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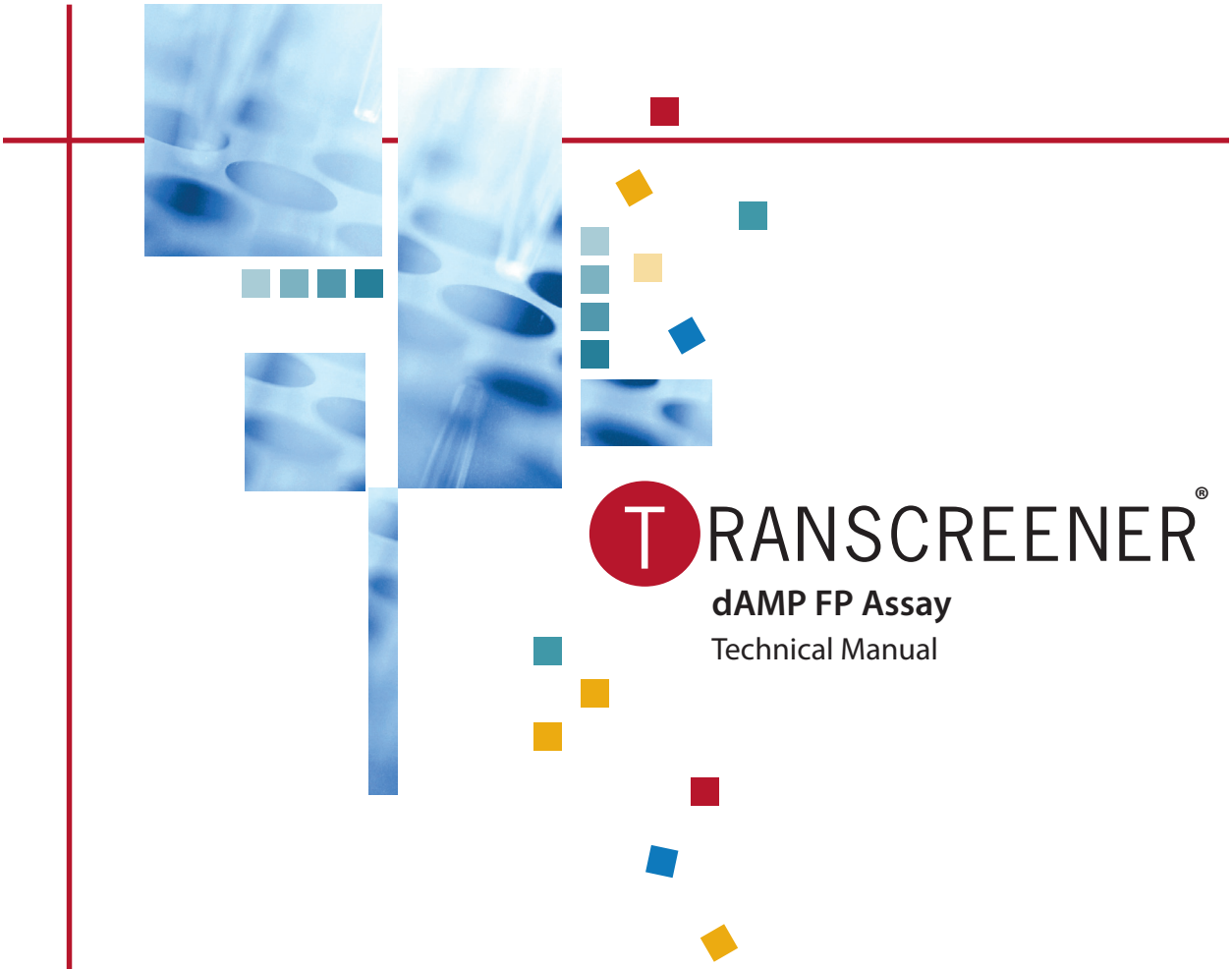
FAR RED FP



TRANSCREENER®

dAMP FP Assay

Technical Manual

Instructions for Part Numbers 3028-1K and 3028-10K

Rev 2022-08

Transcreener® dAMP FP Assay Technical Manual

Contents

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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, 5500 Nobel Drive, Suite 230, Madison, Wisconsin 53711. Phone (608)443-2400. Fax (608)441-2967.

