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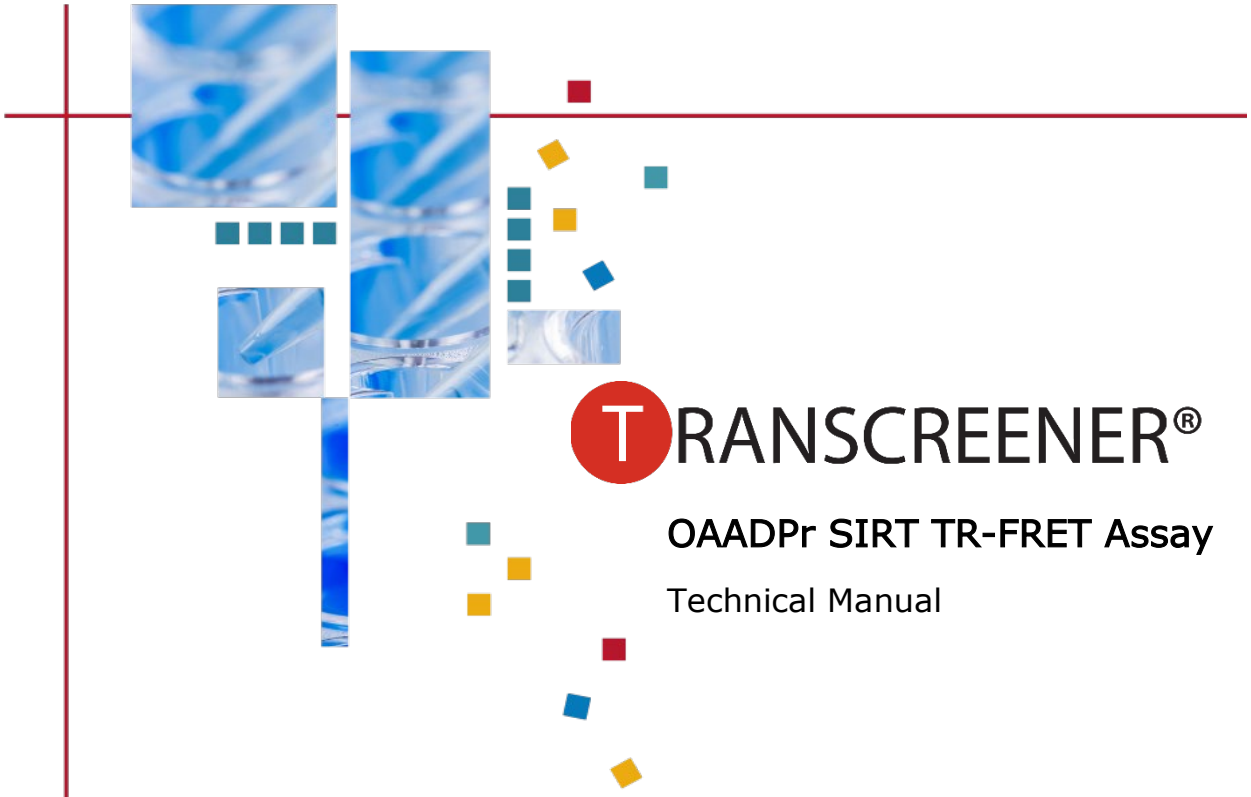

TRANSCREENER®

OAADPr SIRT TR-FRET Assay

Technical Manual

Instructions for Part Numbers 3047-1K and 3047-10K

Transcreener® OAADPr SIRT TR-FRET Assay 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 Dr. Suite 115, Madison, Wisconsin 53717. Phone (608)443-2400. Fax (608)441-2967.

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1.0 Introduction

The Transcreener OAADPr SIRT TR-FRET Assay is a biochemical HTS assay for measuring the production 2'-O-Acetyl-ADP-ribose (OAADPr) by sirtuins (SIRT), NAD-dependent deacetylases (**Figure 1**). The assay relies on a Coupling Enzyme (CE) to convert OAADPr into AMP, which is then detected using a far-red, competitive, time-resolved Förster-resonance-energy-transfer (TR-FRET) assay. As an example, the Transcreener OAADPr SIRT TR-FRET assay can be used to detect the activity of human SIRT1 and SIRT2 using acetylated peptides as substrates.

