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 ectonucleoside 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 nucleotides, 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^2 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 IC_{50}s 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.

Transcreener AMP FP and TR-FRET Assays: Universal Detection of AMP Producing Enzymes

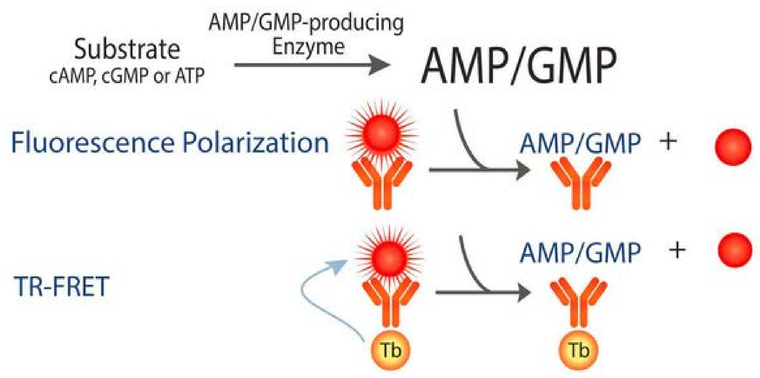
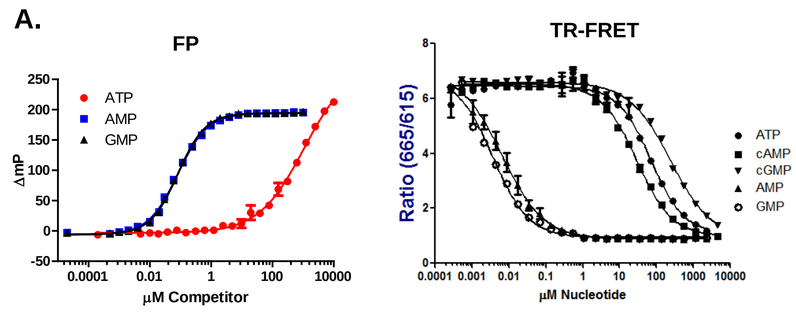


Figure 1. Transcreener AMP/GMP FP and TR-FRET Assays. The Transcreener AMP/GMP assays rely on competitive displacement of fluorescent tracers from a highly selective antibody to produce FP or TR-FRET signals. They use a mix and read format, and can be run in kinetic or endpoint mode. Both assays use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

Highly Selective Antibody Enables Detection of AMP in the Presence of Excess ATP



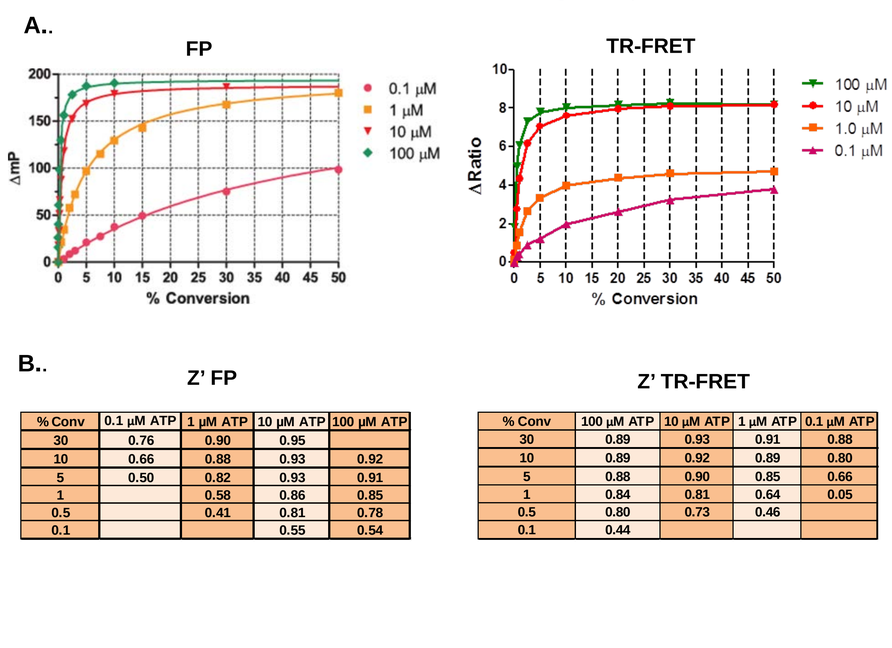
B.

	ATP	cAMP	AMP	cGMP	GMP
IC_{50} (μM)	51.36	21.09	0.006	163.9	0.0032

Selectivity	
AMP vs. ATP	8563
AMP vs. cAMP	3516
GMP vs. cGMP	27326

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

Outstanding Dynamic Range and Sensitivity Accommodates Diverse AMP Producing Enzymes



B..

Z' FP

% Conv	0.1 μM ATP	1 μM ATP	10 μM ATP	100 μM ATP
30	0.76	0.90	0.95	
10	0.66	0.88	0.93	0.92
5	0.50	0.82	0.93	0.91
1		0.58	0.86	0.85
0.5		0.41	0.81	0.78
0.1			0.55	0.54

Z' TR-FRET

% Conv	100 μM ATP	10 μM ATP	1 μM ATP	0.1 μM ATP
30	0.89	0.93	0.91	0.88
10	0.89	0.92	0.89	0.80
5	0.88	0.90	0.85	0.66
1	0.84	0.81	0.64	0.05
0.5	0.80	0.73	0.46	
0.1	0.44			

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.

