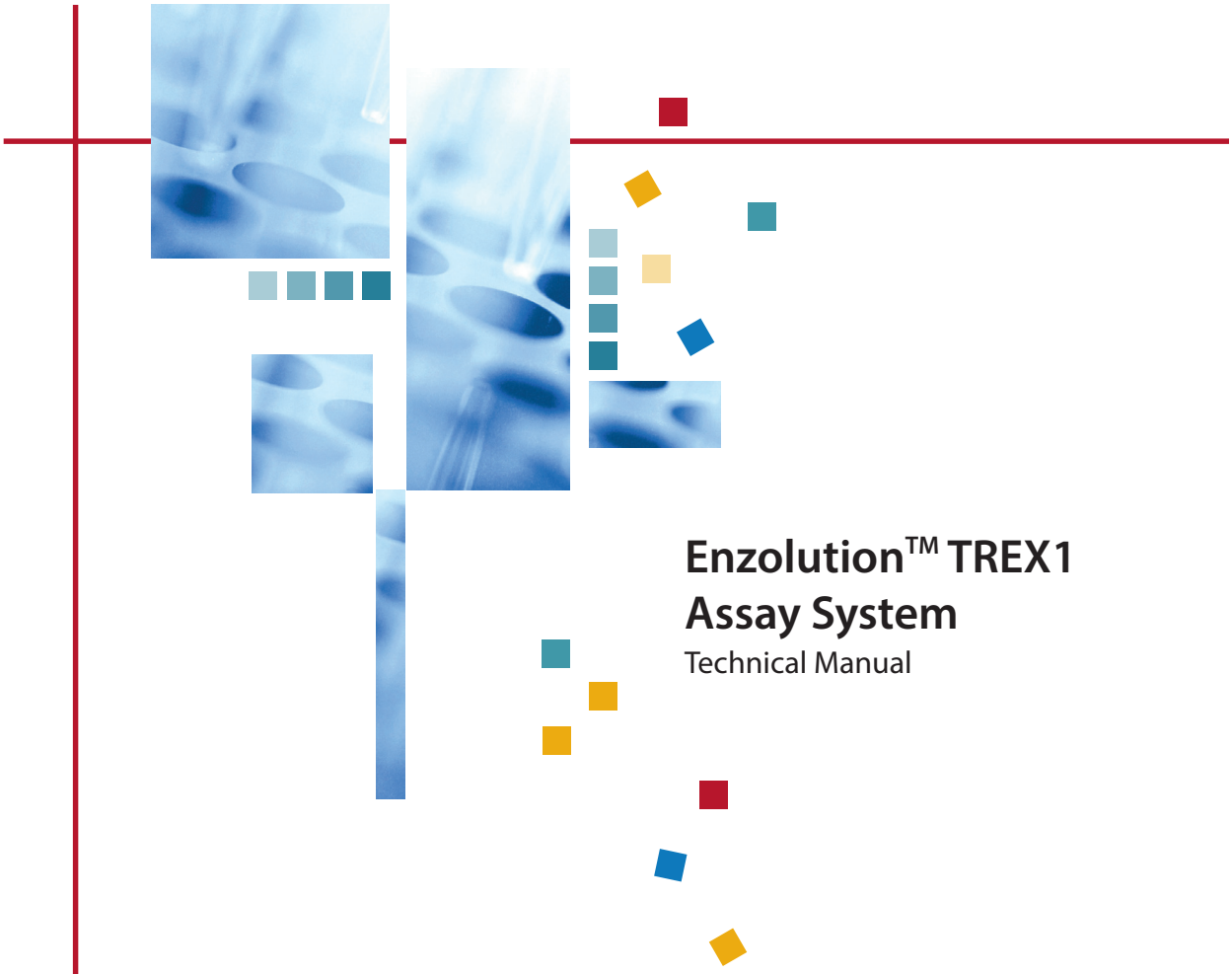
BellBrook Labs

TREX1 Assay System



Enzolution™ TREX1 Assay System

Technical Manual

Instructions for Part Numbers 3029-1K and 3029-10K

Rev 2024-04

Enzolution™ TREX1 Assay System Technical Manual

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U.S. Patent 7,332,278, 7,355,010 and 7,378,505 issued. U.S. Patent Application Nos. 11/353,500, 11/958,515 and 11/958,965, U.S. Divisional Application 12/029,932, and International Patent Application Nos. PCT/US07/088111, European Application Nos. 04706975.2 and 05785285.7, Canadian Application 2,514,877, and Japanese Application 2006-503179 applied. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes other than use of the product or its components to provide a service, information, or data. Commercial Purposes means any activity by a party for consideration other than use of the product or its components to provide a service, information, or data and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (3) resale of the product or its components, whether or not such product or its components are resold for use in research. BellBrook Labs LLC will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use, or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, BellBrook Labs LLC is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, BellBrook Labs LLC, 1232 Fourier Drive, Suite 115, Madison, Wisconsin 53717. Phone (608)443-2400. Fax (608)441-2967.

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1.0 Introduction

The Enzolution™ TREX1 Assay System is intended for use with the Transcreener® dAMP FP Assay Kit (Part #3028) to measure enzymatic activity for TREX1 (also known as Three Prime Repair Exonuclease 1). TREX1 is the major exonuclease responsible for degrading cytosolic DNA, and it plays an important role in preventing aberrant autoimmune