Sensitive Detection of PRMT4 Using Physiological SAM with the AptaFluor® Methyltransferase Assay

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Overview

Histone methyltransferases (HMTs) produce many different methylated products, and assay methods that detect *S*-adenosylhomocysteine (SAH) therefore offer some advantages over methods that detect specific methylation events. However, direct detection of SAH requires a reagent capable of discriminating between SAH and SAM, which differ by a single methyl group. Moreover, HMTs are slow enzymes, and current non-radioactive SAH detection methods are not sufficiently sensitive to allow detection of many HMTs using physiological concentrations of SAM. To overcome this technical gap, we leveraged a naturally occurring SAH-sensing RNA aptamer, or "riboswitch", that binds SAH with nanomolar affinity and exquisite selectivity. We engineered split-aptamer sensors that transduce binding of SAH into positive fluorescence polarization (FP) and time resolved Förster resonance energy transfer (TR-FRET) signals. The AptaFluor Methyltransferase Assay, allows robust detection of SAH (Z' > 0.7) at concentrations below 10 nM, with signal stability of at least six hours in the presence of typical HMT assay components. Here we compare the AptaFluor Methyltransferase Assay to two other HTS-compatible HMT assay methods based on a) immunodetection with an HTRF® readout and b) a coupled enzyme assay with a luminescent readout. We used PRMT4 to compare the assays for detection of HMT activity, as this enzyme as this enzyme is representative of typical low K_m HMTs (K_m = 140 nM) and it has attracted interest as a therapeutic target. All three assay methods allow robust detection of PRMT4 activity using 200 nM SAM in 2 hour reactions. The greater sensitivity of the AptaFluor assay allowed practical detection using significantly less PRMT4 (EC_{50} = 2.8 nM) which can be advantageous for high volume screening and for dose response measurement with high affinity inhibitors. Interestingly, the difference in sensitivity between AptaFluor and the other two assay methods was greater when saturating SAM (2 µM) was used. By enabling direct SAH detection with the sensitivity required for physiological HMT reaction conditions, the AptaFluor Methyltransferase Assay should provide a valuable tool for epigenetic drug discovery.

AptaFluor SAH Methyltransferase Assay:

