# Interrogating TREX1 with the Transcreener dAMP Exonuclease Assay

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## Overview

Three Prime Repair Exonuclease 1 (TREX1) is a crucial component of the innate immune response where it acts as the main exonuclease responsible for degrading cytosolic DNA. Under normal conditions, removal of cytosolic DNA by TREX1 prevents autoimmune conditions caused by inappropriate stimulation of the cGAS/STING Pathway. However, in the context of cancer, TREX1 acts as a brake on induction of an innate immune response to tumors. As a result, TREX1 plays a pro-tumorigenic role in cancer and development of inhibitors is a promising strategy for future monotherapy and combination treatments.

TREX1 cleaves phosphodiester bonds from the 3' termini of dsDNA and ssDNA, releasing mononucleotide 5'-monophosphates. Though TREX1 Inhibitors are being pursued, there are no peer-reviewed reports with quantitative data on specific molecules. To enable pursuit of a robust lead discovery program for TREX1, we developed an HTS assay that directly detects dAMP formation, enabling quantitative measurement of TREX1 enzymatic activity. In this method, TREX1 cleaves Interferon Stimulatory DNA (ISDna) to produce dAMP, which is then detected by the Transcreener dAMP Exonuclease Assay, a far-red, competitive fluorescence polarization (FP) assay.

Here, we demonstrate how the Transcreener dAMP Exonuclease Assay will provide a reliable and robust tool for the discovery of TREX1 inhibitors. We show the sensitivity and selectivity of the assay, followed by the ability to obtain robust assay signals (>100mP) with less than 250 pM of TREX1 and a Z' Value of 0.88. The assay was validated in a pilot screen of 3056 pharmacologically active molecules, which identified several inhibitors. The assay allows easy triaging of non-stoichiometric inhibitors and profiling selectivity against similar targets. The Transcreener dAMP Exonuclease Assay is a powerful tool for discovery of TREX1 antagonists that will accelerate efforts to validate the target pharmacologically, possibly leading to a new class of immune checkpoint inhibitors.

## Transcreener dAMP Exonuclease Assay: Measure TREX1 Enzyme Activity with a Mix-and-Read FP Readout

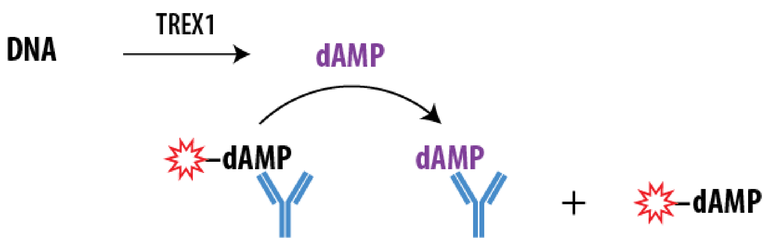


**Figure 1. Schematic Overview of the Transcreener dAMP FP Assay.** The Transcreener dAMP Detection Mixture contains a dAMP AlexaFluor® 633 tracer bound to a dAMP antibody. dAMP produced by the TREX1 enzyme displaces the tracer, which rotates freely, causing a decrease in the FP observed.

## Robust HTS-Ready Assay Procedure

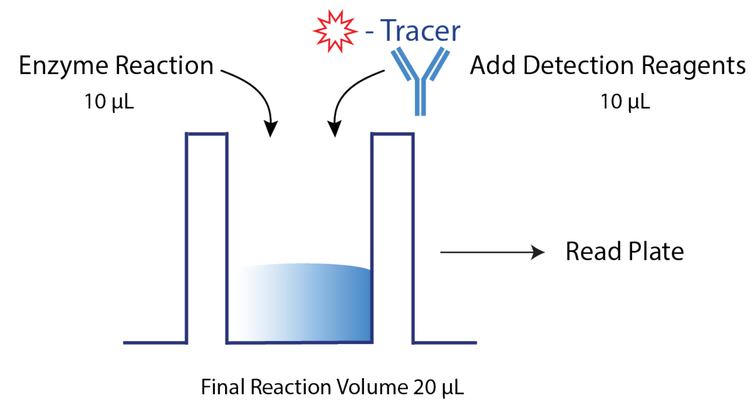


**Figure 2. Transcreener dAMP FP Assay Procedure.** The Transcreener dAMP Exonuclease Assay relies on a simple, but robust, mix-and-read procedure that is compatible with 96, 384, and 1536-well formats. The assay is performed by running an enzyme reaction followed by the addition of detection reagents. Data can then be obtained with a compatible plate reader. Here a 10 µL enzyme reaction was completed, followed by adding 10 µL detection reagents in a 384-well format.