Key Specifications:

- Single-addition, mix-and-read format
- Signal stability > 24 hours
- Excellent data quality (Z' ≥ 0.7)
- Minimum interference from fluorescent compounds and light scattering with far-red tracer

Key Applications:

- Screening for enzyme inhibitors/activators
- Generating dose-response curves and IC_{50} values for inhibitors
- Kinetic and mechanistic analyses

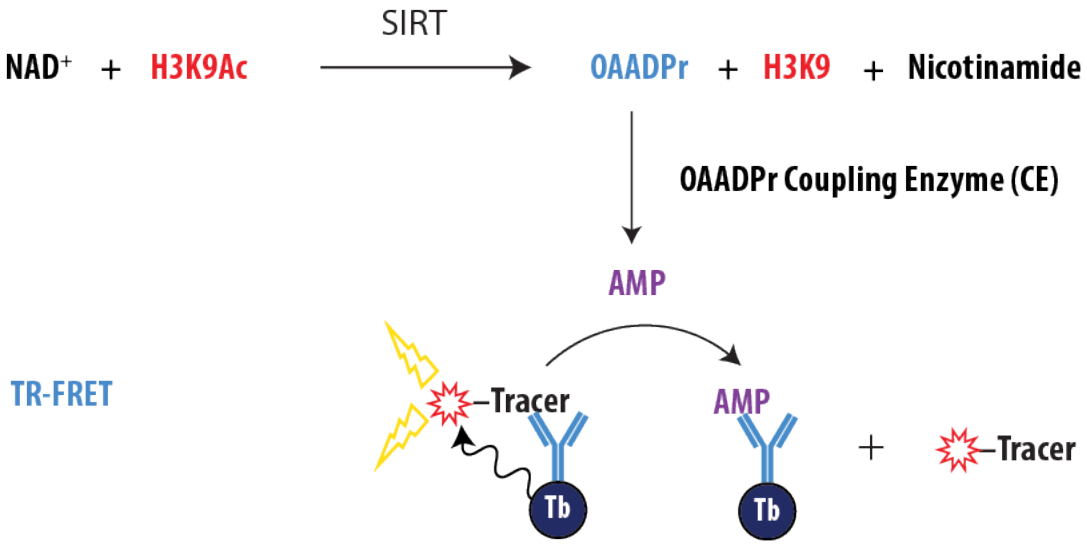

Figure 1. Schematic Overview of the Transcreener OAADPr SIRT TR-FRET Assay. OAADPr produced by the target SIRT Enzyme is completely converted to AMP in real time by the OAADPr Coupling Enzyme. In the detection step, the CE reaction is quenched by EDTA, and AMP displaces a Hilyte 647 Tracer from an AMP[2]/GMP[2] Antibody conjugated to Terbium (Tb), resulting in a decrease in TR-FRET.

2.0 Product Specifications

Product	Quantity	Part#
Transcreener OAADPr SIRT TR-FRET Assay	1,000 assays*	3047-1K
	10,000 assays*	3047-10K

***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.**

IMPORTANT: Antibody centrifugation is required to remove aggregates that can disrupt data quality. Antibodies should be centrifuged at 10,000 x g for 10 minutes before use. Following centrifugation, pipette the solution needed from the top of the aliquot to ensure precipitate is not present in the detection reagents.

Storage

The CE should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that the CE is stable and maintains 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
AMP^2/GMP^2 Antibody-Tb	800 nM solution in 25 mM HEPES buffered saline	The final Antibody-Tb concentration in the 20 µL Complete Assay is 4 nM. Sufficient antibody is included in the kit to complete 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	Sufficient tracer is included in the kit to complete 1,000 assays (Part# 3047-1K) or 10,000 assays (Part# 3047-10K).
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	Sufficient CE for 1,000 assays (Part# 3047-1K) or 10,000 assays (Part# 3047-10K).
Stop & Detect Buffer C, 10X	500 mM HEPES (pH 7.5), 200 mM EDTA, and 0.2% Brij-35	The Stop & Detect Buffer C components quench the CE reactions by chelating metals required for activity.
NAD^+, 5 mM	5 mM in water	Sufficient NAD^+ is included in the kit to complete 1,000 assays (Part# 3047-1K) or 10,000 assays (Part# 3047-10K).
AMP, 5 mM	5 mM in water	The AMP can be used to create a standard curve for conversion of mP values to amount AMP formed, which is equimolar to OAADPr formation.
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.
$MnCl_2$, 1 M	1 M in water	Add to 1X Enzyme Assay Buffer G to a final concentration of 0.5 mM.

