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# Transcreener® OAADPr SIRT TR-FRET Assay Technical Manual

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**We use this protocol and it's working**

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## Abstract

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. 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.

## Image Attribution

Figure 1. Schematic Overview of the Transcreener OAADPr SIRT TR-FRET Assay.

## Guidelines

**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.

**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.

## Safety warnings

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

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

## Before start

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

## Introduction

1 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

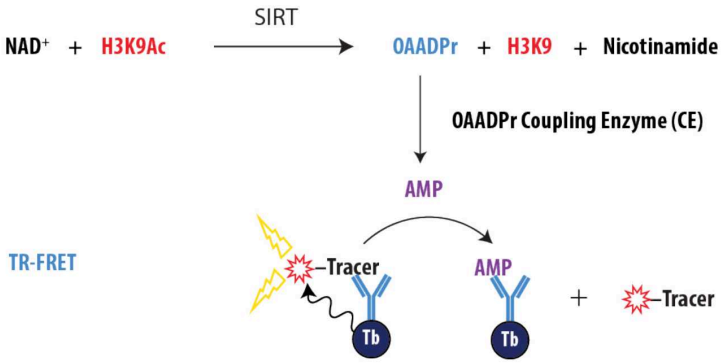


**Figure 1. Schematic Overview of the Transcreener OAADPr SIRT TR-FRET Assay.**
OAADPr produced by the target SIRT Enzyme is completely converted to AMP in real time by the OAADPr Coupling Enzyme. In the detection step, the CE reaction is quenched by EDTA, and AMP displaces a Hilyte 647 Tracer from an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

## Product Specifications

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| | 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.

## Materials Provided

2.1

| | 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. |

## 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 |
| | 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.<br>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. |

## Before You Begin

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

## Plate Reader Configuration

3.1 **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 |
|---|---|---|---|---|---|
| | AMP$^2$/GMP$^2$ Hilyte 647 Tracer | 10 µM | 100 nM | 6 µL* | |
| | AMP$^2$/GMP$^2$ 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 |
|---|---|---|---|---|---|
| | AMP$^2$/GMP$^2$ Hilyte 647 Tracer | 10 µM | 100 nM | 6 µL* | |
| | AMP$^2$/GMP$^2$ 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.**

## Calculate the Optimal AMP$^2$/GMP$^2$ Hilyte 647 Tracer Concentration

3.2 **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 AMP$^2$/GMP$^2$ 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 AMP$^2$/GMP$^2$ 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 AMP$^2$/GMP$^2$ Hilyte 647 Tracer concentration for 10 µL of 1X AMP Detection Mix would be 149.4 nM.

## Protocol

4

## Performing an Enzyme Assay

4.1

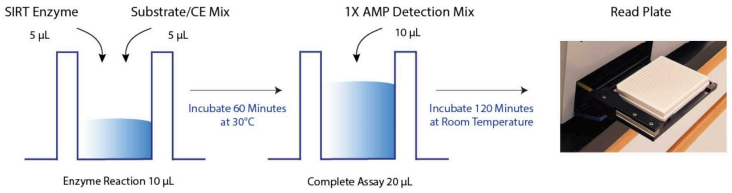


**Figure 2. Simple Mix-and-Read Format.** The Sirt Enzyme Reaction is run in the presence of CE, so that OAADPr is converted to AMP in real time. After the Enzyme Reaction incubation is complete, 1X AMP Detection Mix is added, which contains EDTA to quench the Enzyme Reaction. Plates are allowed to sit 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:**

**1X AMP Detection Mix - Add 10 µL Per Well**

| | Component | 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:

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

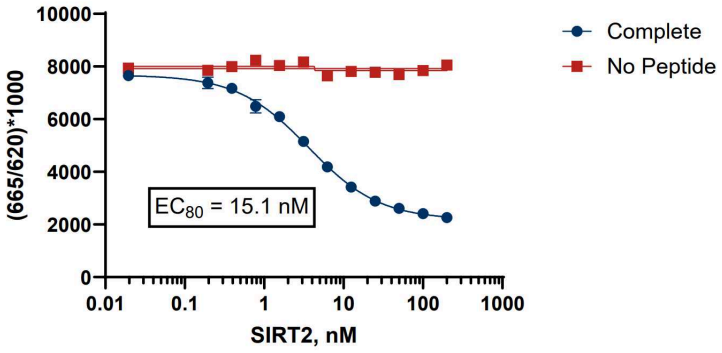


**Figure 3. Enzyme Titration Curve.** Example SIRT2 Enzyme titration. For screening and profiling inhibitors, the ideal range of enzyme concentrations is between $EC_{50}$ and $EC_{80}$.

## 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.**

## Performing an AMP 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.

| | 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 |

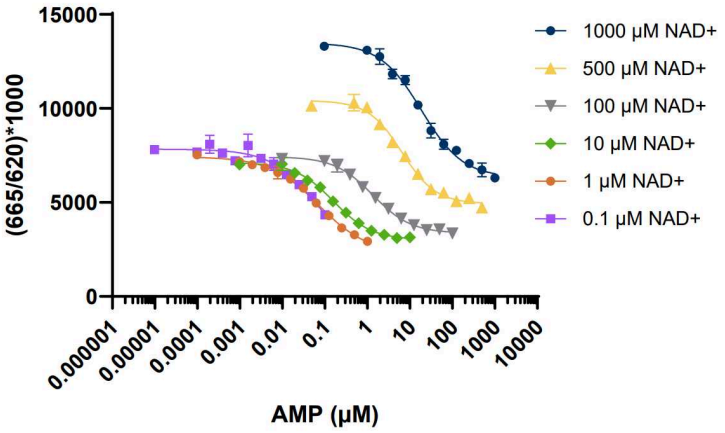


**Figure 4. AMP Standard Curves.** Example concentrations for a standard curve corresponding to 50 µM $NAD^+$ and sample standard curves for 0.1 µM, 1 µM, 10 µM, 100 µM, 500 µM, and 1,000 µM $NAD^+$ used in a 10 µL SIRT Enzyme Reaction.

## General Considerations

5

## Reagent and Signal Stability

5.1 **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.

## Enzyme Assay Controls

5.2 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. |

## Frequently Asked Questions

6

| | Question | Possible Solutions |
|---|---|---|
| | Low Selectivity | Suboptimal tracer concentration<br>▪ 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.<br>▪ 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<br>▪ 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.<br>▪ Some compounds may interfere with the detection mixture, causing a change in signal.<br>▪ 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. |

## Appendix

7

## Using the Assay with Different Volumes and Plate Formats

7.1 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 |

| | Component | Total Volume | Enzyme Reaction Volume | 1X AMP Detection Mix Volume |
|---|---|---|---|---|
| | 1536 Well Low Volume Plate | 8 µL | 4 µL | 4 µL |

## Links to Applicable Application Notes

7.2
- 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

## Contact Information

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