responses in normal cells by acting as a checkpoint for the cGAS/STING pathway. It is a 3'-5' exonuclease that cleaves single bases from the 5' termini of double stranded or single stranded DNA, releasing deoxynucleoside monophosphates, including deoxy-AMP (dAMP). The Transcreener® dAMP FP assay enables sensitive detection of dAMP in a single addition, mix-and-read format that has been optimized and extensively validated for high throughput screening (HTS) and inhibitor dose response measurements using most multimode plate readers.

The Enzolution™ TREX1 Assay System provides all reagents required to screen and profile TREX1 inhibitors when used with the Transcreener® dAMP FP Assay Kit, including purified human TREX1 (amino acids 1-286, C-terminal 6xHis) and DNA Substrate. TREX1 is relatively non-specific for its DNA Substrate; the Enzolution™ TREX1 Assay System uses interferon stimulatory DNA (ISD), a 45bp dsDNA oligomer from the Listeria monocytogenes genome that induces a strong type I IFN response in mammalian cells. Note that we have optimized the assay using a saturating concentration of ISD, which makes the assay relatively insensitive to DNA-competitive inhibitors; use of lower ISD concentrations will require adjustment of the TREX1 concentration accordingly. Also, the protocol is configured for 384-well plates; use of different multiwell plate formats will require some additional optimization.

Key Applications:

- Screening for TREX1 inhibitors
- Generating dose response curves and IC_{50} values for TREX1 inhibitors
- Kinetic and mechanistic analyses

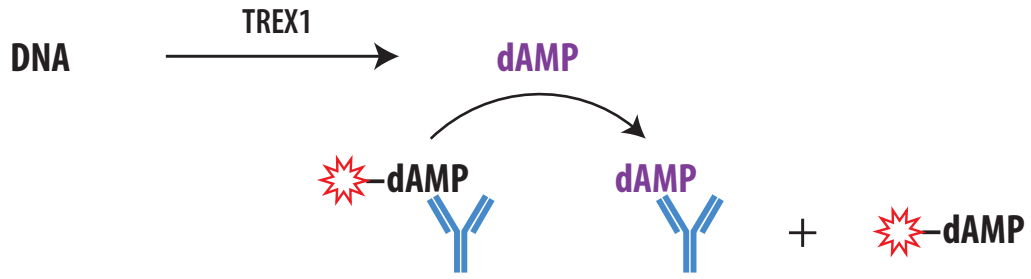


Figure 1. Schematic Overview of the Enzolution™ TREX1 Assay System with the Transcreener dAMP FP Assay. dAMP formed by TREX1 displaces an Alexa Fluor® 633 tracer from the dAMP antibody, resulting in decreased fluorescence polarization.