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
Enzyme	The Transcreener OAADPr SIRT TR-FRET Assay is designed for use with purified SIRT enzymes, which are available from BellBrook as stand-alone products (Part# 2340, 2341, 2342, 2343) and in SIRT1 and SIRT2 Enzolution Assay kits (Part# 3048, 3049). It is not recommended for use with cell lysates or impure enzyme preparations as contaminating enzymes, such as phosphatases or nucleotidases, can interfere with the assay and reduce the assay window.
Acetylated Peptide	The assay has been validated using H3K9Ac (1-21), an acetylated peptide derived from the N-terminus of Histone H3. H3K9Ac (1-21) is supplied in SIRT1 and SIRT2 Enzolution Assay kits (Part# 3048 and 3049).
Plate Reader	A multimode microplate reader configured to measure 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.
Assay Plates	It is important to use assay plates that are entirely white with a nonbinding surface. We recommend Corning® 384-well plates (Cat.# 4513). The suggested plate has a square well top that enables easier robotic pipetting and a round bottom that allows good Z' factors. It has a recommended working volume of 15–20 µL.

3.0 Before You Begin

NOTE: Read the entire protocol and note any reagents or equipment needed (see Section 2.2).

3.1 Plate Reader Configuration

3.1.1 Filters and Settings

To confirm that your plate reader is capable of measuring TR-FRET (not simply fluorescence intensity) of the terbium:Hilyte 647 donor:acceptor pair (Ex320/Em615/Em665), please see our Instrument Compatibility page, which has links for instrument-specific application notes. Please contact BellBrook Labs Technical Support if you have questions about settings and filter sets for a specific instrument.

3.1.2 Define the Maximum Signal Window for the Instrument

Measuring high (tracer + antibody-Tb) and low (tracer + antibody-Tb + AMP) TR-FRET values will define the maximum assay window of your specific instrument. Prepare High and Low TR-FRET controls in quantities sufficient to perform at least 6 replicates for each condition, as described below, and measure the TR-FRET values.

High FRET Control:

Component	As Provided	Final Concentration in 20 µL Complete Assay	Example: 25 Assays	Your Numbers
AMP²/GMP² Hilyte 647 Tracer	10 µM	100 nM	6 µL*	
AMP²/GMP² Antibody-Tb	800 nM	4 nM	3 µL	
Stop & Detect Buffer C	10X	0.5X	30 µL	
Water			561 µL	
Total			600 µL	

***NOTE: Pipetting small sample volumes accurately requires the correct equipment and proper technique. An extra dilution step may be required to ensure accuracy.**

Low FRET Control:

Component	As Provided	Final Concentration in 20 µL Complete Assay	Example: 25 Assays	Your Numbers
AMP²/GMP² Hilyte 647 Tracer	10 µM	100 nM	6 µL*	
AMP²/GMP² Antibody-Tb	800 nM	4 nM	3 µL	
Stop & Detect Buffer C	10X	0.5X	30 µL	
AMP	5 mM	100 µM	12 µL	
Water			549 µL	
Total			600 µL	

3.1.3 Measure the Ratio of High and Low Signal

Calculate the Z'-Factor using the equation below; values greater than 0.7 are acceptable.

$$Z' = 1 - \frac{[(3 \text{ x High FRET Mixture Stan. Dev.}) + (3 \text{ x Low FRET Mixture Stan. Dev.})]}{(mean\ of\ High\ FRET\ Mixture\ ratio_{\frac{665}{615}} - mean\ of\ Low\ FRET\ Mixture\ ratio_{\frac{665}{615}})}$$

CAUTION: Contact BellBrook Labs Technical Service for assistance if your calculated Z'-factor is less than 0.7.

3.2 Calculate the Optimal AMP²/GMP² Hilyte 647 Tracer Concentration

NOTE: This step is not required if you are using one of BellBrook's Enzolution SIRT Assay Systems.

The sensitivity and dynamic range of the Transcreener OAADPr SIRT TR-FRET Assay are determined by the concentration of the Transcreener AMP²/GMP² Hilyte 647 Tracer. A lower tracer concentration increases the sensitivity but may reduce the dynamic range, while a higher tracer concentration results in lower sensitivity but may extend the dynamic range. The optimal Transcreener AMP²/GMP² Hilyte 647 Tracer concentration for the 1X AMP Detection Mix can be calculated based on the concentration of NAD^+ in the Enzyme Reaction:

[Tracer] (nM) = 2.68 x [NAD^+] (µM) + 15.4

For example, if 50 µM NAD^+ is used in a 10 µL Enzyme Reaction, the optimal AMP²/GMP² Hilyte 647 Tracer concentration for 10 µL of 1X AMP Detection Mix would be 149.4 nM.

