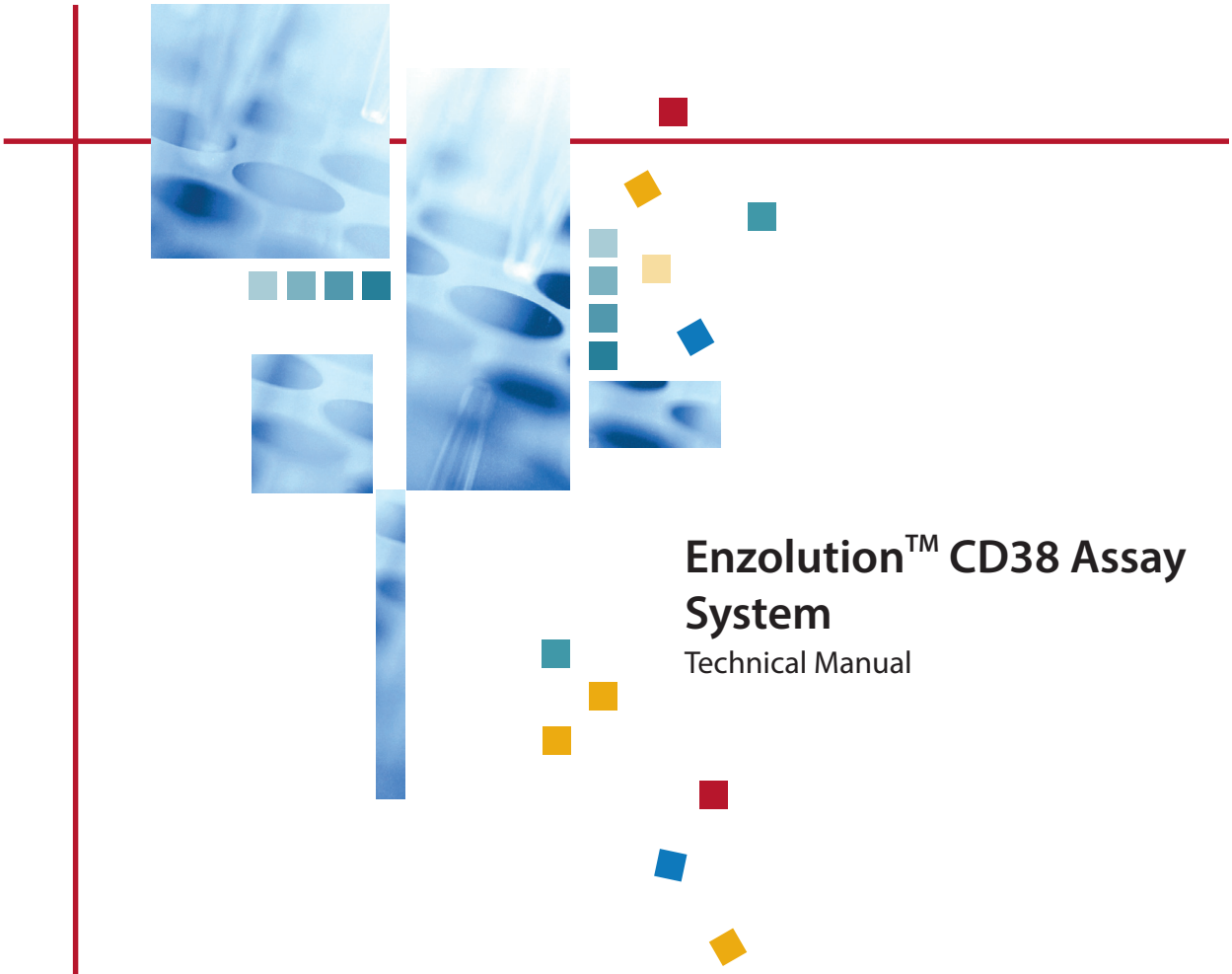
BellBrook Labs

CD38 Assay System



Enzolution™ CD38 Assay System

Technical Manual

Instructions for Part Numbers 3031-1K and 3031-10K

Rev 2024-04

Enzolution™ CD38 Assay System Technical Manual

Contents

1.0 Introduction

The Enzolution™ CD38 Assay System is intended for use with the Transcreener® ADPR FP Assay Kit (Part #3031) to measure enzymatic activity for CD38 (Also known as Cluster of Differentiation 38). CD38 is a multifunctional, membrane-bound enzyme that modulates β-Nicotinamide adenine dinucleotide (NAD) levels by degrading it to adenosine diphosphate ribose (ADPR) and nicotinamide. The Transcreener® ADPR FP assay uses a Coupling Enzyme to convert ADPR to AMP, which is then detected using a far-red, competitive fluorescence polarization (FP) assay. It is in a single addition, endpoint mix-and-read format in which enzyme reactions are quenched by addition of the detection reagents. 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™ CD38 Assay System provides all reagents required to screen and profile CD38 inhibitors when used with the Transcreener® ADPR FP Assay Kit, including purified human CD38 (amino acids 43-300, C-terminal 6xHis) and NAD Substrate. Note that the assay has been optimized to minimize interference of test compounds with the Coupling Enzyme (excess Coupling Enzyme is present), however, we recommend counter screening against the detection reagents to triage false positives. Additionally, the protocol is configured for 384-well plates; use of different multiwell plate formats will require adjustment of reagents concentrations utilized in the assay.

Key Applications:

- Screening for CD38 inhibitors
- Generating dose response curves and IC_{50} values for CD38 inhibitors
- Kinetic and mechanistic analyses

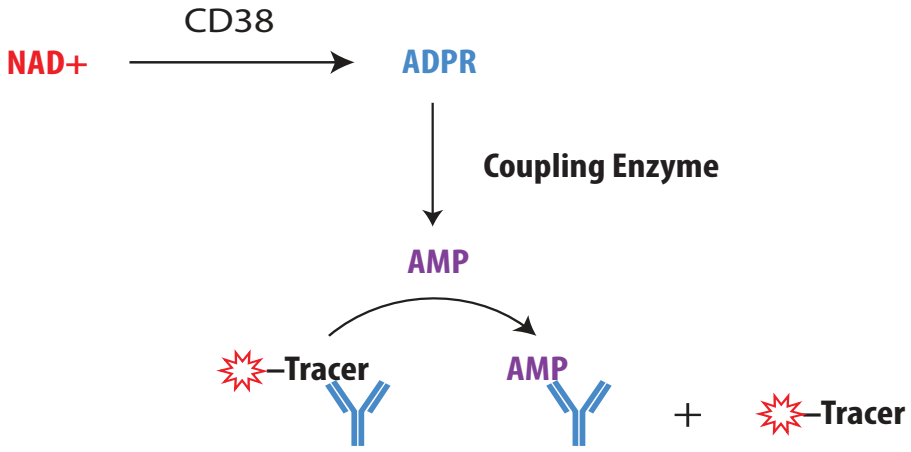