2.0 Product Specifications

Product	Quantity	Part #
Enzolution™ TREX1 Assay System	1,000 assays*	3029-1K
	10,000 assays*	3029-10K

*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 volumes.

Storage

TREX1 should be stored at -80°C; other reagents can be stored at –20°C. Though we have confirmed that TREX1 is stable up to 2 freeze-thaw cycles, we recommend aliquoting the enzyme and snap-freezing for multiple uses to minimize loss of activity.

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

2.1 Materials Provided

Component	Composition	Notes
TREX1 Enzyme	0.1 mg/mL (3.2 µM)* solution in 50 mM TRIS (pH 8.0), 500 mM NaCl with 20% glycerol	Amino acids 1-286, C-terminal 6xHis, 31.3 kDa. Sufficient enzyme is included in the kit to complete 1,000 assays (Part # 3029-1K) or 10,000 assays (Part # 3029-10K).
Interferon Stimulatory DNA	25 µM in H_2O	Supplied as a 45-bp annealed duplex interferon stimulatory DNA (ISD) oligomer.
TREX1 Assay Buffer, 10X	100 mM TRIS (pH 7.5), 75 mM $MgCl_2$, 0.05% BSA, and 0.1% Brij-35	Use the TREX1 Assay Buffer in the Enzyme Reaction and for inhibitor incubation. Changes to the assay buffer could affect TREX1 activity and/or detection of dAMP.
384-Well Low Volume Black Assay Plates	Corning #4514	Black polystyrene non binding 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 plate formats.

*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 nucleases that can degrade both nucleotide substrates and products, reducing assay performance. Use nuclease free water such as: Invitrogen Part # AM9930
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. 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.
Laboratoy Incubator	An incubator model that is capable of maintaining temperature stability at 30°C is required.

Transcreener® dAMP Exonuclease Assay - SOLD SEPARATELY		
Component	**Composition**	**Notes**
dAMP Antibody	3.5 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 EDTA in the Stop & Detect Buffer B quenches the Enzyme Reaction by chelating Mg^{2+}. Therefore, the assay should work as an end-point assay as long as the EDTA is at least equimolar to the Mg^{2+}. The final concentrations of Mg^{2+} and EDTA in the Complete Assay are 7.5 mM and 20 mM, respectively.
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.

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 Transcreener® dAMP Assay Technical Manual Section 4.1**).
3. Please read and understand the Transcreener® dAMP Assay Technical Manual prior to using with this kit.

4.0 Protocol

The methods described below are for single-addition, endpoint detection in 384 well plates using 10 µL TREX1 reactions and 10 µL of detection/quench reagents (final volume 20 µL when the plates are read). TREX1 reactions are initiated by the addition of the ISD Substrate and quenched by the addition of EDTA in the dAMP Detection Mix (see **Figure 2**) (TREX1 is a Mg-dependent enzyme). The use of different plate densities or reaction volumes will require changes in reagent quantities (see **Section 5.1** for example reaction volumes). Additionally, the methods were designed for initial velocity detection of dAMP formation by TREX1 over a range of 0.2 to 20 µM dAMP; if desired, the dynamic range can be changed by adjusting the concentration of dAMP Ab, as described in the Transcreener® dAMP FP Assay Technical Manual.