## Nanomolar Sensitivity and Outstanding Selectivity

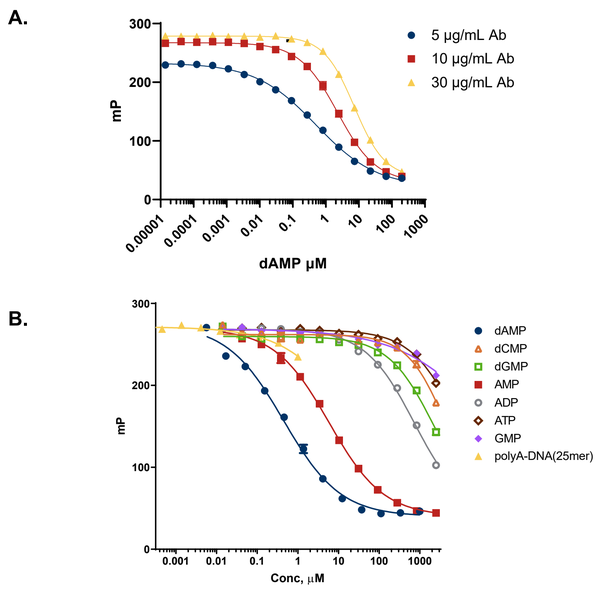


**Figure 3. Assay Sensitivity and Specificity of dAMP Antibody. A.** Competition curves indicate displacement of tracer by dAMP and show dependence of antibody concentration on the dynamic range. A lower limit of detection of 1 nM can be achieved by using 5 µg/mL Ab. **B.** Competition curves show outstanding selectivity for dAMP vs. dCMP, dGMP, and related molecules; cross-reactivity with AMP is not functionally relevant because it is not present in TREX1 reactions.

## Detection of TREX1 Under Initial Velocity

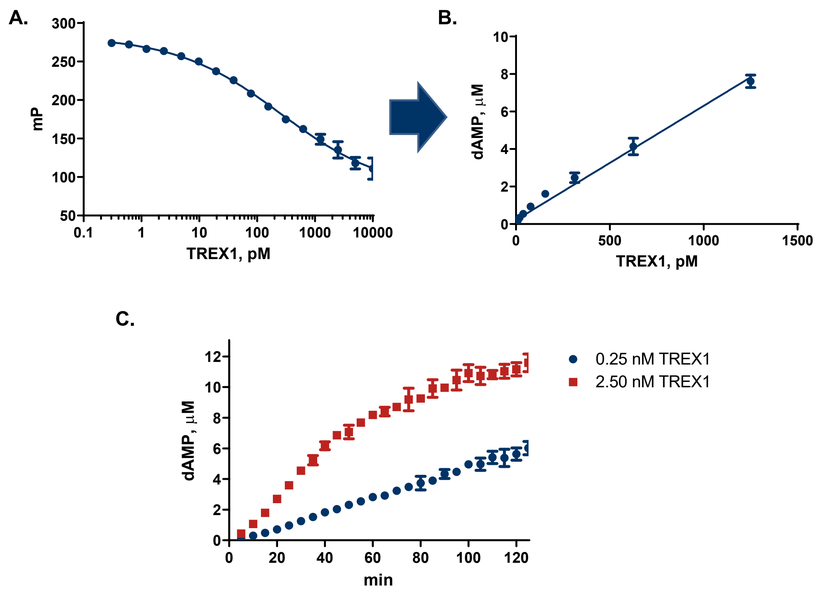


**Figure 4. TREX1 Enzyme Titration and Kinetics. A.** Dependence of assay response on TREX1 concentration. **B.** Conversion of mP to dAMP using a standard curve demonstrates that dAMP formation is linear with enzyme. **C.** Continuous detection of dAMP formation by TREX1 showing linearity over time until substrate DNA is depleted (10x enzyme, 2.5 nM), indicating initial velocity in the screening condition (350 nM ISDna and 250 pM TREX1 for 60 min).

## DNA Dependence and Z' Determination

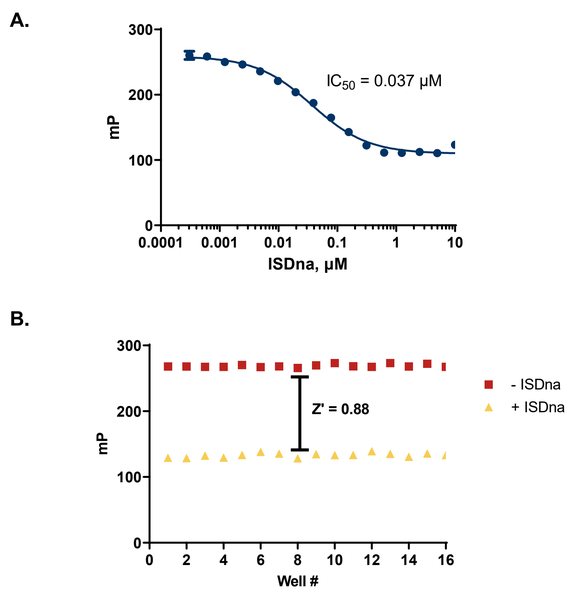


**Figure 5. Assay Robustness for HTS Screening.** A. Dependence TREX1 activity on ISDna concentration; half-maximal response of 37 nM. B. Z' measurement using optimized TREX1 assay conditions (n=16). A Z' of 0.88 demonstrates a robust assay method amenable to HTS.