Figure 1. Schematic Overview of the Enzolution™ CD38 Assay System with the Transcreener ADPR FP Assay. ADPR produced by CD38 is converted to AMP by the Coupling Enzyme in real time. In the detection step, the CD38 and the Coupling Enzyme are quenched by EDTA, and AMP displaces an Alexa Fluor® 633 tracer from the AMP²/GMP² antibody, resulting in decreased fluorescence polarization.

2.0 Product Specifications

Product	Quantity	Part #
Enzolution™ CD38 Assay System	1,000 assays*	3031-1K
	10,000 assays*	3031-10K

*The exact number of assays depends on the enzyme reaction conditions. The kits are designed for use with 384-well plates, using 10 µL Enzyme Reaction and 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 the CD38 Enzyme is stable up to 3 freeze-thaw cycles, we recommend aliquoting the enzyme for multiple uses to minimize loss of activity. We recommend aliquoting NAD to avoid the break down of the molecule into detectable products.

Use the reagents provided in this kit within 6 months from date of receipt.

2.1 Materials Provided

Component	Composition	Notes
CD38 Enzyme	0.1 mg/mL (3.2 µM) solution in 50 mM MES (pH 5.0), 100 mM NaCl with 10% glycerol*	Amino acids 43-300, C-terminal 6xHis, 31.2 kDa. Sufficient enzyme is included in the kit to complete 1,000 assays (Part # 3031-1K) or 10,000 assays (Part # 3031-10K).
NAD, 5mM	5 mM in H_2O	Ensure the NAD/Coupling Enzyme Mix (Table 1) is used immediately after preparation to avoid the degradation of the substrate as this may result in an increase in the background signal and reduction of the assay window.
Enzyme Assay Buffer B, 10X	500 mM TRIS (pH 7.5), 100 mM $MgCl_2$, 0.01% BSA, and 0.1% Brij-35	Use Enzyme Assay Buffer B in the Enzyme Reaction and for preincubation with inhibitors. Changes to the assay buffer could affect CD38 activity and/or detection of AMP.
384-Well Low Volume Black Assay Plates	Corning #4514	Black 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.