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1.0 Introduction

The Transcreener® dAMP FP Assay is a far-red, competitive fluorescence polarization (FP) assay (**Figure 1**). The assay is designed to be used with exonuclease enzymes such as TREX1 (also known as Three Prime Repair Exonuclease 1), that produce the product dAMP (deoxyadenosine monophosphate, deoxyadenylic acid, or deoxyadenylate) by cleaving sources of DNA. An example is the human enzyme TREX1 which is the major exonuclease responsible for degrading cytosolic DNA.

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 dAMP FP Assay provides the following benefits:

- A simple single addition exonuclease activity assay capable of HTS.
- Excellent data quality (Z′ ≥ 0.7) and signal (≥85 mP polarization shift) at dAMP ranges between 0.1-10 µM.
- Sensitive detection of dAMP down to 10 nM and as high as 100 µM.
- Far-red tracer further minimizes interference from fluorescent compounds and light scattering.

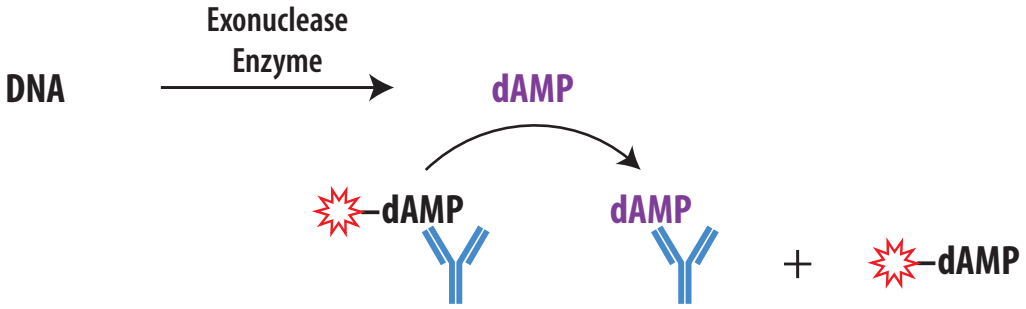


Figure 1. Schematic overview of the Transcreener dAMP FP Assay. The Transcreener dAMP Detection Mixture contains an dAMP AlexaFluor® 633 tracer bound to an dAMP antibody. dAMP produced by an exonuclease enzyme displaces the tracer, which rotates freely, causing a decrease in FP.

2.0 Product Specifications

Product	Quantity	Part #
Transcreener® dAMP FP Assay	1,000 assays*	3028-1K
	10,000 assays*	3028-10K

*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. The assay has exhibited little or no signal change with up to 5 freeze thaw cycles. 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
dAMP Antibody	3.45 mg/mL solution in PBS with 5% glycerol*	Mouse polyclonal antibody. Sufficient antibody is included in the kit to complete 1,000 assays (Part # 3028-1K) or 10,000 assays (Part # 3028-10K).
dAMP 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 # 3028-1K) or 10,000 assays (Part # 3028-10K).
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 will stop enzyme reactions that require Mg^{2+}. To ensure that the enzyme reaction is stopped completely, confirm that the EDTA concentration is at least equimolar to the magnesium ion concentration in the reaction. The final concentration of Stop & Detect Buffer B at the time of FP measurement is 0.5X.
dAMP	5 mM in H_2O (pH 7.0)	The dAMP in this kit can be used to generate a standard curve which can be used to convert mP values to dAMP product formed.

