Development of a High Throughput Transcreener® Assay to Explore the Ectonucleotidase Enzyme Family

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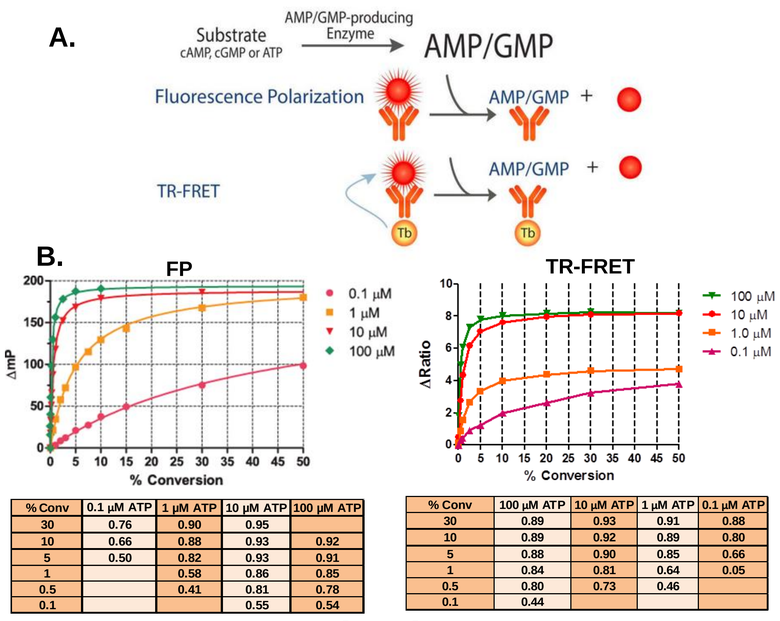
Abstract

There has been an increasing focus in understanding cancer and tumor growth by studying the role of tumor immunosurveillance. Cancer cells employ several elegant ways to avoid the antitumor immune response. One well-studied mechanism is generation of adenosine, an important signaling molecule involved in antitumor T cell response suppression. Adenosine is generated by the hydrolysis of extracellular ATP released by dying tumor cells. The conversion of ATP into adenosine is mediated by the family of ectonucleotidases. These membrane bound enzymes hydrolyze nucleotides to nucleosides and are crucial for maintaining immune homeostasis. The subfamily includes ectonucleoside triphosphate diphosphohydrolase-1, also known as CD39, ENTPD1, or NTPDase1 that hydrolyzes ATP and ADP to AMP and CD73 or 5` Nucleotidase, that hydrolyze AMP to adenosine. These enzymes are emerging as extremely prominent immune-oncology targets for drug discovery. 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 Transcreener GDP^2 Assay was coupled with an Adenosine Kinase enzyme to detect the production of adenosine using CD73. Adenosine Kinase converts the adenosine to GDP which can be detected using the GDP^2 antibody. The availability of HTS-compatible enzyme assay methods will accelerate the discovery of inhibitors for CD39 and CD73 that play a role in tumor immunity and other diseases impacted by adenosine signaling.

Transcreener AMP^2 FP and TR-FRET Assays: Universal Detection of AMP Producing Enzymes

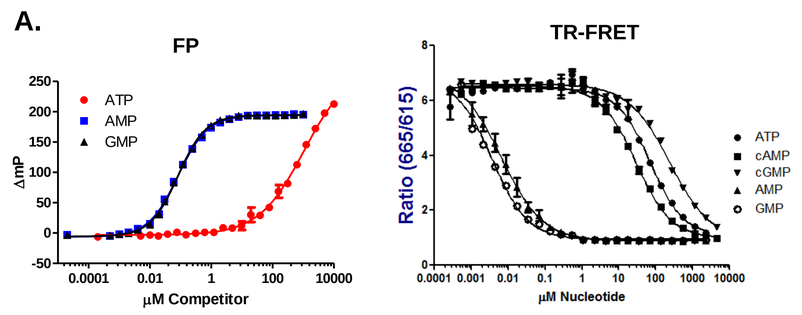


% Conv	0.1 µM ATP	1 µM ATP	10 µM ATP	100 µM ATP
30	0.76	0.90	0.95	
10	0.66	0.88	0.93	0.92
5	0.50	0.82	0.93	0.91
1		0.58	0.86	0.85
0.5		0.41	0.81	0.78
0.1			0.55	0.54

% 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 1. Transcreener AMP^2/GMP^2 FP and TR-FRET Assays. A. The Transcreener AMP^2/GMP^2 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. **B.** Standard curves mimicking enzyme reactions: ATP at indicated initial concentrations (0.1–100 µM) is reduced as AMP is increased proportionally. **C.** Z' values were calculated from the standard curves (n = 16) to demonstrate the robustness and sensitivity of the assay.