*The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration.

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	A multimode microplate reader configured to measure FP of the AMP^2/GMP^2 AlexaFluor® 633 Tracer is required. Transcreener® FP Assays have been successfully used 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 a submicroliter volumes into 384-well plates.
Laboratory Incubator	An incubator model that is capable of maintaining temperature stability at 30°C is required.

Transcreener ADPR FP Assay - SOLD SEPARATELY		
Component	**Composition**	**Notes**
AMP^2/GMP^2 Antibody	1.26 mg/mL solution in PBS with 10% glycerol*	Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3030-1K) or 10,000 assays (Part # 3030-10K).
AMP^2/GMP^2 Alexa Fluor ® 633 Tracer	800 nM 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 # 3030-1K) or 10,000 assays (Part # 3030-10K).
ADPR-AMP Coupling Enzyme	400X ADPR-AMP Coupling Enzyme in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 10% glycerol	Sufficient for 1,000 assays (Part # 3030-1K) or 10,000 assays (Part # 3030-10K) with coupling enzyme present in excess to ensure ADPR is completely converted to AMP.
Stop & Detect Buffer B, 10X	200 mM HEPES (pH 7.5), 400 m EDTA, and 0.2% Brij-35	The Stop & Detect Buffer B components quench the Coupling Enzyme Reaction by chelating Mg^{2+}. Therefore, it should work for any target enzyme, as long as the EDTA is at least equimolar to the Mg^{2+}. In the case of CD38, EDTA quenches both the CD38 and Coupling Enzyme reactions. The final concentrations of Mg^{2+} and EDTA in the Complete Assay are 5mM and 20mM, respectively.
ADPR	5 mM ADPR in deionized water, pH 7.0	The ADPR in this kit can be used to create a standard curve to convert mP values to ADPR product formed.

3.0 Before You Begin

1. Read the entire protocol and note any reagents or equipment needed (see **Section 2.2**).
2. Check the FP instrument and verify that it is compatible with the assay being performed (see Full list of compatible plate readers and settings)
3. Please read and understand the Transcreener ADPR Assay Technical Manual prior to using with this kit.

4.0 Protocol

The methods described below are for single-addition, endpoint detection: CD38 and Coupling Enzyme reactions are quenched by the addition of EDTA along with the detection reagents (see **Figure 2**) (Both enzymes are Mg-dependent). The methods were designed for 384-well plates using 10 µL CD38 Enzyme Reaction and 10 µL of detection/quench reagents (final volume 20 µL when the plates are read). The use of different plate densities or reaction volumes will require changes in reagent quantities (see **Section 5.1** for example reaction volumes).

The methods were optimized for initial velocity detection of ADPR formation by CD38 over a range of 0.3 to 3 µM ADPR. These utilize 15 µM NAD, at or below the reported K_m values (15-45 µM), to ensure sensitive detection of inhibitors that compete with NAD. The use of higher NAD concentrations may increase the background signal due to contaminating ADPR and/or AMP. Additionally, significant changes in NAD concentration may require optimization of the AMP²/GMP² Ab concentration to adjust the dynamic range as described in the Transcreener ADPR Assay Manual.

Note: Tracer concentrations remain constant at 4 nM in the 20 µL Complete Assay regardless of changes to other reaction conditions. Additionally, the coupling enzyme is present in at least 5x excess over what is required for complete conversion of ADPR to AMP in real time over a range of initial NAD concentrations; it is not recommended that this parameter be changed.

