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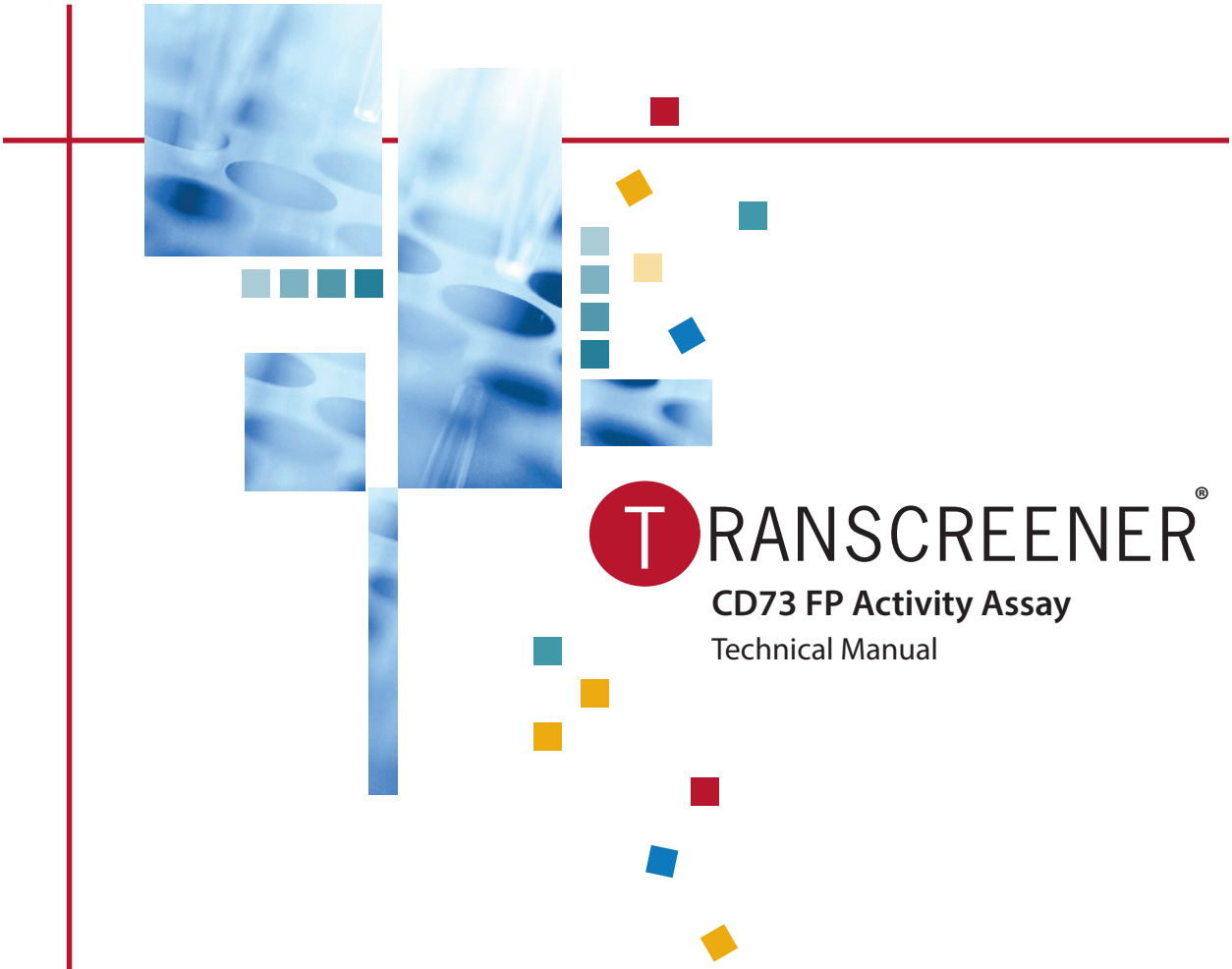
BellBrook Labs

FAR RED FP



TRANSCREENER®

CD73 FP Activity Assay

Technical Manual

Instructions for Part Numbers 3026-1K and 3026-10K

Rev 2020-03

Transcreener® CD73 FP Assay Technical Manual

Contents

1.0 Introduction

The Transcreener® CD73 FP Assay is a far-red, competitive fluorescence polarization (FP) assay (**Figure 1**). The assay is designed to be used with enzymes such as ecto-5'-nucleotidase (also known as 5'-nucleotidase, NT5E, Cluster of Diferentiation 73, or CD73), that produce the product adenosine (ADO) The Transcreener CD73 FP Assay is simple biochemical assay for measuring CD73 activity based on the Transcreener ADP^2 Assay. The assay uses a coupling enzyme to convert adenosine into AMP and ADP in the presence of ATP.