*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 dAMP assays are designed for use with purified enzyme preparations. Contaminating enzymes, can produce background signal and reduce the assay window.
- ***Enzyme Buffer Components***—User-supplied enzyme buffer components include enzyme, buffer, and test compounds.
- ***Plate Reader***—A multidetection microplate reader configured to measure FP of the dAMP 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® Plus and CLARIOstar® Plus; Molecular Devices SpectraMax™ Paradigm; 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 0.5 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 dAMP 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 7.3** 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.

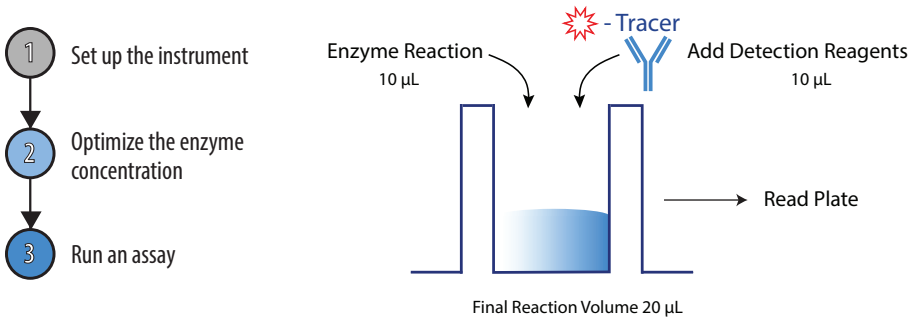


Figure 2. An outline of the procedure. The procedure consists of 3 main steps with a mix-and-read assay format.

4.1 Set Up the Instrument

Becoming familiar with ideal instrument settings for FP is essential to the success of the Transcreener dAMP 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 dAMP 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 dAMP Alexa Fluor 633 Tracer at 1 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 2 nM tracer. After adding this to the enzyme reaction, the concentration in the final 0.5X 20 µL reaction volume would be 1 nM.

High FP Mixture

Prepare the following solution.

Component	Stock Concentration	Final Concentration	Example: 25 Assays	Your Numbers
dAMP Antibody	3.45 mg/mL*	10.0 µg/mL	1.45 µL**	
dAMP Alexa Flour 633 Tracer	400 nM	1 nM	1.25 µL**	
Enzyme Buffer			495.9 µL	
Total			**500.0 µL**	

*Please note dAMP 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
dAMP Alexa Fluor 633 Tracer	400 nM	1 nM	1.25 µL	
Enzyme Buffer			498.75 µL	
Total			**500.0 µL**	

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

4.1.3 Measure the FP

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 Determine Optimal dAMP Antibody Concentration

The antibody is the only assay component that requires adjustment for difference reaction conditions. Its concentration will define the dynamic range of the assay, and it should be adjusted based on the dAMP concentration produced in the enzyme reactions. We have determined optimal dAMP Antibody concentrations for up to 100 µM dAMP.

Using the dAMP Antibody concentration calculated in the chart below will produce excellent results for most users. If it does not produce the results you require a dAMP antibody titration might be required. Please refer to **Section 7.2** for instructions on preparing the dAMP Antibody titration in the buffer system ideal for you enzyme target.

dAMP Concentration in 10 µL Enzyme Reaction	dAMP Antibody Concentration in 10 µL 1X dAMP Detection Mixture
0.2 - 2 µM	10.0 µg/mL
2 - 10 µM	20.0 µg/mL
>10 µM	60.0 µg/mL

4.3 Optimize the Enzyme Concentration

Perform an enzyme titration to identify the optimal enzyme concentration for the Transcreener dAMP 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 have used an enzyme buffer containing 10 mM TRIS (pH 7.5), 7.5 mM $MgCl_2$, 0.01% Brij-35, and 1% DMSO (test compound solvent). Run your enzymatic reaction at its requisite temperature and time period.