4.0 Protocol

4.1 Performing an Enzyme Assay

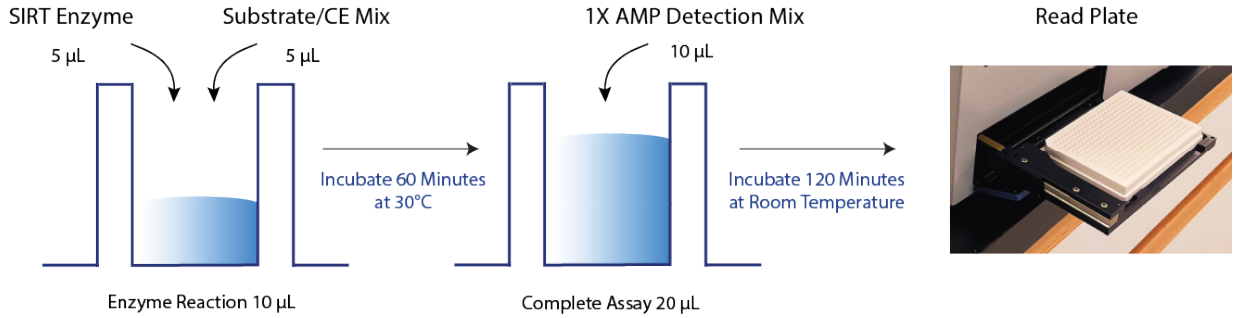

Figure 2. Simple Mix-and-Read Format. The Sirt Enzyme Reaction is run in the presence of CE, so that OAADPr is converted to AMP in real time. After the Enzyme Reaction incubation is complete, 1X AMP Detection Mix is added, which contains EDTA to quench the Enzyme Reaction. Plates are allowed to sit for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

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 format will require changes in reagent quantities (see **Section 6.1**). 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 SIRT enzyme to 2X the desired concentration in Complete Assay Buffer.

   c. Prepare Substrate/CE Mix by combining 2X CE, 2X the desired concentration of NAD^+ and peptide in Complete Assay Buffer.

2. **Run the SIRT Enzyme Reaction**

   a. Add 5 µL of 2X SIRT 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-Tb 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 combining 1X Stop & Detect Buffer C, 8 nM AMP^2/GMP^2 Antibody-Tb, and an appropriate concentration of AMP^2/GMP^2 Hilyte 647 Tracer (see **Section 3.2**) in Ultrapure Nuclease-Free Water.

   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.

1x AMP Detection Mix based on 50 µM NAD^+ in the 10 µL Enzyme Reaction:

Component	1X AMP Detection Mix - Add 10 µL Per Well			
	As Provided	Detection Mix Concentration	Final Concentration in 20 µL Complete Assay	Final Volume in AMP Detection Mix (1X)
AMP^2/GMP^2 Antibody-Tb	800 nM	8 nM	4 nM	100 µL
AMP^2/GMP^2 Hilyte 647 Tracer	10 µM	150 nM	75 nM	150 µL
Stop & Detect Buffer C	10X	1X	0.5X	1000 µL
Water				8,750 µL
Total				**10,000 µL**

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

For inhibitor screening and profiling, we recommend using an enzyme concentration that produces 50–80% of the maximal signal (EC_{50} to EC_{80}) (see **Figure 3**). Meeting these parameters will ensure **a)** a robust signal, resulting Z' values >0.7 and good sensitivity to inhibition and **b)** initial velocity conditions for the enzyme reaction, which corresponds to the linear phase of the reaction (after conversion of mP values to AMP formed). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

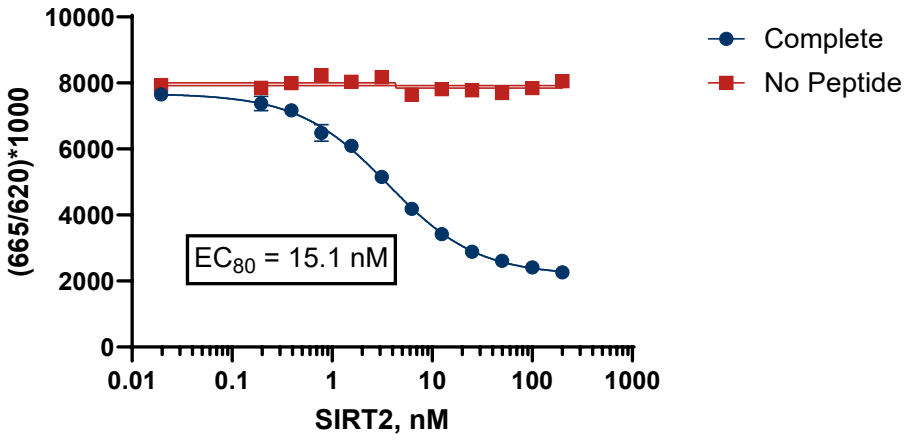

Figure 3. Enzyme Titration Curve. Example SIRT2 Enzyme titration. For screening and profiling inhibitors, the ideal range of enzyme concentrations is between EC_{50} and EC_{80}.