Ectonucleotidases are plasma membrane-bound enzymes with externally-oriented active sites that metabolize nucleotides to nucleosides and are crucial for maintaining immune homeostasis. CD73 is a GPI anchored cell surface protein that plays a critical role in adenosinergic signaling. As an enzyme, CD73 catalyzes the hydrolysis of AMP into ADO and phosphate. Recent studies have shown a key role for adenosine in immunosuppression in the tumor microenvironment with ectonucleotidases emerging as promising immuno-oncology targets.

The Transcreener assay is designed specifically for high throughput screening (HTS), with a single-addition, mix-and-read format. It offers reagent stability and compatibility with commonly used multimode plate readers.

The Transcreener CD73 FP Assay provides the following benefits:

- A simple single addition CD73 activity assay capable of HTS.
- Excellent data quality (Z' ≥ 0.7) and signal (≥85 mP polarization shift) at ADO ranges between 0.3 µM and 3 µM.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

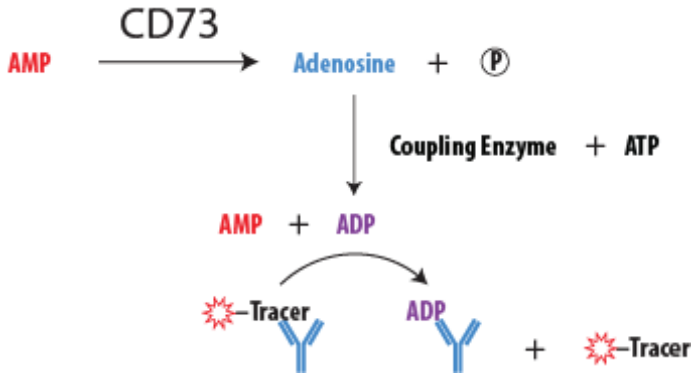


Figure 1. Schematic overview of the Transcreener CD73 FP Assay. The Transcreener ADO Detection Mixture contains a coupling enzyme that generates ADP from ADO, and an ADP AlexaFluor® 633 tracer bound to an ADP antibody. ADP produced by the coupling enzyme displaces the tracer, which rotates freely, causing a decrease in FP.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® CD73 FP Assay	1,000 assays*	3026-1K
	10,000 assays*	3026-10K

*The exact number of assays depends on enzyme reaction conditions. The kits are designed for use with 384-well plates, using 20 µL reaction volumes.

Storage

Store all reagents at –20°C upon receipt.

Please recommend avoiding freeze thaw cycles for the best result. Please aliquot and store if not using multiple reagents at one time.

Use the reagents provided in this kit within 1 year from date of receipt.

2.1 Materials Provided

Component	Composition	Notes
ADP[2] Antibody	3.2 mg/mL solution in PBS with 10% glycerol*	Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3026-1K) or 10,000 assays (Part # 3026-10K).
ADP Alexa Fluor 633 Tracer	400 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 # 3026-1K) or 10,000 assays (Part # 3026-10K).
Coupling Enzyme	1.0 mg/mL solution in 50% glycerol.	Sufficient coupling enzymes to ensure ADO is converted to ADP for detection by ADP detection reagents.
ATP	5 mM	The ATP supplied in this kit can be used for the detection mixture. It is used by the coupling enzyme to convert ADO to AMP and ADP.
AMP	5 mM	The AMP supplied in this kit can be used for the ADO/AMP standard curve
ADO	5 mM	The ADO supplied in this kit can be used for a standard curve.

*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

Note: Contact BellBrook Labs Technical Service for suppliers and catalog numbers for buffer components, and additional information regarding setup of FP instruments.