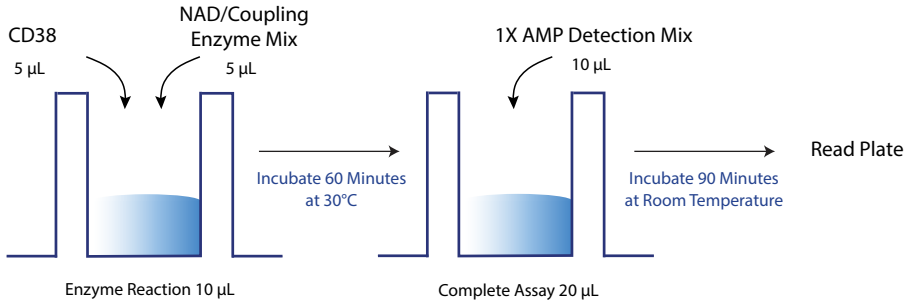


Figure 2. An Outline of the Procedure. The CD38 Enzyme Reaction is run in the presence of Coupling Enzyme, so that ADPR is converted to AMP in real time. After the Enzyme Reaction incubation is completed, AMP detection reagents are added (Transcreener® AMP²/GMP² Antibody and Tracer) along with EDTA to quench the CD38 and Coupling Enzyme.

Component	10 µL Enzyme Reaction Components	
	Working Stock	Final Concentration in 10 µL
Enzyme Assay Buffer B, 10X	1X in Nuclease Free Water	1X (50 mM TRIS pH 7.5, 10 mM $MgCl_2$, 0.001% BSA, and 0.01% Brij-35)
CD38 Enzyme, 0.1 mg/mL (3.2 µM)	2X in 1X Enzyme Assay Buffer B	5 pM - 50 pM*
NAD, 5 mM	30 µM in 1X Enzyme Assay Buffer B (with 2x Coupling Enzyme)	15 µM
Coupling Enzyme, 400x	2X in 1X Enzyme Assay Buffer B (with 30 µM NAD)	1X

Table 1. CD38 Enzyme Reaction Components. Concentrations are provided for the standard protocol using 5 µL CD38 Enzyme Mix and 5 µL NAD/Coupling Enzyme Mix for the Enzyme Reaction.

*See Section 4.1 for Determining the Optimal Enzyme Concentration.

Table 2. 1X AMP Detection Mix Components. Volumes provided in the table are based on preparation of a 10mL solution; adjust these appropriately for the desired volume, including 10% extra for pipetting dead volume.

	1X AMP Detection Mix - Add 10 µL Per Well			
Component	As Provided	Detection Mix Concentration	Final Concentration in 20 µL Complete Assay	Example
AMP²/GMP² Antibody*	1.26 mg/mL	10.0 µg/mL	5 µg/mL	79.4 µL
AMP²/GMP² Alexa Fluor® 633 Tracer	800 nM	8 nM	4 nM	100.0 µL
Stop & Detect Buffer B, 10X	10X	1X	0.5X	1,000.0 µL
Nuclease Free Water	-	-	-	8,820.6 µL
Total Volume	-	-	-	10,000 µL

*The exact concentration may vary from batch to batch. Please refer to the Certificate of Analysis for an accurate concentration.

4.1 Determining the Optimal Enzyme Concentration

Using the enzyme concentration suggested in the CD38 Enzyme Certificate of Analysis should provide a robust signal that is within the linear range for ADPR formation. However, for best results, we suggest performing an enzyme titration to identify the optimal enzyme concentration (EC_{50} to EC_{80}), especially when running the assay in a different buffer system or with a different substrate concentration. The enzyme titration should be performed in duplicate and this example uses a 2X serial dilution. If a compound screen is planned, you should include the solvent (e.g., DMSO) at its final assay concentration.

Note: CD38 is a labile protein that is easily denatured; rapid or prolonged mixing should be avoided to preserve enzymatic activity.