	10 µL Enzyme Reaction Components		
Component	As Provided	Working Stock	Final Concentration in 10 µL
TREX1 Assay Buffer, 10X	10X	1X, Nuclease Free Water	1X (10 mM TRIS pH 7.5, 7.5 mM $MgCl_2$, 0.005% BSA, and 0.01% Brij-35)
TREX1 Enzyme	0.1 mg/mL (3.2 µM)	2X in 1X TREX1 Assay Buffer	1X (0.1 nM - 1.2 nM)
Interferon Stimulatory DNA	25 µM	0.70 µM in 1X TREX1 Assay Buffer	0.35 µM

Table 1. TREX1 Enzyme Reaction Components. Concentrations are provided for the standard protocol using 5 µL of TREX1 Enzyme Mix and 5 µL of ISD Substrate Mix for the Enzyme Reaction.

	1X dAMP Detection Mix - Add 10 µL Per Well			
Component	As Provided	Detection Mix Concentration	Final Concentration in 20 µL Complete Assay	Example Amounts for 10 mL Stock
dAMP Antibody*	3.5 mg/mL	20 µg/mL	10 µg/mL	57.1 µL
dAMP Alexa Fluor® 633 Tracer	400 nM	2 nM	1 nM	50.0 µL
Stop & Detect Buffer B, 10X	10X	1X	0.5X	1,000.0 µL
Nuclease Free Water	-	-	-	8,892.9 µL

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

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

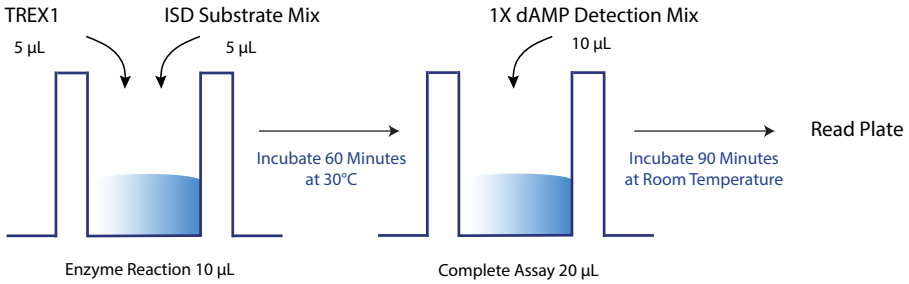


Figure 2. An Outline of the Procedure. The TREX1 Enzyme Reaction is initiated by the addition of ISD. After the Enzyme Reaction incubation is completed, dAMP detection reagents are added (Transcreener® dAMP Antibody and Tracer) along with EDTA to quench the TREX1 reaction.

4.1 Setting Up a Standard Curve

Use of a standard curve for conversion of mP values to amount of dAMP formed allows quantitative measurement of TREX1 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 3X serial dilution from 200 µM to 0.0034 µM dAMP, which encompasses the appropriate range for this application.

Note: These concentrations represent the dAMP levels in the 10 µL TREX1 Enzyme Reaction; they will be diluted 2-fold by addition of detection reagents. The reagent volumes indicated below are sufficient for running the standard curve in duplicate plus excess for pipetting dead volume.

4.1.1 Detailed Methods

1. Prepare 600 µL 1X TREX1 Assay Buffer: dilute 60 µL TREX1 Assay Buffer, 10X in 540 µL Ultrapure Nuclease Free Water.
2. Prepare 30 µL of 400 µM dAMP: dilute 2.4 µL 5 mM dAMP stock in 27.6 µL 1X TREX1 Assay Buffer.
3. Prepare 160 µL of ISD Substrate Mix: dilute 4.5 µL Interferon Stimulatory DNA in 155.5 µL 1X TREX1 Assay Buffer.
4. Prepare 300 µL 1X dAMP Detection Mix based on the concentrations provided in **Table 2**: 30 µL 10X Stop & Detect Buffer B, 1.5 µL dAMP Alexa Fluor® 633 Tracer and 20 µg/mL dAMP Antibody in Ultrapure Nuclease Free Water.