- ***Ultrapure Water***—Some deionized water systems are contaminated with nucleases that can degrade both nucleotide substrates and products, reducing assay performance. Careful handling and use of ultrapure water eliminates this potential problem.
- ***Enzyme***—Transcreener CD73 assays are designed for use with purified CD73 enzyme preparations. Contaminating enzymes, such as phosphatases or nucleotidases, can produce background signal and reduce the assay window.
- ***Enzyme Buffer Components***—User-supplied enzyme buffer components include enzyme, buffer, and test compounds. We use a buffer comprised of 25mM Tris (pH7.5), 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.01% Brij.
- ***Plate Reader***—A multidetection microplate reader configured to measure FP of the ADP Alexa Fluor 633 tracer is required. Transcreener FP Assays have been successfully used on the following instruments: BioTek Synergy™2 and Synergy™4; BMG Labtech PHERAstar and PHERAstar Plus; Molecular Devices Analyst GT; Perkin Elmer EnVision® and ViewLux; and Tecan Infinite® F500, Safire2™, and M1000.
- ***Assay Plates***—It is important to use assay plates that are entirely black with a nonbinding surface. We recommend Corning® 384-well plates (Cat. # 4514). 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.
- ***Liquid Handling Devices***—Use liquid handling devices that can accurately dispense a minimum volume of 2.5 µL into 384-well plates.

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 **Section 4.1**).

4.0 Protocol

The Transcreener CD73 FP Assay protocol consists of 3 steps (**Figure 2**). The protocol was developed for a 384-well format, using a 10 µL enzyme reaction and 20 µL final volume when the plates are read. The use of different densities or reaction volumes will require changes in reagent quantities (see **Section 7.2** for example reaction volumes). Once the instrument parameters and enzyme optimization are complete, the assay itself consists of a single step, simply add detection reagents to your enzyme reaction and read the plate.

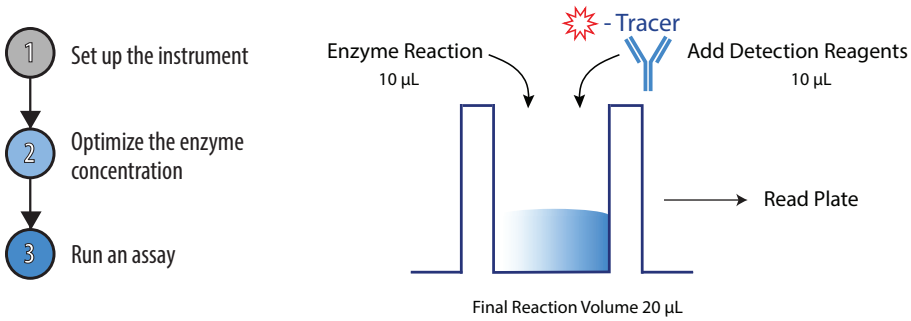


Figure 2. An outline of the procedure. The procedure consists of 3 main steps with a mix-and-read assay format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FP is essential to the success of the Transcreener CD73 FP Assay.

Note: A complete list of instruments and instrument-specific application notes can be found online at: https://www.bellbrooklabs.com/technical-resources/instrument-compatibility Contact BellBrook Labs Technical Service if you have questions about settings and filter sets for a specific instrument.

4.1.1 Verify That the Instrument Measures FP

Ensure that the instrument is capable of measuring FP (not simply fluorescence intensity) of ADP AlexaFluor 633 Tracer.

4.1.2 Define the Maximum mP Window for the Instrument

Measuring high (tracer + antibody) and low (free tracer) FP will define the maximum assay window of your specific instrument. Prepare High and Low FP Mixtures in quantities sufficient to perform at least 6 replicates for each condition.

Use ADP Alexa Fluor 633 Tracer at 2 nM in your enzyme buffer in a 20 µL final reaction volume. This mimics the 2-fold dilution when adding an equal volume of detection mixture to an enzyme reaction. As an example, the 1X detection mixture may contain 4 nM tracer. After adding this to the enzyme reaction, the concentration in the final 0.5X 20 µL reaction volume would be 2 nM.

High FP Mixture

Prepare the following solution.

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
ADP² Antibody	3.2 mg/mL*	10.0 µg/mL	1.6 µL**	
ADP Alexa Flour 633 Tracer	400 nM	2 nM	2.5 µL	
Enzyme Buffer			495.9 µL	
Total			**500.0 µL**	

*Please note ADP² Antibody concentration varies by lot number. This is an example and should be adjusted based on stock concentration accordingly.

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

Low FP Mixture

Prepare the following solution.