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^2/GMP^2 antibody in both FP and TR-FRET formats, as reflected in selectivity ratios of more than 1000 **(B).**

Optimization of Transcreener AMP^2/GMP^2 FP Assay for CD39

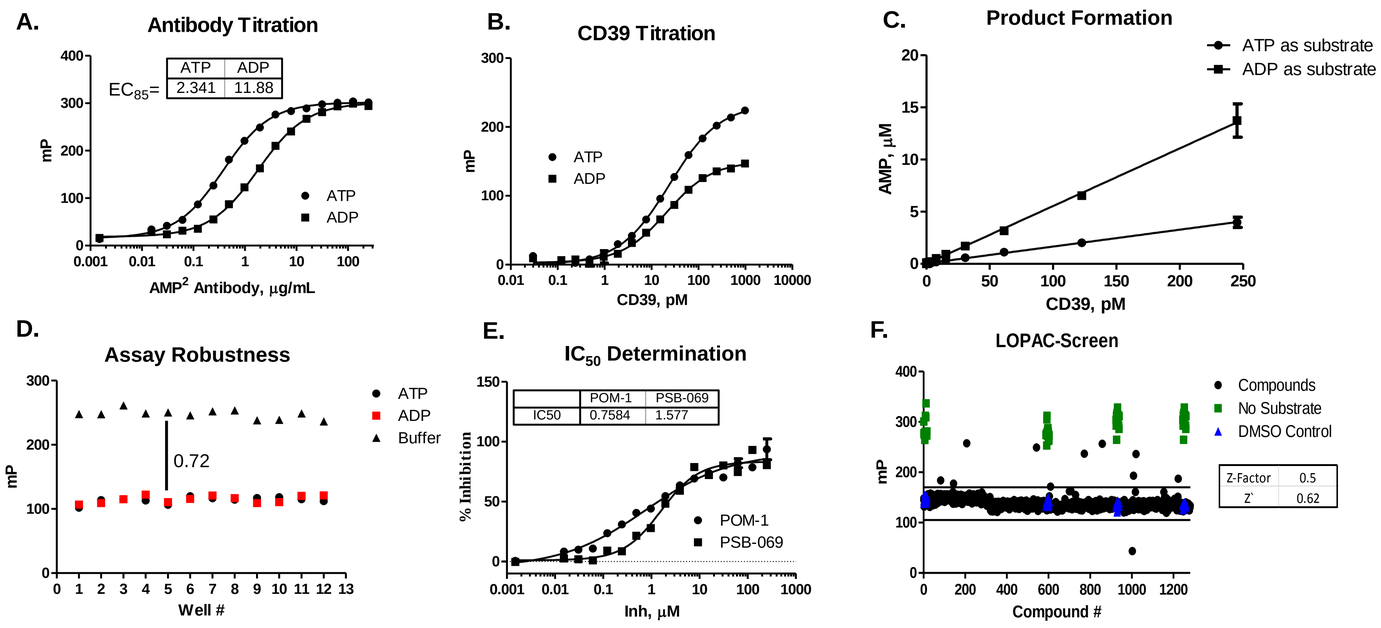


Figure 3. CD39 Activity Optimization and Screening in FP. A. Antibody/tracer binding analysis in CD39 reaction conditions (ATP or ADP as substrate) are used to determine the optimal antibody concentration; the EC_{85} concentration provides a good combination of sensitivity and signal magnitude. **B.** 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. Most of the identified hits were known ATPase inhibitors.