4.1.1 Enzyme Titration Steps

1. Prepare 700 µL 1X Enzyme Assay Buffer B: dilute 70 µL 10X Enzyme Assay Buffer B in 630 µL Ultrapure Nuclease Free Water.
2. Prepare 100 µL of 32 nM CD38 Enzyme: dilute 1 µL of 3.2 µM CD38 Enzyme in 99 µL 1X Enzyme Assay Buffer B.
3. Prepare 160 µL of CD38 Enzyme Mix: dilute 10 µL of 32 nM CD38 Enzyme in 150 µL 1X Enzyme Assay Buffer B.
4. Add 10 µL of the CD38 Enzyme Mix to well 1 (including replicates).
5. Add 5 µL of 1X Enzyme Assay Buffer B to wells 2-12, DO NOT add the Assay Buffer to well 1.
6. Transfer 5 µL from well 1 to well 2 and mix by pipetting, then transfer 5 µL from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 11 has received CD38 Enzyme. Well 12 is to be used as a blank and should not include enzyme.
   IMPORTANT: After mixing the last well (11) in the dilution series, remove 5 µL from that well only and discard, so that all the wells contain 5 µL final volume.
7. Prepare 200 µL of NAD/Coupling Enzyme Mix: dilute 1 µL 400X Coupling Enzyme and 1.2 µL 5 mM NAD in 197.8 µL 1X Enzyme Assay Buffer B.
   Note: Prepare the NAD/Coupling Enzyme Mix right before use to avoid degradation of the substrate and possible reduction of the assay window.
8. Start the enzyme reaction by adding 5 µL of the NAD/Coupling Enzyme Mix to every well (1-12). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
9. Prepare 430 µL 1X AMP Detection Mix based on the concentrations provided in **Table 2**: 43 µL 10X Stop & Detect Buffer B, 4.3 µL AMP²/GMP² Alexa Fluor® 633 Tracer and 10 µg/mL AMP²/GMP² Antibody in Ultrapure Nuclease Free Water.
10. Add 10 µL of 1X AMP Detection Mix to every well (1-12), in replicate.
11. Gently mix on a plate shaker for 40 to 60 seconds and then allow it to incubate at room temperature for 90 minutes before reading.
    Note: The reagent volumes indicated above are sufficient for running the enzyme titration in duplicate plus excess for pipetting dead volume. Scaling of volumes can be performed if necessary.

For detection of inhibitors at single concentration or in dose response mode, we recommend selecting an enzyme concentration that produces a 50–80% change in FP signal (EC_{50} to EC_{80}) (see **Figure 3**) and an assay window of at least 100 mP. This will result in initial velocity conditions, which correspond to the linear phase of the reaction after conversion of mP values to ADPR formed (see **Figure 7**). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

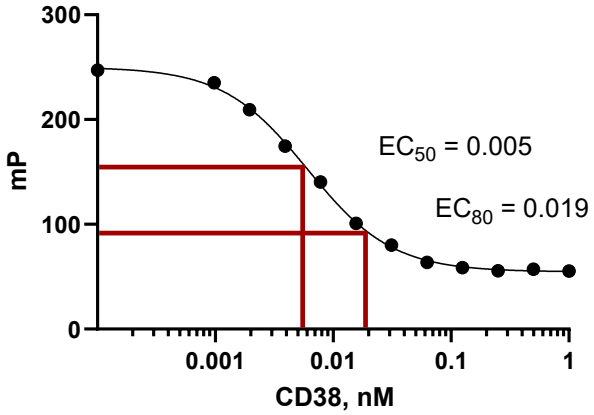
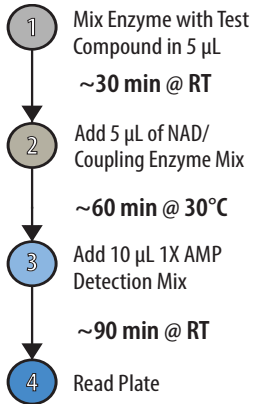


Figure 3. Enzyme Titration Curve. Example enzyme titration with CD38. The ideal range of enzyme concentrations is between EC_{50} and EC_{80}; the specific concentration may vary depending on the enzyme lot.

4.2 Performing Single Compound Screening and Dose-Response Assays

4.2.1 Experimental Samples

1. Perform a serial dilution of test compounds with your method of choice. Add the CD38 Enzyme to the test compounds at the desired concentration so that the total volume of this mixture is 5 µL. Mix gently on a plate shaker for 40 to 60 seconds. Preincubate the Enzyme Inhibitor Mix for the desired time (typically at least 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.
2. Start the enzyme reaction by adding 5 µL of the NAD/Coupling Enzyme Mix. It is recommended to incubate the enzyme reaction at 30°C for 60 minutes.
   Note: The final volume of the enzyme reaction mixture should be 10 µL for 384 well plates. See Section 5.1 for a list of other plate formats.
3. After the incubation, add 10 µL of 1X AMP Detection Mix to the 10 µL Enzyme Reaction and mix the 20 µL Complete Assay using a plate shaker for 40 to 60 seconds.
4. Incubate at room temperature (20–25°C) for 90 minutes and measure FP.