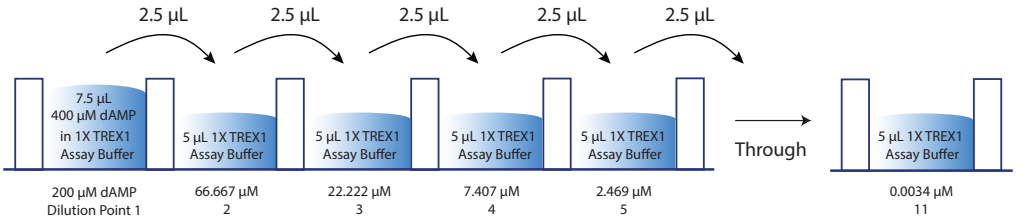


Figure 3. Performing a Serial Dilution. Example 3-fold serial dilution of dAMP to generate a standard curve.

5. Add 7.5 µL of the 400 µM dAMP to well 1 (including replicates).
6. Add 5 µL of 1X TREX1 Assay Buffer to wells 2-12, **DO NOT** add the 1X TREX1 Assay Buffer to well 1.
7. Transfer 2.5 µL from well 1 to well 2 and mix by pipetting, then transfer 2.5 µL from well 2 to well 3 and mix by pipetting; repeat this serial dilution process until well 11 has received dAMP. Well 12 is to be used as a blank and should correspond to 0 µM of dAMP on the standard curve.
   IMPORTANT: After mixing the last well in the dilution series, remove 2.5 µL from that well only and discard, so that all the wells contain 5 µL final volume.
8. Add 5 µL of the ISD Substrate Mix to every well (1-12) then add 10 µL of 1X dAMP Detection mix to every well (1-12).
9. 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.

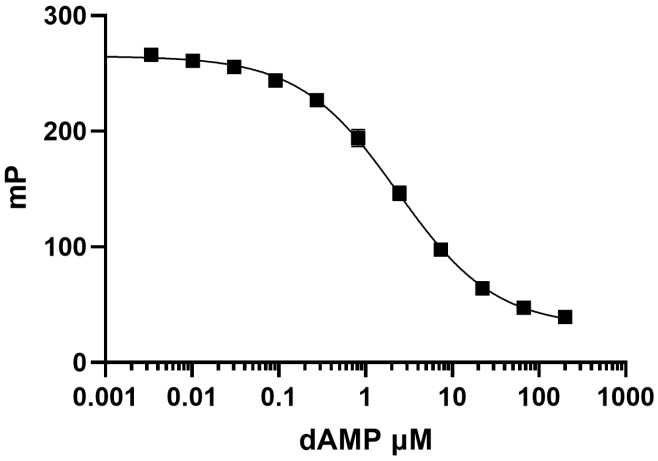


Figure 4. dAMP Standard Curve. Standard curve using 1X dAMP Detection Mix as described in **Step 4** and **Table 2**.

4.2 Determining the Optimal Enzyme Concentration

Using EC_{80} enzyme concentration shown in the TREX1 Enzyme Certificate of Analysis should provide a robust signal that is within the linear range for dAMP formation. However, for best results, we suggest performing an enzyme titration to identify the optimal enzyme concentration, 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 diultion. If a compound screen is planned, you should include the solvent (e.g., DMSO) at its final assay concentration. The Transcreener® dAMP FP Assay Kit has been shown to be able to tolerate up to 5% DMSO.

Note: TREX1 is a labile protein that is easily denatured; rapid or prolonged mixing should be avoided to preserve enzymatic activity.

