A High Throughput Assay for the Ectonucleotidase CD39 Based on the Transcreener® Assay Platform

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Abstract
Ectonucleotidases are plasma membrane-bound enzymes with externally oriented active sites that metabolize nucleotides to nucleosides and are crucial for maintaining immune homeostasis. The ectonucleotidase triphosphate diphosphohydrolase-1, also known as CD39, ENTPD1, or NTPDase1 hydrolyzes ATP and ADP to AMP. AMP can further be processed to adenosine leading to a significant impact on many disease states. Recent studies have shown a key role for adenosine in immunosuppression in the tumor microenvironment, and ectonucleotidases are emerging as promising immuno-oncology targets. As the only HTS method capable of direct detection of AMP, the Transcreener platform is uniquely suited for measuring ectonucleotidase activity with the high sensitivity and low levels of interference required for a successful HTS campaign. The homogenous assays use a far red fluorescence polarization (FP) or TR-FRET readout and they can be broadly applicable to ectonucleotidases. We developed a simple biochemical assay for measuring CD39 activity using the Transcreener AMP/GMP Assay. The assay provides robust detection of AMP production (Z’ > 0.6) with sub nanomolar amounts of CD39. Initial pilot screens have demonstrated robust assay performance (Z’ = 0.6 – 0.7) and IC₅₀ values determined for tool compounds of CD39 were consistent with published values. The availability of HTS-compatible enzyme assay methods will accelerate the discovery of inhibitors for CD39 and related ectonucleotidases that play a role in tumor immunity and other diseases impacted by adenosine signaling.

Outstanding Dynamic Range and Sensitivity Accommodates Diverse AMP Producing Enzymes

Optimization of Transcreener AMP/GMP FP Assay for CD39

Figure 1. Transcreener AMP/GMP FP and TR-FRET Assays. The Transcreener AMP/GMP assays rely on competitive displacement of fluorescent tracers from a highly selective antibody to produce FP or TR-FRET signals. They use a mix and read format, and can be run in kinetic or endpoint mode. Both assays use far red fluros to minimize compound interference.

Figure 2. Competition Curves with Various Nucleotides. Equilibrium binding curves demonstrate the exquisite selectivity of the Transcreener AMP/GMP antibody in both FP and TR-FRET formats, as reflected in selectivity ratios of more than 1000 (B).

Optimization of Transcreener AMP/GMP TR-FRET Assay for CD39

Figure 3. ATP/AMP Standard Curves and Robustness. A. Standard curves mimicking enzyme reactions: ATP at indicated initial concentrations (0.1–100 µM) is reduced as AMP is increased proportionally. B. Z’ values were calculated from the standard curves (n = 16) to demonstrate the robustness and sensitivity of the assay.

Figure 4. CD39 Activity Optimization and Screening in FP. A. Equilibrium binding curves demonstrate the exquisite selectivity of the Transcreener AMP/GMP antibody in both FP and TR-FRET formats, as reflected in selectivity ratios of more than 1000 (B).

Figure 5. CD39 Enzyme Optimization and Screening in TR-FRET. A. CD39 was titrated to determine optimal enzyme concentration. B. Raw data was converted to product formation to show a linear correlation of the enzyme with the product formed. D. Z’ measurements using optimized CD39 reaction conditions indicate a robust assay. E. Assay robustness for CD39 probe compounds yielded IC₅₀ values that correlated with literature values. F. A pilot screen with a 1,200 compound LOPAC library showed tight clustering around the mean and clear separation of potential hits; the Z’ was 0.62.

Conclusions
- The Transcreener AMP FP and TR-FRET Assays are the only HTS assays that enable direct detection of AMP in a homogenous format.
- The exquisite selectivity for AMP vs. ATP or ADP allows robust detection of CD39 under initial velocity conditions.
- The assays provide a biochemical platform for lead discovery and optimization with CD39 and other members of the ectonucleotidase family.