4.3.1 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_{80} has been used with enzymes. To determine the EC_{80} enzyme concentration based on the EC_{50}, use the following equation:

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

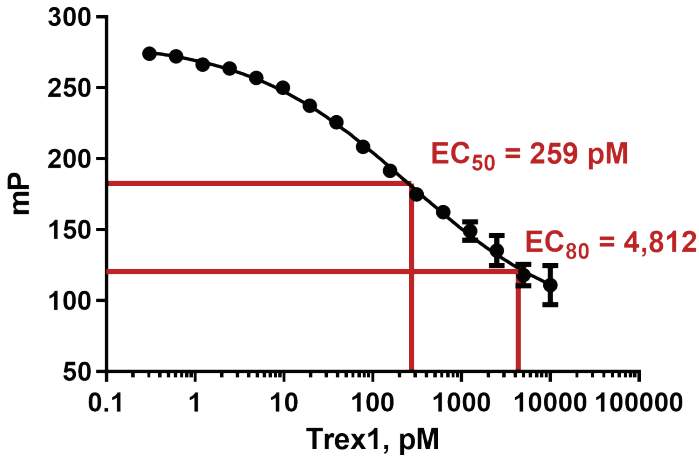


Figure 3. Enzyme titration curve. Example enzyme titration with TREX1. The ideal range of enzyme concentrations is shown in red.

4.3.2 Enzyme Assay Controls

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

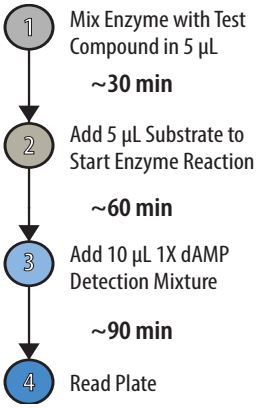
Component	Notes
No Inhibitor Control	This control consists of the dAMP Detection Mixture, the enzyme reaction components with enzyme, and no inhibitor. This control will provide the dAMP formed from the reaction when screening.
No Enzyme Control	This control consists of the dAMP Detection Mixture, the enzyme reaction components without enzyme. It defines the upper limit of the assay window.
Minus-DNA Control	To verify that the enzyme does not interfere with the detection module, perform an enzyme titration in the absence of DNA.
dAMP Standard Curve	Although optional, a standard curve can be useful to ensure day-to-day reproducibility. 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 .

4.4 Run an Assay

4.4.1 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 substrate, then mix. 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 substrate, in 5 µL to achieve the appropriate final concentration. See **Section 7.3** 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 dAMP Detection Mixture as follows:

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.



Component	1X dAMP Detection Mixture			
	Stock	Detection Mix Conc.	Example Volume 2-10 µM dAMP	Your Numbers
dAMP Antibody	3.45 mg/mL	20.0 µg/mL	58 µL	
dAMP Alexa Fluor 633 Tracer	400 nM	2 nM	50 µL	
10X Stop & Detect Buffer B	10X	1X	1,000 µL	
Water	-	-	8,892 µL	
Total			**10,000 µL**	

5. Add 10 µL of 1X dAMP 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 (0.5X Stop & Detect Buffer B, 1 nM tracer, and 10 µg/mL dAMP Antibody).
6. Incubate at room temperature (20–25°C) for 90 minutes and measure FP.

4.4.2 dAMP 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 dAMP Tracer without the dAMP Antibody and is set to low mP, typically between 20-50 mP depending on the instrument.
Minus Tracer Control	This control contains the dAMP Antibody without the dAMP Tracer and is used as a sample blank for all wells. It contains the same dAMP Antibody concentration in all wells.

5.0 General Considerations

5.1 Assay Types

5.1.1. Endpoint Assay

The Transcreener dAMP Assay Kit is designed for endpoint readout. The Stop & Detect Buffer B contains EDTA to stop Mg^{2+} dependent enzyme reactions by chelating available Mg^{2+}.