4.2 Performing Single Compound Screening and Dose-Response Assays

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.

4.3 Performing an AMP Standard Curve

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.

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

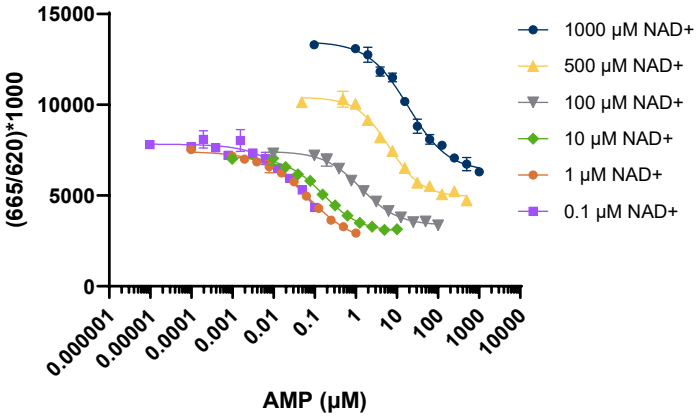

Figure 4. AMP Standard Curves. Example concentrations for a standard curve corresponding to 50 µM NAD^+ and sample standard curves for 0.1 µM, 1 µM, 10 µM, 100 µM, 500 µM, and 1,000 µM NAD^+ used in a 10 µL SIRT Enzyme Reaction.

5.0 General Considerations

5.1 Reagent and Signal Stability

5.1.1 Signal Stability

The stability of the TR-FRET assay window at 10% substrate conversion was determined after the addition of the 1X AMP Detection Mix to the standard samples. The TR-FRET assay window at 10% substrate conversion remained constant (<10% Change) for at least 24 hours at room temperature (20–25°C). If you plan to read TR-FRET on the following day, seal the plates to prevent evaporation.

5.1.2 AMP Detection Mix Stability

The AMP Detection Mix is stable for at least 16 hours at room temperature (20–25°C). If you prepare the AMP Detection Mix more than 30 minutes before addition, store it on ice or at 4°C until needed to help decrease the equilibration time.

5.2 Enzyme Assay Controls

The enzyme reaction controls define the limits of the enzyme assay.

Component	Notes
No Inhibitor Control	This is a complete Enzyme Reaction with all detection components. It provides the maximum signal (minimal TR-FRET value) for an uninhibited enzyme reaction.
No Enzyme Control	This contains all Enzyme Reaction components in the absence of the target enzyme. It provides the minimal signal (maximal TR-FRET value), mimicking 100% Inhibition.
Minus-Substrate Control	This is an alternative to a No Enzyme Control; it may be more appropriate for enzymes that produce some background signal that is not substrate-dependent.

6.0 Frequently Asked Questions

Question	Possible Solutions
Low Selectivity	Suboptimal tracer concentration • To achieve maximum sensitivity and assay window, the AMP^2/GMP^2 Hilyte 674 Tracer concentration must be optimized for each starting NAD^+ concentration.
No change in signal observed	Low antibody/tracer activity or signal. • The tracer and antibody are stable for up to 6 freeze-thaw cycles. For frequent use aliquot the antibody and tracer and store the aliquots at -20°C. Use a minimum of 20 µL aliquots.
Is a standard curve required?	No, it is not required to run a standard curve. We recommend running the AMP standard curve if you want to convert raw signal ratio values to product formed. While designing a standard curve, make sure that most of the points are within the area of interest (initial velocity conditions). We do not recommend using a standard curve from previous experiments, rather generate a new curve with each experiment to achieve the most accurate result.
Can this assay be used with cell lysates?	The assay will only work with purified recombinant protein. The presence of nucleases in the lysates prohibits the use of Transcreener assays with lysates.
High background signal or change in signal after incubation with detection mixture.	Interference from impurities • Nuclease contamination in the buffer can cause the assay window to collapse, causing a change in signal. We recommend using nuclease-free water and freshly prepared buffer for each assay. • Some compounds may interfere with the detection mixture, causing a change in signal. • Bovine serum albumin (BSA) at concentrations >1% interferes with the detection reagents. Detergents, such as Brij-35, can be substituted for BSA in the Enzyme Reaction to prevent nonspecific binding of enzymes and substrates to the plate.

7.0 Appendix

7.1 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

7.2 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

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