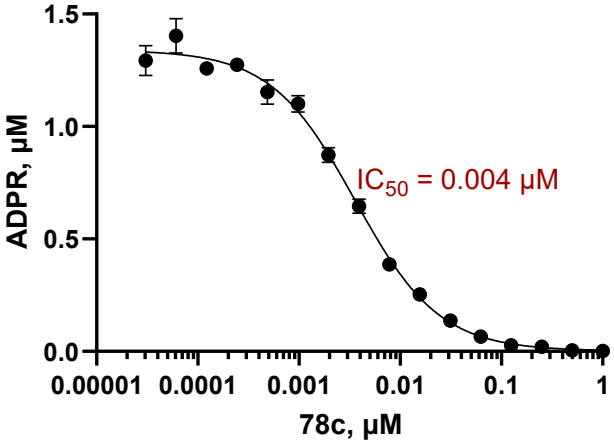


Figure 4. Dose-Response Curve. Example dose response curve with probe inhibitor 78c.

4.3 Setting Up a Standard Curve

Use of a standard curve for conversion of mP values to amount of ADPR formed allows quantitative measurement of CD38 activity and accurate IC_{50} determinations; it is not typically done for screening at single concentrations. Here we describe preparation of a standard curve using 2X serial dilution from 50 µM to 0.05 µM ADPR, which encompasses the appropriate range for this assay using 15 µM NAD.

Note: The reagent volumes indicated below are sufficient for running the standard curve in duplicate plus excess for pipetting dead volume.

1. Prepare 600 µL 1X Enzyme Assay Buffer B: dilute 60 µL 10X Enzyme Assay Buffer B in 540 µL Ultrapure Nuclease Free Water.
2. Prepare 50 µL of 100 µM ADPR: dilute 1 µL 5 mM ADPR stock in 49 µL 1X Enzyme Assay Buffer B.
3. Prepare 200 µL of NAD/Coupling Enzyme Mix: dilute 1 µL 400X Coupling Enzyme and 1.2 µL 5 mM NAD in 197.8 µL 1X Enzyme Assay Buffer B.
   Note: Prepare the NAD/Coupling Enzyme Mix right before use to avoid degradation of the substrate and possible reduction of the assay window.

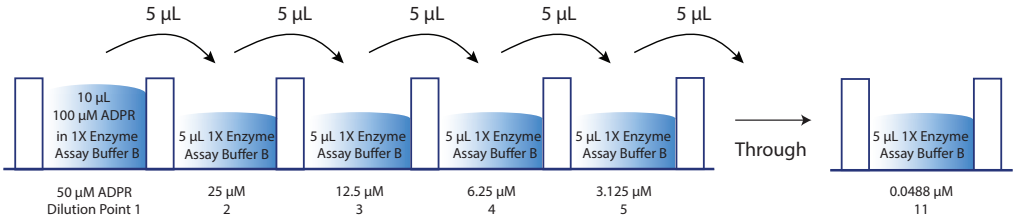


Figure 5. Performing a Serial Dilution. Example 2-fold serial dilution of ADPR to generate a standard curve.

4. Add 10 µL of the 100 µM ADPR to well 1 (including replicates).
5. Add 5 µL of 1X Enzyme Assay Buffer B to wells 2-12, **DO NOT** add the Assay Buffer to well 1.
6. Transfer 5 µL from well 1 to well 2 and mix by pipetting, then transfer 5 µL from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 11 has received ADPR. Well 12 is to be used as a blank and should correspond to 0 µM of ADPR on the standard curve.
   IMPORTANT: After mixing the last well in the dilution series, remove 5 µL from that well only and discard, so that all the wells contain 5 µL final volume.
7. Add 5 µL of the NAD/Coupling Enzyme Mix to every well (1-12). Gently mix for 40 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
8. Prepare 430 µL 1X AMP Detection Mix based on the concentrations provided in **Table 2**: 43 µL 10X Stop & Detect Buffer B, 4.3 µL AMP²/GMP² Alexa Fluor® 633 Tracer and 10 µg/mL AMP²/GMP² Antibody in Ultrapure Nuclease Free Water.
9. Add 10 µL of 1X AMP Detection Mix to every well (1-12).
10. Gently mix on a plate shaker for 40 seconds and then allow it to incubate at room temperature for 90 minutes before reading.