5.1.2 Real-Time Assay

Real-time experiments can be performed by substituting the Stop & Detect Buffer B, 10X (provided) with a detection buffer that does not contain EDTA. However, the equilibration time for the tracer, dAMP Antibody, and dAMP is at 80% after 15 minutes and stabilizes after 90 minutes. Real-time assay can be achieved but with precaution when converting mP to dAMP formation. Running a standard curve side by side with your real-time assay is a way to achieve accurate conversion. We recommend reading the plate after 90 minutes for screening and potency profiling for best results.

5.2 Reagent and Signal Stability

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

5.2.1 Signal Stability

The mP value at 5 µM dAMP remained constant (<10% change) for at least 24 hours at room temperature (20–25°C). If you plan to read FP on the following day, seal the plates to prevent evaporation.

5.2.2 dAMP Detection Mixture Stability

The dAMP Detection Mixture is stable for up to 8 hours 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
No change in FP observed	Low antibody/tracer activity or Δ mP signal. • The tracer and antibody are stable for up to 5 freeze-thaw cycles. For frequent use aliquot the antibody and tracer and store the aliquots at -20°C. Use a minimum of 10 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 an enzyme reaction?	No, it is not required to run a standard curve. We recommend running the dAMP standard curve, if you want to convert the raw mP values to product formed (dAMP) or if you are determining Km or Vmax. 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 ATPases and nucleases in the lysates prohibits the use of Transcreener assays with lysates.
Why is my window with the enzyme very small?	Example reasons for a diminished window • Be sure that the protein has activity. We recommend using enzyme from a commercial source as a positive control as needed. • Since the equilibration time of the assay is 90 minutes, start reading the plate at 90 min. Earlier time points may lead to diminished signal.

7.0 Appendix

7.1 dAMP Standard Curve

The standard curve mimics an enzyme reaction in which dAMP is formed. The standard curve allows calculation of the concentration of dAMP produced in the enzyme reaction. In this example, a 16-point standard curve was prepared using the concentrations of dAMP shown in **Table 1**. Commonly, 12- to 16-point standard curves are used.

Data Point	dAMP (µM)
1	200.000
2	66.667
3	22.222
4	7.407
5	2.469
6	0.823
7	0.274
8	0.091
9	0.030
10	0.010
11	0.0034
12	0.0011
13	0.00038
14	0.00013
15	0.00004
16	0.000

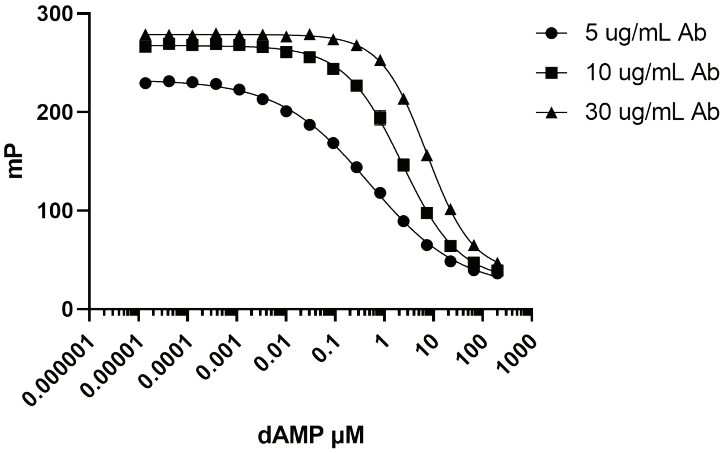


Figure 4. dAMP Standard Curve. Standard curve with varying dAMP Antibody concentrations to obtain ideal sensitivity.