Direct, Homogenous SAH Detection with a TR-FRET Readout

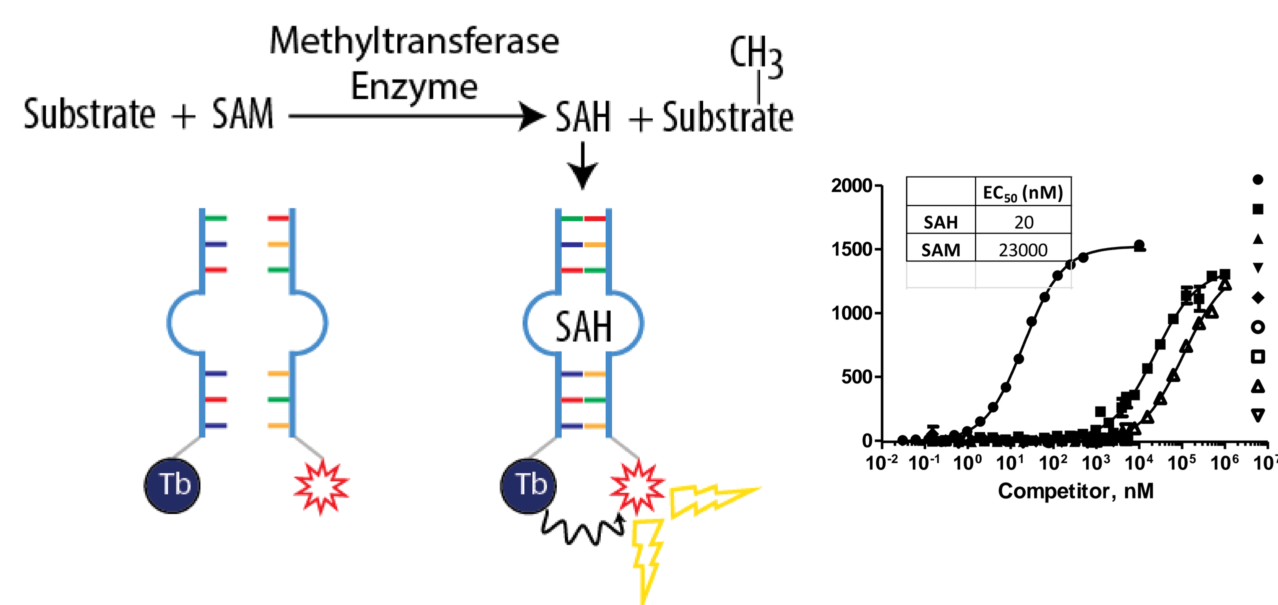
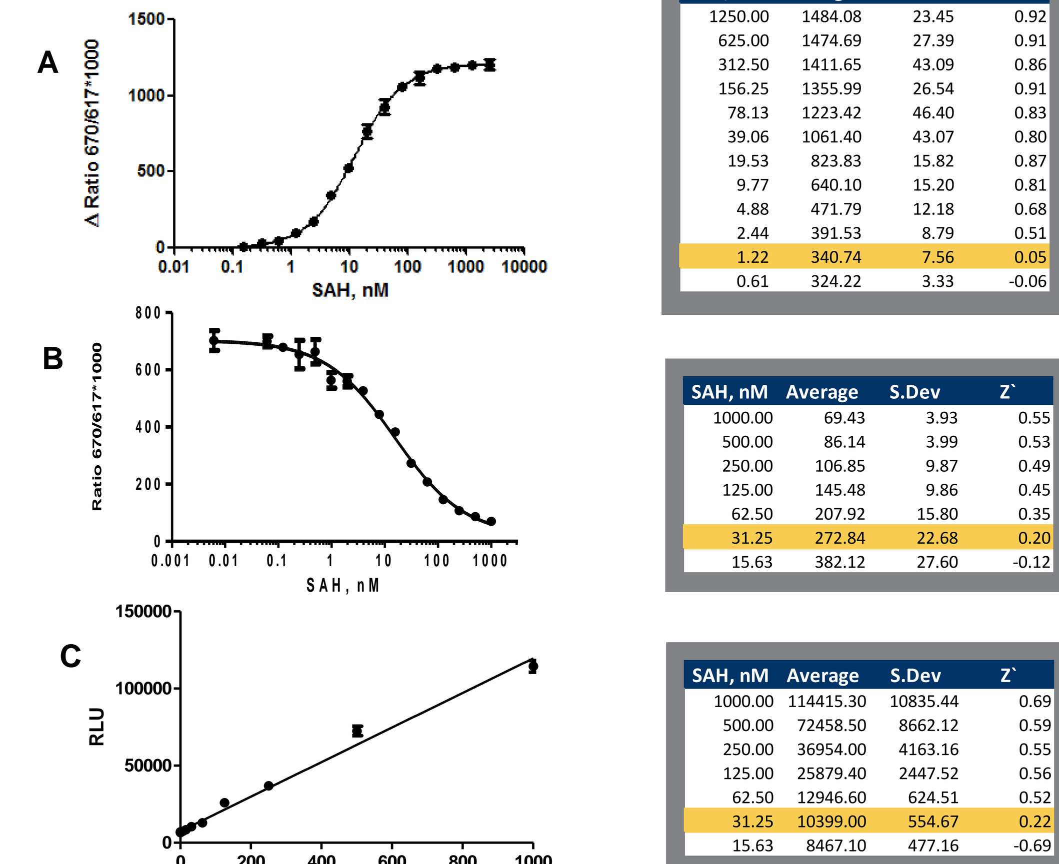


Figure 1. AptaFluor SAH Methyltransferase Assay Principle: A. SAH-driven assembly of a split aptamer allows FRET between a Terbium chelate donor and a DyLight 650 acceptor**. B. Selectivity analysis.** The modified SAH riboswitch discriminates between SAH and related nucleotides, including SAM, which differs by a single methyl group. This makes unambiguous detection of SAH possible in the presence of excess SAM.

Sensitivity



SAH, nM	Average	S.Dev	Z`
1250.00	1484.08	23.45	0.92
625.00	1474.69	27.39	0.91
312.50	1411.65	43.09	0.86
156.25	1355.99	26.54	0.91
78.13	1223.42	46.40	0.83
39.06	1061.40	43.07	0.80
19.53	823.83	15.82	0.87
9.77	640.10	15.20	0.81
4.88	471.79	12.18	0.68
2.44	391.53	8.79	0.51
1.22	340.74	7.56	0.05
0.61	324.22	3.33	-0.06

SAH, nM	Average	S.Dev	Z`
1000.00	69.43	3.93	0.55
500.00	86.14	3.99	0.53
250.00	106.85	9.87	0.49
125.00	145.48	9.86	0.45
62.50	207.92	15.80	0.35
31.25	272.84	22.68	0.20
15.63	382.12	27.60	-0.12

SAH, nM	Average	S.Dev	Z`
1000.00	114415.30	10835.44	0.69
500.00	72458.50	8662.12	0.59
250.00	36954.00	4163.16	0.55
125.00	25879.40	2447.52	0.56
62.50	12946.60	624.51	0.52
31.25	10399.00	554.67	0.22
15.63	8467.10	477.16	-0.69

Figure 2. SAH titrations for A. AptaFluor®, B. EPIgeneous™ (CisBio) assays, and C. MTase-Glo™ (Promega). All assays were run in recommended 384-well plates, n=8 and read using appropriate settings in a BMG Labtech Pherastar plate reader. LLD = lowest concentration that produced a positive Z' value.

