to 1X Enzyme Assay Buffer G to a final concentration of 0.5 mM.

	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 µM 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

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- 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

4 The methods described below are for endpoint detection of OAADPr formation by SIRT2 under initial velocity conditions (≤ 10% conversion of NAD^+ to OAADPr) with a sub-saturating concentration of NAD^+ (50 µM) and H3K9Ac peptide (100 µM). 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**).

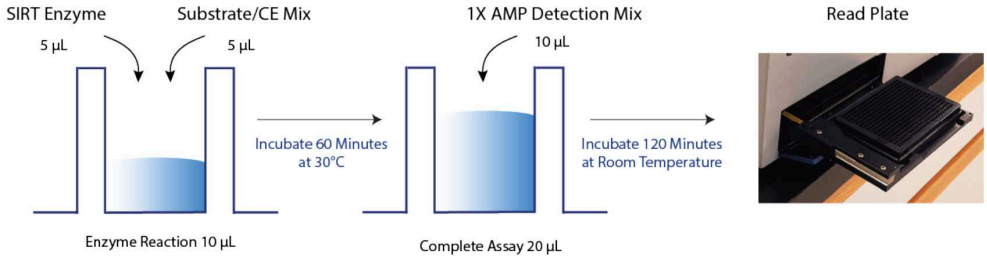

Figure 2. Simple Mix-and-Read Format. The SIRT Enzyme Reaction is run in the presence of CE, so that OAADPr is converted to AMP in real time. After the Enzyme Reaction incubation is complete, 1X AMP Detection Mix is added, which contains EDTA to quench the Enzyme Reaction. Plates are allowed to sit for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

10 µL Enzyme Reaction Components:

	Component	Stock	Working Stock	Final Concentration in 10 µL
	Complete Assay Buffer	10X Enzyme Assay Buffer G, 1 M DTT, 1 M $MnCl_2$	1X Enzyme Assay Buffer G, 1 mM DTT, 0.5 mM $MnCl_2$ in Nuclease Free Water	50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM DTT, 0.01% Triton X-100
	SIRT2 Enzyme	0.5 mg/mL (11.36 µM) SIRT2	2X in Complete Assay Buffer	8 nM – 30 nM (see **Section 4.1**)
	Substrate/ CE Mix	5 mM NAD^+, 10 mM H3K9Ac peptide, 200X CE	100 µM NAD^+, 200 µM H3K9Ac peptide, 2X CE in Complete Assay Buffer	50 µM NAD^+, 100 µM H3K9Ac peptide, 1X CE

Table 1. SIRT2 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 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 G, 1 mM DTT, and 0.5 mM $MnCl_2$ in Ultrapure Nuclease-Free Water.

b. Dilute SIRT2 enzyme to 2X the desired concentration* in Complete Assay Buffer.

c. Prepare Substrate/CE Mix by combining 2X CE, 100 µM NAD^+, 200 µM H3K9Ac peptide in Complete Assay Buffer.

2. Run the SIRT2 Enzyme Reaction

a. Add 5 µL of 2X SIRT2 enzyme to each well, followed by 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.

***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 SIRT2 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:

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

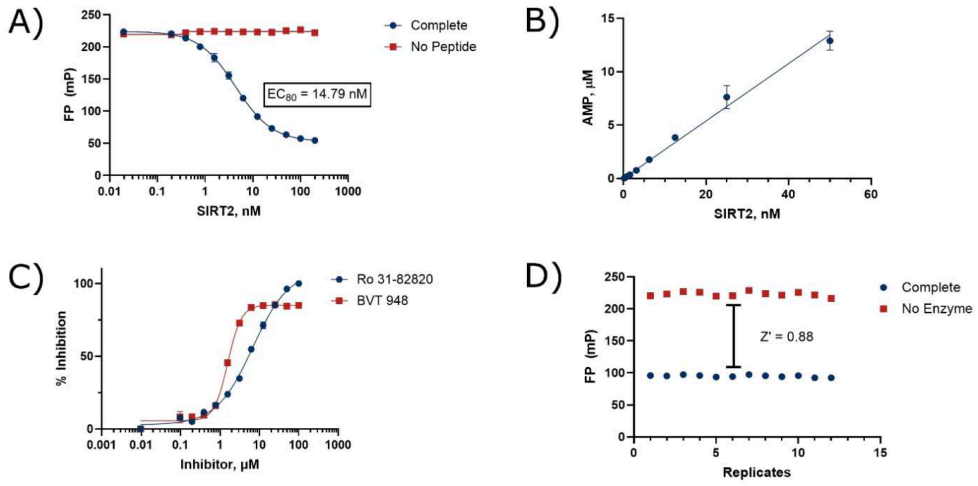

Figure 3. Sample Data for FP Readout Mode. (**a**) Enzyme titration curve. (**b**) Raw polarization in enzyme titration curve is converted to AMP formed using the standard curve in **Section 4.3**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism (see **Section 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 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 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 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 100 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

a. Prepare the AMP Detection Mix as described in **Section 4.1** and add 10 µL to

each well. Mix gently on a plate shaker and incubate at room temperature for 120 minutes.

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

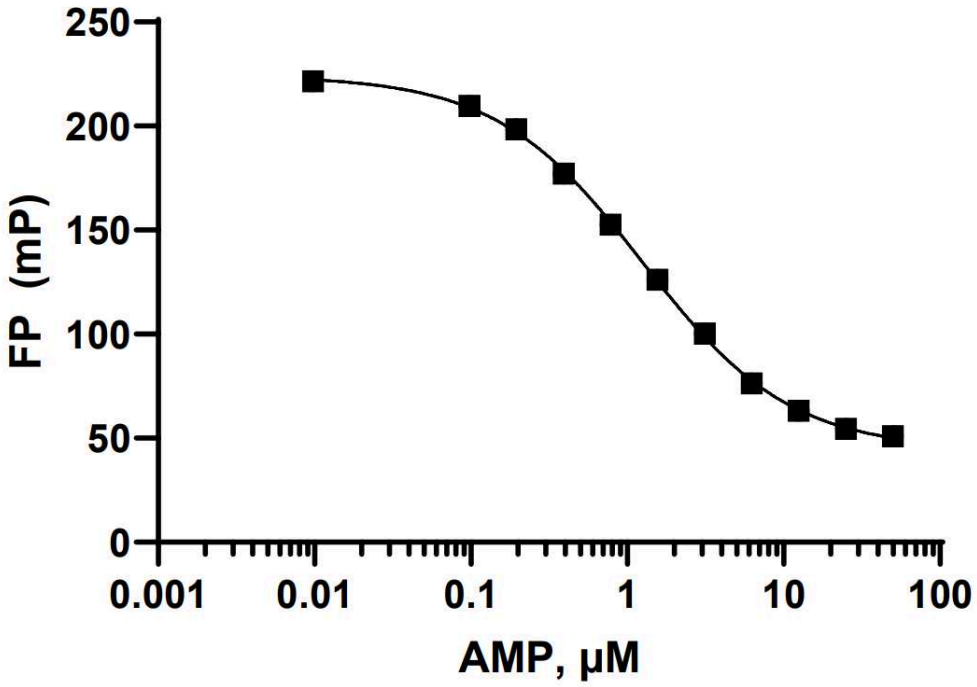

Figure 4. AMP Standard Curves. Example concentration and data for a standard curve corresponding to 50 µM NAD^+ in SIRT Enzyme Reaction.

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:

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

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
- 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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