Standard curves illustrate tuning the assay to the appropriate levels for the sensitivity desired. In this example 200 µM dAMP standard curves were completed using 5, 10, and 30 µg/mL final concentrations of dAMP antibody. Using the correct dAMP antibody for the amount of dAMP produced in the assay is imperative to achieving quality results. Use the following equations to calculate the Z′ factor:

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

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

7.2 Optimizing the dAMP Antibody Concentration

Using an antibody concentration from the chart in **Section 4.2** will produce excellent results for most users. If it does not produce the results you require, we recommend that you perform an dAMP Antibody titration in the buffer system ideal for your enzyme target. This titration will determine the optimal antibody concentration for your assay conditions. The nucleotide substrate concentration in the enzyme reaction generally determines the appropriate concentration of dAMP Antibody. We recommend using the EC_{70} for 0.2 - 2 µM, EC_{85} for 2 - 20 µM, and EC_{95} for >10 µM range of dAMP produced by the enzyme for the selected dAMP concentration of antibody

7.2.1. Titrate the dAMP Antibody

1. Prepare the reaction buffer. Example: 10 mM TRIS, 7.5 mM $MgCl_2$, and 0.01% Brij-25. Include desired substrate but omit the enzyme.
2. Dispense 10 µL of the reaction buffer into each well of columns 2–16.
3. Dispense 20 µL of dAMP Antibody (at 2 mg/mL concentration in the same reaction buffer) into each well of column 1.
4. Remove 10 µL from each well of column 1 and add it to the corresponding well of column 2.
5. Repeat step 4 for the remaining columns, thereby performing a 2-fold serial dilution across the plate to column 16.
6. Add 10 µL of 2 nM dAMP Alexa Fluor® 633 Tracer in 1X Stop & Detect Buffer B to each well.
7. Mix the plate, equilibrate at room temperature for 1.5 hours, and measure FP.

7.2.2 Calculate the Optimal dAMP Antibody Concentration

To determine the dAMP antibody concentration for EC_X, input the EC_{50} and hillslope values from a sigmoidal dose-response curve fit into the equation below.

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

7.3 Using the Assay with Different Volumes and Plate Formats

Component	Total Volume	Enzyme Reaction Volume	dAMP 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.

7.4 Summary of Additive Effects on the Transcreener dAMP 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. The maximum tolerance is defined as the mP reading at 0 µM and 10 µM dAMP where there was <3 standard deviation change observed at the listed concentration below. Signal was measured at 1.5 hours and 24 hours after dAMP detection mix was added.

Component	1.5-Hour Tolerance[a]	24-Hour Tolerance[a]
Solvents		
DMSO	5.00%	5.00%
Ethanol	5.00%	1.25%
Methanol	5.00%	5.00%
Glycerol	0.50%	0.50%
Detergents		
Brij-35	0.07%	0.07%
CHAPS	0.03%	0.01%
NP40	0.03%	0.03%
Triton X-100	0.06%	0.06%
Metal chelates		
EDTA	>50 mM	>50 mM
EGTA	>50 mM	>50 mM
Reductants		
Dithiothreitol	7 mM	12.5 mM
Salts		
Magnesium acetate	15.5 mM	15.5 mM
Magnesium chloride	15.5 mM	15.5 mM
Magnesium sulfate	15.5 mM	15.5 mM
Potassium chloride	100 mM	100 mM
Sodium azide	62.5 mM	62.5 mM
Sodium chloride	>250 mM	>250 mM
Zinc chloride	8 mM	8 mM

Component	1.5-Hour Tolerance[a]	24-Hour Tolerance[a]
Phosphatase Inhibitors		
Imidazole	62.5 mM	62.5 mM
Sodium orthovanadate	8 mM	8 mM
Carrier Proteins/Coactivators		
BSA	0.2 mg/mL	0.2 mg/mL
BGG	1.25 mg/mL	1.25 mg/mL
ISDNA	>10 µM	>10 µM

a. mP at 0 µM or 10 µM dAMP where the mP signal increased or decreased <3 standard deviations of the plate controls at the listed concentration.

Not all combination of these components have been tested together. Results may vary depending on your assay conditions.

5500 Nobel Drive, Suite 230
Madison, Wisconsin 53711 USA
Email: info@bellbrooklabs.com
Phone: 608.443.2400
Toll-Free: 866.313.7881
FAX: 608.441.2967