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
ADP Alexa Fluor 633 Tracer	400 nM	2 nM	2.5 µL	
Enzyme Buffer			497.5 µL	
Total			**500.0 µL**	

4.1.3 Measure the FP

Caution: Contact BellBrook Labs Technical Service for assistance if the assay window is <100 mP.

Subtract the Low FP Mixture readings from the corresponding High FP Mixture readings. The difference between the low and high FP values should be >100 mP.

4.2 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener CD73 FP Assay. Use enzyme buffer conditions and substrate concentrations that are optimal for your enzyme and experimental goals. If a compound screen is planned, you should include the library solvent at its final assay concentration. We routinely use enzyme buffer containing 25 mM TRIS (pH 7.5), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.01% Brij-35, and 1% DMSO (test compound solvent). Run your enzymatic reaction at its requisite temperature and time period.

4.2.1 Enzyme Titration Steps

To achieve the most robust assay and a high signal, the quantity of enzyme required to produce a 50–80% change in FP signal is ideal (EC_{50} to EC_{80}) for screening of large compound libraries and generating inhibitor dose-response curves (see **Figure 3**). It is recommended to have at least a 100 mP shift to achieve a good assay window. Typically, an EC_{70} to EC_{80} has been used with the CD73 enzyme. To determine the EC_{80} enzyme concentration, use the following equation:

$$EC_{80} = (80 \div (100 - 80))^{(1 \div \text{hillslope})} \times EC_{50}$$

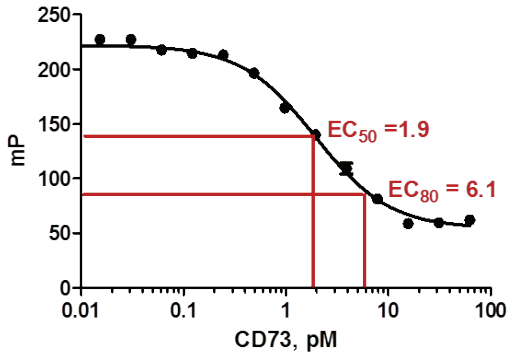


Figure 3. Enzyme titration curve. The ideal range of enzyme concentrations is shown in red.

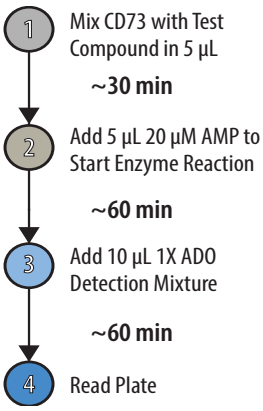
4.2.2 Enzyme Assay Controls

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

Component	Notes
0% ADO Conversion Control	This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme), 0 µM ADO and 10 µM AMP. It defines the upper limit of the assay window.
30% ADO Conversion Control	This control consist of the ADP Detection Mixture, the enzyme reaction components (without enzyme, 3 µM ADO and 7 µM AMP.
100% ADO Conversion Control	This control consists of the ADP Detection Mixture, the enzyme reaction components (without enzyme), 10 µM ADO and 0 µM AMP. It defines the lower limit of the assay window.
Minus-Nucleotide Control	To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of AMP.
AMP/ADO Standard Curve	Although optional, a standard curve can be useful to ensure day-to-day reproducibility and that the assay conditions were performed using initial rates. It can also be used to calculate product formed and inhibitor IC_{50} values. See **Section 7.1** for a description of how to run the standard curve.
Background Control	Use only 0.5X enzyme reaction conditions .

4.3 Run an Assay

4.3.1 Experimental Samples



1. Add the enzyme to the test compounds at the desired concentration. The total volume of this mixture is 5 µL. Mix on a plate shaker. Incubate the enzyme inhibitor mixture for the desired time (typically at least 30 minutes).
2. Start the enzyme reaction by adding 5 µL of AMP, then mix. It is recommended to use concentrations of 10 µM AMP, in the 10 µL final enzyme reaction mixture. Concentrations may vary based on your experiment.
   Note: The final volume of the enzyme reaction mixture should be 10 µL for 384 well plates. Use 2X AMP, in 5 µL to achieve the appropriate final concentration. See **Section 7.2** for a list of other plate formats.
3. It is recommended to incubate the enzyme reaction for 1 hour at room temperature. Please incubate at a temperature and time ideal for your experiment.
4. Prepare 1X ADO Detection Mixture as follows: **The detection mixture should be made and as fresh as possible. It has a deck stability up to 1 hour after being prepared.**