4.2.1 Detailed Methods

1. Prepare 600 µL 1X TREX1 Assay Buffer: dilute 60 µL TREX1 Assay Buffer, 10X in 540 µL Ultrapure Nuclease Free Water.
2. Prepare 160 µL of 20 nM TREX1 Enzyme: dilute 1 µL TREX1 Enzyme in 159 µL 1X TREX1 Assay Buffer with gentle mixing.
3. Prepare 160 µL of ISD Substrate Mix: dilute 4.5 µL Interferon Stimulatory DNA in 155.5 µL 1X TREX1 Assay Buffer.
4. Add 10 µL of the 20 nM TREX1 to well 1 (including replicates).
5. Add 5 µL of 1X TREX1 Assay Buffer to wells 2-12, DO NOT add the 1X TREX1 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 TREX1 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. Start the enzyme reaction by adding 5 µL of the ISD Substrate Mix to every well (1-12). Gently mix for 40 to 60 seconds on a plate shaker. Incubate at 30°C for 60 minutes.
8. Prepare 300 µL 1X dAMP Detection Mix based on the concentrations provided in **Table 2**: 30 µL 10X Stop & Detect Buffer B, 1.5 µL dAMP Alexa Fluor® 633 Tracer and 20 µg/mL dAMP Antibody in Ultrapure Nuclease Free Water.
9. Add 10 µL of 1X dAMP Detection mix to every well (1-12), in replicate.
10. 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 an 50-80% change in FP signal (EC_{50} to EC_{80}) (see **Figure 5**) 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 dAMP formation (see **Figure 6**). The EC_{50} is provided by common graphing programs; the EC_{80} enzyme concentration can be calculated from the EC_{50}, as follows:

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

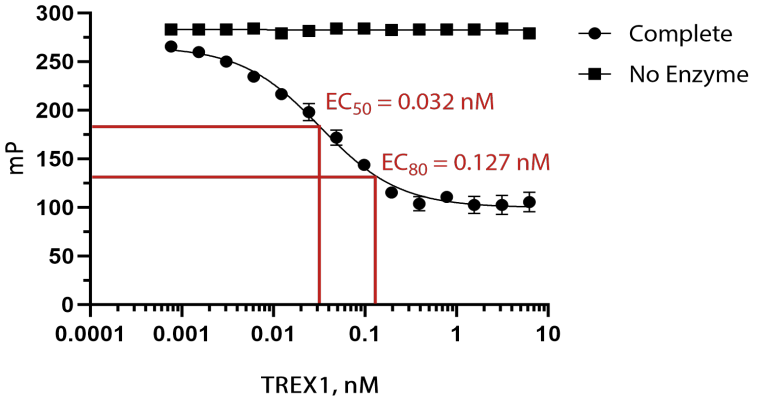


Figure 5. Enzyme Titration Curve. Example TREX1 Enzyme titration. The ideal range of enzyme concentrations is between EC_{50} and EC_{80}; the specific concentration may vary depending on the enzyme lot.

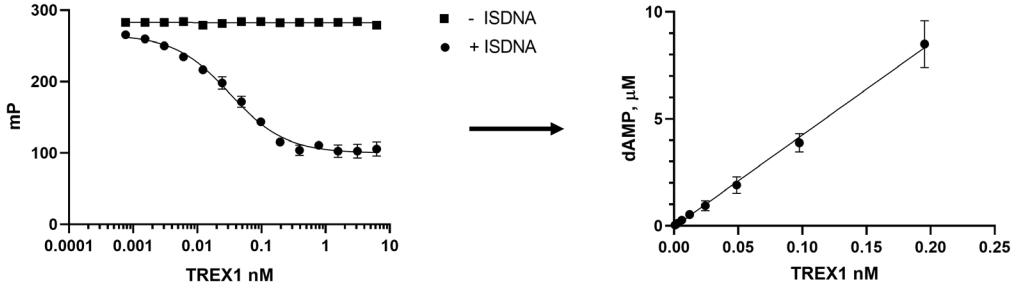
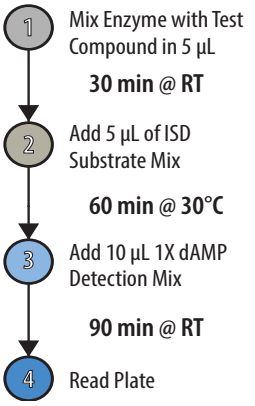


Figure 6. Enzyme Titration Curve converted to dAMP formed. Raw polarization signal (mP) is converted to dAMP formed using a standard curve as described in **Section 4.1**. Only the linear portion of the graph is shown; interpolation was performed using GraphPad Prism.