Optimization of Transcreener AMP^2/GMP^2 TR-FRET Assay for CD39

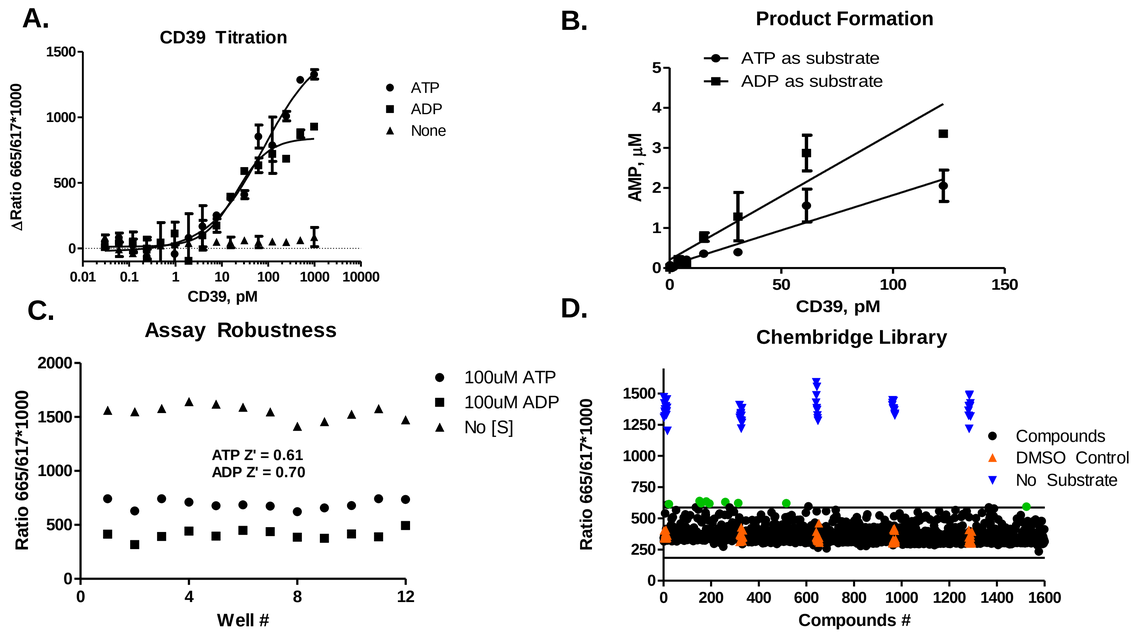


Figure 4. CD39 Enzyme Optimization and Screening in TR-FRET: A. CD39 was titrated to determine optimal enzyme concentration. **B.** Raw data was converted to product formation to show a linear correlation of the enzyme with the product formed (AMP) **C.** Conditions determined in **A** and **B** were 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.

Transcreener GDP FP Assay for Detection of Adenosine Produced by CD73

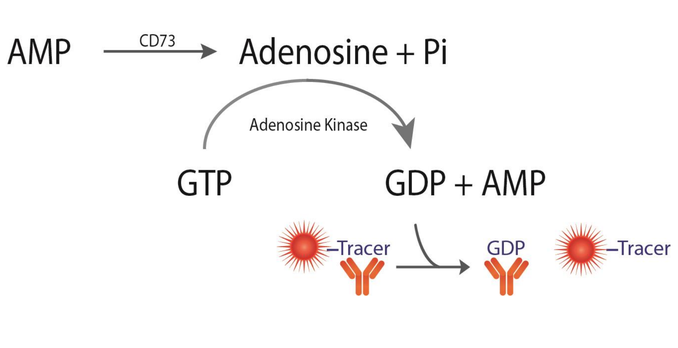


Figure 5. Transcreener GDP Assay for Detection of Adenosine: Transcreener GDP assay coupled with Adenosine kinase could be used to detect adenosine. Adenosine, the product of CD73 reaction gets converted to GDP which can be detected using the Transcreener GDP FP assay.

Assay Development for Measurement of Adenosine

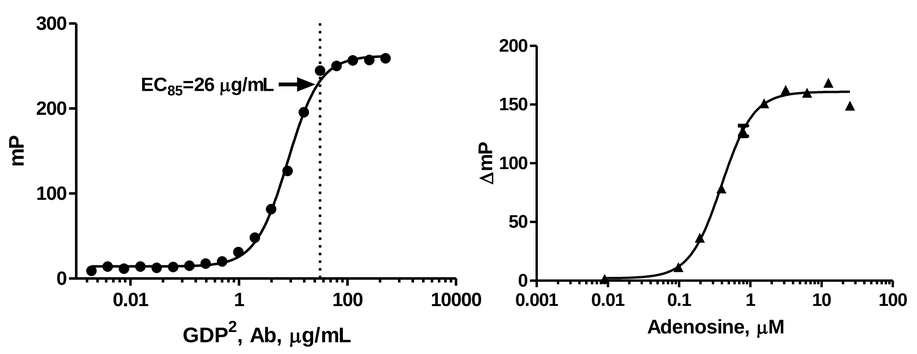


Figure 6. Assay Development for Adenosine Measurement: A. GDP Antibody was titrated in the presence of 25 µM GTP , 10 ng/µL of adenosine Kinase-1. Plate was mixed well and incubated for an hour and read in Tecan Safire. The optimal antibody (EC_{85}) concentration was determined to be around 26 µg/mL for monoclonal GDP antibody. B. 25 µM adenosine/AMP standard curve using Transcreener GDP assay.

Measuring CD73 Activity Using the Transcreener GDP Assay

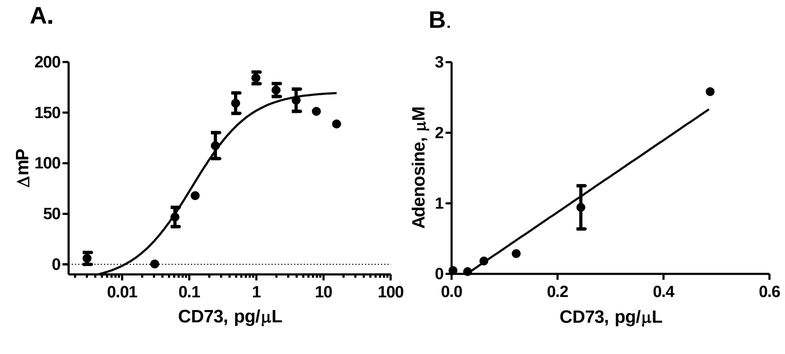


Figure 7. Measuring CD73 Activity: A. CD73 (From R&D systems) was titrated in the system described. **B.** There is a linear correlation between CD73 enzyme and adenosine demonstrating initial velocity conditions.

Conclusions

- ***The Transcreener AMP^2/GMP^2 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.***
- ***Coupling Adenosine Kinase 1 to the Transcreener GDP assay allows robust detection of adenosine, a product of CD73 enzyme.***

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