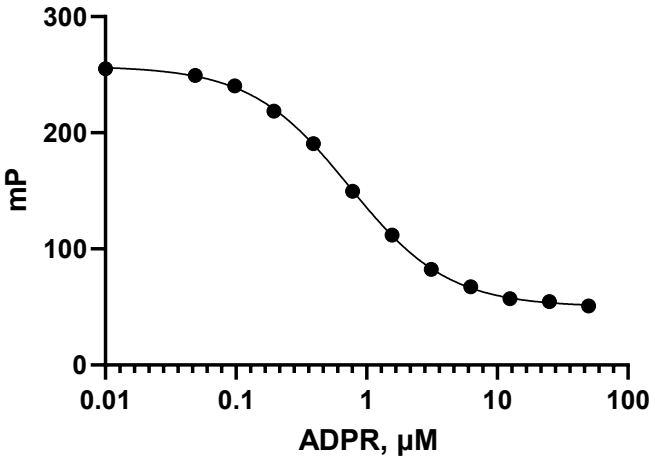


Figure 6. ADPR Standard Curve. Standard curve using 1X AMP Detection Mix as shown in **Table 2.**

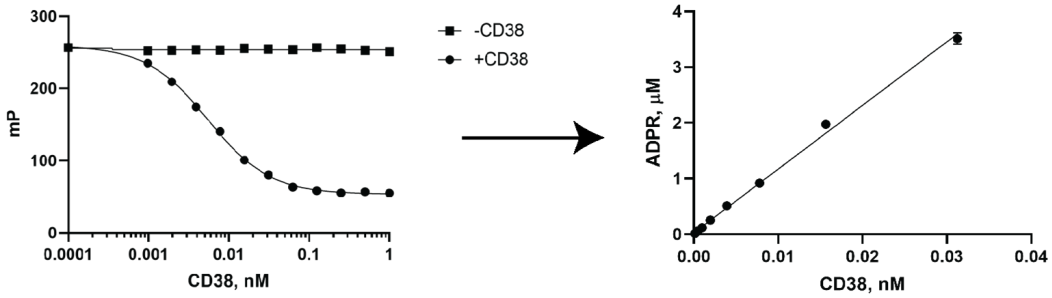


Figure 7. Enzyme titration curve converted to ADPR formed. Raw polarization signal (mP) is converted to ADPR formed using a standard curve as described in **Section 4.3**. Only the linear portion of the graph is shown after interpolation which is performed through GraphPad Prism.

4.4 Measuring Assay Robustness with Z'

By taking into account both dynamic range and data variability at the high and low ranges of the assay, the Z' statistic provides a measure of what is of most interest when considering the suitability of an assay for HTS: the usable screening or "assay window." It is a dimensionless coefficient for the quality of the screening window that is relevant for any assay, regardless of detection method or readout, without the intervention of test compounds. As a guideline , a Z' value of 0.5 or greater is generally considered to be indicative of a very good screening window for a biochemical assay. When running a Z' statistic use the controls with and without enzyme (no test compound) to achieve final results. Use the following formula to determine Z'.

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

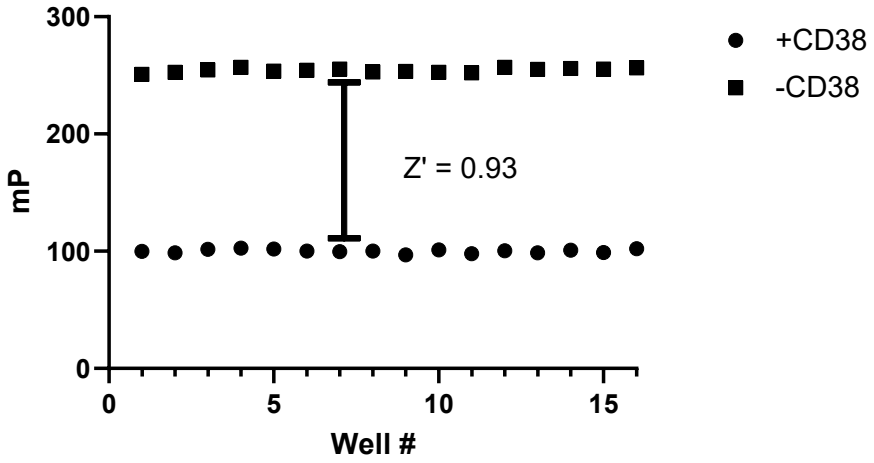


Figure 8. Z' Measurement. Complete assay is performed with and without CD38 Enzyme (n=16). Z' is then calculated based on the formula shown in **Section 4.4.**

5.0 Appendix

5.1 Using the Assay with Different Volumes and Plate Formats

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

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

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

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