## Pilot Screen of 3056 Small Molecules

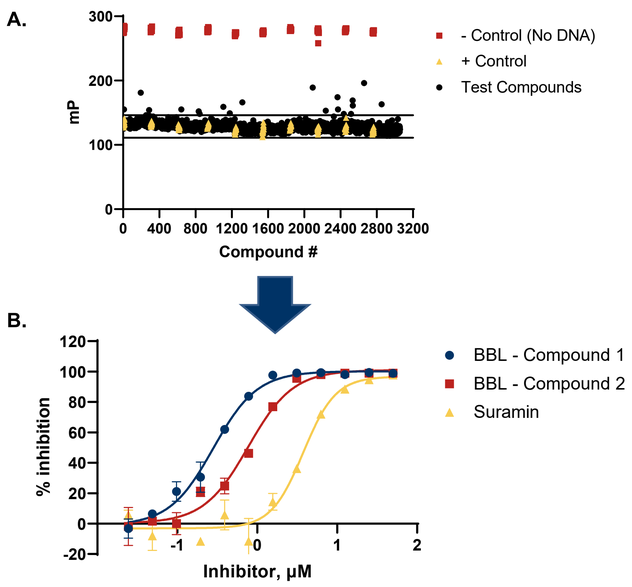


**Figure 6. Pilot Screen of 3056 Small Molecules. A.** 3056 compounds were screened from the Enamine Discovery Diversity set. A total of 15 potential inhibitors were identified with polarization values ≥ 3 standard deviations above the mean. **B.** Selected hits from the pilot screen were tested in dose-response mode (12 concentrations, n = 2). Additionally, a dose-response curve for the control Suramin is shown ($IC_{50}$ = 3.0 µM).

## Triaging and Selectivity Profiling

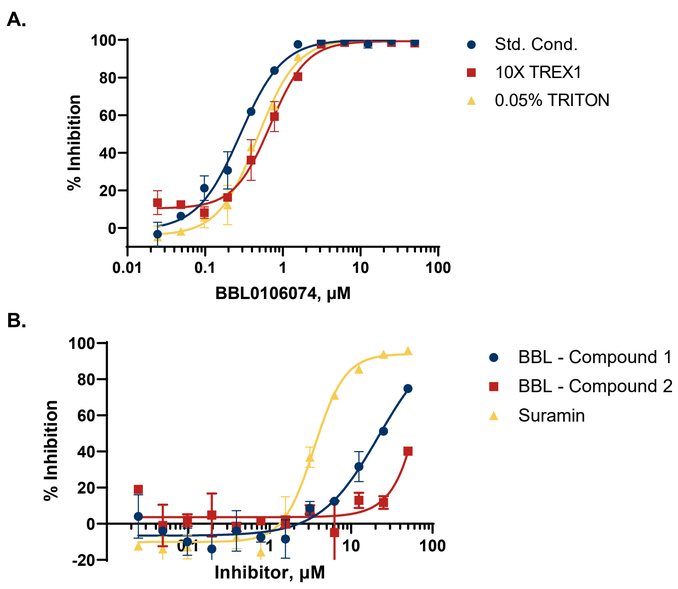


**Figure 7. Triaging Assays and Selectivity Profiling. A.** A representative hit was tested in triaging assays: 0.05% Triton X-100 disperses aggregated inhibitors, 10X enzyme assay titrates out covalent and/or non-stoichiometric inhibitors. **B.** The Transcreener dAMP Exonuclease Assay allows selectivity profiling versus TREX2. Both compounds are more than 50-fold less potent for TREX2, while Suramin, a positive control, inhibits TREX1 and TREX2 at similar $IC_{50}$ (3.0 µM for TREX1 and 3.5 µM for TREX2).

## Conclusions

- **A far-red, FP homogenous immunoassay for dAMP allows direct detection of product formation by TREX1 over a concentration range of 0.1 to 100 µM.**
- **The assay provides excellent data quality (Z'>0.7) and signal (>100 mP polarization shift), providing a robust TREX1 HTS assay.**
- **Ability to use the assay in continuous mode will enable kinetic and MOA studies, e.g., measurement of inhibitor residence times using jump dilution.**
- **Pilot screens validate the assay for discovery of TREX1 inhibitors, triaging hits, and profiling selectivity against similar targets.**
- **The Transcreener dAMP Exonuclease Assay will facilitate rapid discovery of inhibitors for TREX1 and related 3'-5' exonucleases.**


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