	1X ADO Detection Mixture			
Component	**Stock**	**Detection Mix Conc.**	**Example Volume**	**Your Numbers**
ADP² Antibody	3.2 mg/mL	20.0 µg/mL	62.5 µL	
ADP Alexa Fluor 633 Tracer	400 nM	4 nM	100 µL	
Coupling Enzyme	1.0 mg/mL	20.0 µg/mL	200 µL	
ATP	5 mM	10 µM	20 µL	
Enzyme Buffer	-	-	9,617.5 µL	
Total			**10,000 µL**	

Note: This is an example of running an assay for HTS or to obtain a dose response. Your volumes and concentrations may vary. It is important to have a 1:1 ratio of enzyme mix and detection mix for the final assay readout.

5. Add 10 µL of 1X ADO Detection Mixture to 10 µL of the enzyme reaction. Mix using a plate shaker.
   Note: After detection mixture is added to enzyme reaction the final concentration of components in a 20 µL will be 0.5X the Detection Mixture (2 nM tracer, 10 µg/mL ADP Antibody 10 µg/mL Coupling Enzyme, and 5 µM ATP).
6. Incubate at room temperature (20–25°C) for 1 hour and measure FP.

4.3.2 ADO Detection Controls

These controls are used to calibrate the FP plate reader and are added to wells that do not contain enzyme.

Component	Notes
Minus Antibody (Free Tracer) Control	This control contains the ADP Tracer without the ADP² Antibody and is set to low mP, typically between 20-50 mP depending on the instrument.
Minus Tracer Control	This control contains the ADP² Antibody without the ADP Tracer and is used as a sample blank for all wells. It contains the same ADP² 10 µg/mL Antibody concentration in all wells.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener CD73 Assay Kit does not have a stop solution currently available. The assay has to be done in real time to determine enzymatic activity.

5.1.2 Real-Time Assay

You can perform real-time experiments with the Transcreener CD73 Assay however, the equilibration time for the tracer and ADP² Antibody is greater than 5 minutes, making it difficult to quantitate ADP produced during short-term enzyme reactions. We recommend reading the plate after 30 minutes for the best results.

5.2 Reagent and Signal Stability

Transcreener technology provides a robust and stable assay method to detect ADO.

5.2.1 Signal Stability

Since the assay is a Real-Time assay the signal will change as the enzyme produces more product. We recommend reading the assay between 30 and 60 minutes after addition of the ADO Detection Mixture for the best results. It is important to read plates at the same interval to avoid plate to plate variability when performing high throughput screens.

5.2.2 ADO Detection Mixture Stability

The ADO Detection Mixture is stable for up to 1 hour at room temperature (20–25°C) before addition to the enzyme reaction (i.e., when stored on the liquid handling deck).

6.0 Frequently Asked Questions

Question	Possible Solutions
Other Transcreener Assays require adjustment of antibody concentration. Is that something I need to do for the CD73 FP Assay?	Unlike other Transcreener assays, the CD73 FP Assay does not require adjustment to the antibody concentration. The ADP[2] antibody used for ADO detection in the CD73 assay does not show any selectivity for ADO. One could therefore use the same antibody concentration up to mM amounts of ADO. However, since this assay is a coupled, high concentrations of ADO (>100 uM) can inhibit the coupling enzyme. We have optimized the conditions for 10 uM ADO detection.
No change in FP observed	Low antibody/tracer activity or Δ mP 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. • Other components of the detection mix should not have multiple freeze-thaw cycles. Aliquot reagents and store at -20°C for future use.
Is a standard curve required every time I run the CD73 reaction?	No, it is not required to run a standard curve. We recommend running the AMP/ADO standard curve, if you want to convert the raw mP values to product formed (ADO) or if you are determining Km or Vmax. While designing a standard curve, make sure that most of the points are between 0% and 30% conversion (initial velocity conditions) with fewer points close to 100% conversion. This would ensure that "hook effect" is not an issue while converting mP values to ADO. 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.
Do I need to add both ADO and AMP to my standard curve?	It is best to run a standard curve that mimics your enzyme reaction, to estimate the ADO more accurately. We routinely run the standard curves with ADO and AMP (please note ATP is required by the coupling enzyme in the detection mix).
Can this assay be used with cell lysates?	The assay will only work with purified recombinant CD73 protein. The presence of ATPases and nucleases in the lysates prohibits the use of Transcreener assays with lysates.
High background signal or change in signal after incubation with detection mixture.	• The ATP used in the detection mixture has not been freeze thawed more than 4 times. Non-specific degradation of ATP can also cause a small window. • Buffers should be made fresh with nuclease free water to prevent non-specific hydrolysis of nucleotides. • Be sure to not leave the plate in the instrument between the reads as this may cause evaporation leading to high background. • Use non-binding black plates. Medium binding or high binding plates will cause increased assay variability.
Why is my window with CD73 very small?	Example reasons for a diminished window • Be sure that CD73 protein has activity. We recommend using CD73 from a commercial source as a positive control as needed. • Handle the coupling enzyme with care. We recommend storing the enzyme at -20°C and aliquoting to prevent loss of activity. • Since the equilibration time of the assay is 5-10 minutes, start reading the plate at 30 min. Earlier time points may lead to diminished signal.