Optimization of Transcreener AMP/GMP FP Assay for CD39

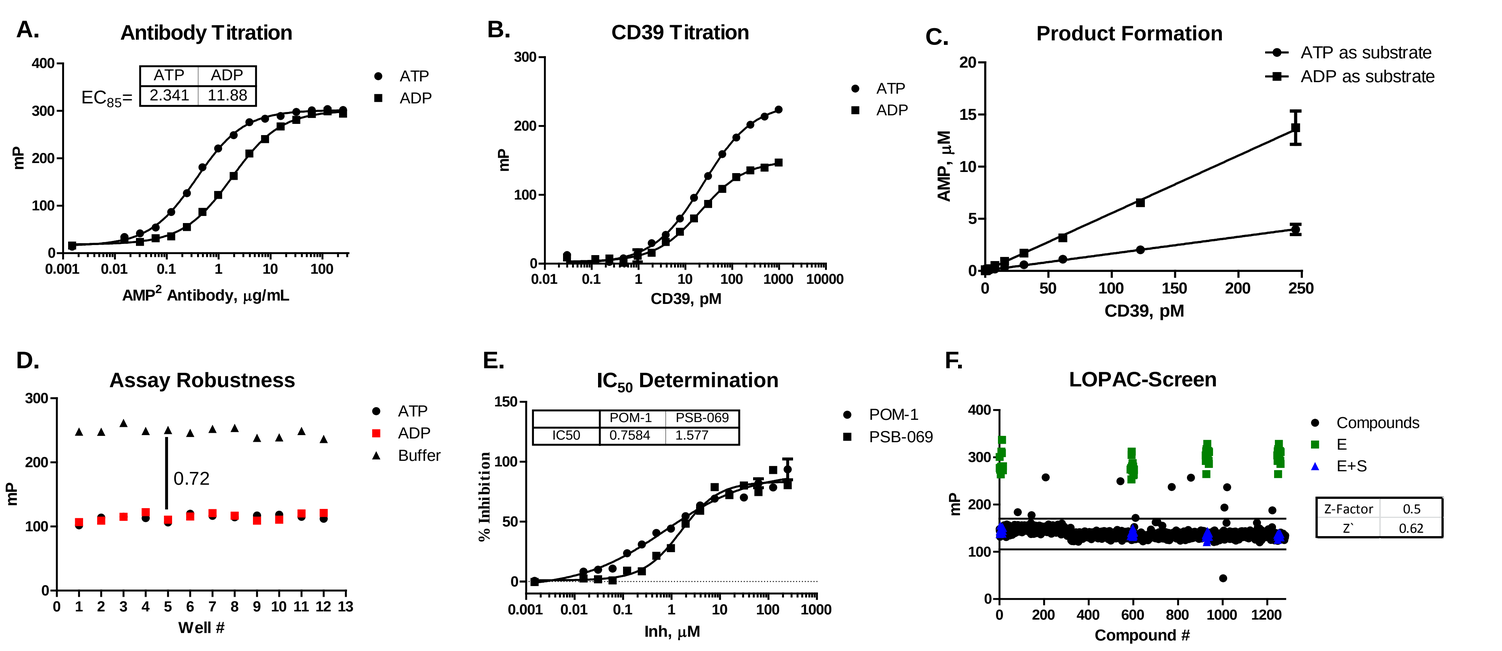


Figure 4. CD39 Activity Optimization and Screening in FP. A. Antibody/tracer binding analysis in CD39 reaction conditions (ATP or ADP as substrate) are used to determine the optimal antibody concentration; the EC_{85} concentration provides a good combination of sensitivity and signal magnitude. **B.** CD39 was titrated to determine optimal enzyme concentration. **C.** 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.** Dose response curves for CD39 probe compounds yielded IC_{50} 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.

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

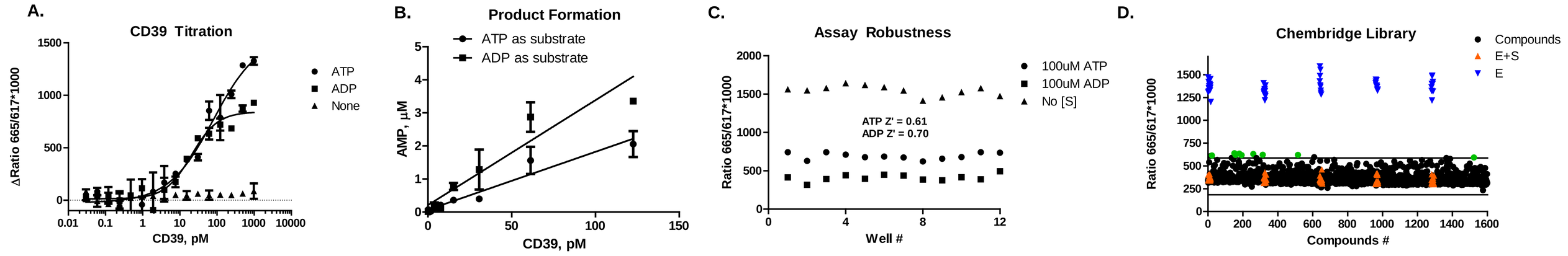


Figure 5. CD39 Enzyme Optimization and Screening in TR-FRET: A. CD39 was titrated to determine optimal enzyme concentration. **B.** Raw data was converted to product formation to show a linear correlation of the enzyme with the product formed (AMP) **C.** Conditions determined in **A** and **B** were used to determine Z' of the assay. **D.** A 1600 compound pilot screen was done using a subset from the Chembridge Library, with potential hits identified.

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.***

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