4.3 Performing Single Compound Screening and Dose-Response Assays

4.3.1 Detailed Methods

1. Perform a serial dilution of test compounds with your method of choice. Add the TREX1 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 should be based on the volume of the Enzyme Reaction.
2. Start the enzyme reaction by adding 5 µL of the ISD Substrate 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. Following the 60 minute incubation, add 10 µL of 1X dAMP 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.

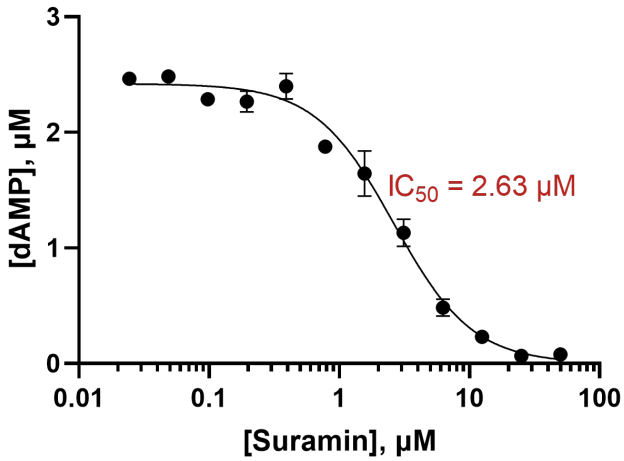


Figure 7. Dose-Response Curve. Example dose response curve with probe inhibitor Suramin.

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 the overall quality of an assay for HTS based on the usable signal or "assay window." It is a dimensionless coefficient for the quality of the assay 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. Z′ is determined using following formula, where complete reactions are untreated TREX1 enzymatic assays and No Enzyme are negative controls containing all components except TREX1; assays should be performed at n =16 for statistical significance.

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

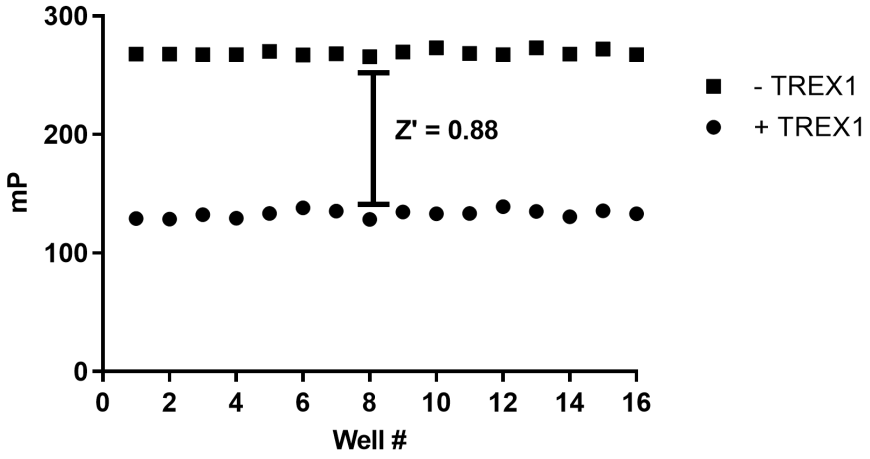


Figure 8. Z′ Measurement. Complete assay is performed with and without TREX1 (n=16). Z′ is then calculated based on the formula from 4.4.1.

5.0 Appendix

5.1 Using the Assay with Different Volumes and Plate Formats

Component	Total Volume	Enzyme Reaction Volume	dAMP Detection Mixture 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

1232 Fourier Drive, Suite 115
Madison, Wisconsin 53717 USA
Email: TechSupport@bellbrooklabs.com
Phone: 608.443.2400
Toll-Free: 866.313.7881
FAX: 608.441.2967