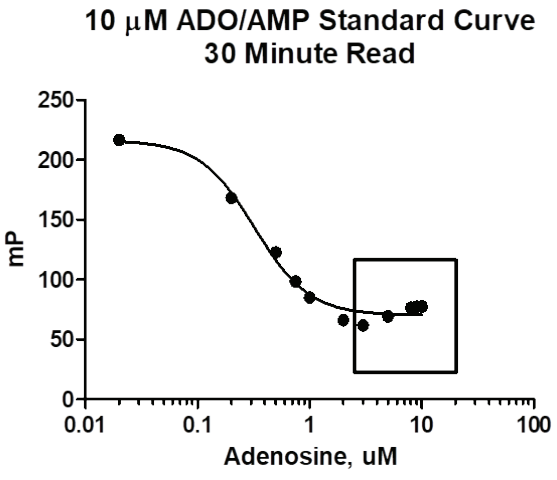
7.0 Appendix

7.1 ADO Standard Curve

The standard curve mimics an enzyme reaction in which ADO is formed. The standard curve allows calculation of the concentration of ADO produced in the enzyme reaction. In this example, a 12-point standard curve was prepared using the concentrations of AMP and ADO shown in **Table 1**. Commonly, 8- to 12-point standard curves are used.

AMP (µM)	ADO (µM)
10	0
9.9	0.1
9.8	0.2
9.7	0.3
9.5	0.5
9.25	0.75
9	1
8	2
7	3
5	5
2	8
1	9
0	10

In this assay, coupling enzyme (in the presence of ATP) converts the adenosine produced by CD73 into AMP while ATP becomes ADP. This AMP feeds into the CD73 reaction (as the assay is designed as a real time assay) preventing depletion of the substrate. The AMP concentration therefore increases gradually as ADP is produced. This causes the mP to increase slightly at 5 µM ADO and higher (>50% conversion) causing a "hook effect." This should not pose any problems to the users as the assay is still able to perform well at initial velocities (<30% conversions) . An example of the "hook effect" is shown below.



ADO (µM)	mP	S. Dev	Z'
0	216.6	8.0	NA
0.2	168.2	5.5	0.16
0.5	122.9	4.6	0.60
0.75	98.4	8.8	0.57
1	85.2	6.3	0.67
2	66.2	4.5	0.75
3	61.7	4.5	0.76
5	69.3	5.5	0.73
8	76.6	3.5	0.76
9	77.5	5.8	0.70
10	77.6	8.5	0.64

Use the following equations to calculate the Z' factor:

$$Z' = 1 - \frac{[(3 \times SD_{0\ \mu M\ ADO}) + (3 \times SD_{sample})]}{|(mP_{0\ \mu M\ ADO}) - (mP_{sample})|}$$

$$\Delta mP = mP_{0\ \mu M\ ADO} - mP_{sample}$$

7.2 Using the Assay with Different Volumes and Plate Formats

Component	Total Volume	Enzyme Reaction Volume	ADO 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.

7.3 Summary of Additive Effects on the Transcreener CD73 FP Assay

The assay window was determined to have limited effect with certain components when used under the recommended conditions. To determine the additive affects of a buffer component please test by titrating the component in the known concentration range. Use only the detection mix and a standard curve to determine the effect on assay performance. Contact BellBrook Labs Technical Support for further reagent compatibility information.

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