Measuring PRMT4 Enzymatic Activity @ 200 nM SAM

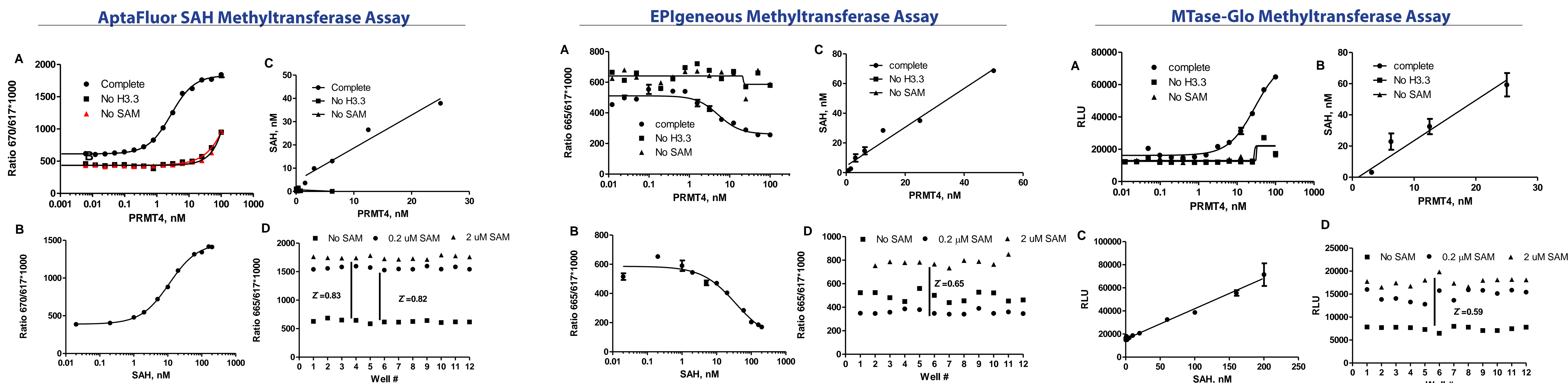


Figure 3. Comparison of AptaFluor with other homogenous methyltransferase assay methods for measuring PRMT4 initial velocity at 200 nM SAM. For each method: A. PRMT4 titration, B. Standard curve mimicking conversion of SAM to SAH, C. Conversion of raw data to SAH formation, D. Scatterplot for Z' determination using PRMT4 at 10 nM that produced around 25 nM SAH. AptaFluor, Mtase-Glo and EPIgeneous assays were run in LV 384 well white plates, final volume of 20 µL after addition of detection reagents and read on a Pherastar PLUS. PRMT4 enzyme reactions contained 10 µM H3.3 peptide in addition to the indicated amount of PRMT4.

Measuring PRMT4 Enzymatic Activity @ 2 µM SAM

Figure 4. Comparison of AptaFluor with other homogenous methyltransferase assay methods for measuring PRMT4 initial velocity at 2 µM SAM. A titration of PRMT4 enzyme and SAH formation is shown for each method: **A., B. AptaFluor assay; C., D. EPIgeneous assay; E., F. MTase-Glo assay.**

Summary

	AptaFluor	EPIgeneous	MTase-Glo
Detection Method	Split Aptamer	Antibody	Enzyme-Coupled
Signal	TR-FRET (Pos.)	TR-FRET (Neg.)	Luminescent (Pos.)
Reagent Additions	1	1	2
LLD for SAH	1.22 nM	31.25 nM	31.25 nM
PRMT4 Detection @ 200 nM SAM			
EC_{50}	2.75 nM	29.02 nM	5.27 nM
Z' @ 10%	0.82	0.65	0.59
PRMT4 Detection @ 2 µM SAM			
EC_{50}	0.91 nM	10.0 nM	33.54 nM

Conclusions

- *The AptaFluor SAH Methyltransferase Assay leverages the exquisite affinity and selectivity of a microbial riboswitch for direct detection of SAH with a TR-FRET signal.*
- *The AptaFluor assay provides more than 20-fold greater sensitivity for SAH detection than the two other homogenous SAH detection methods tested, MTase-Glo and EPI-generous.*
- *All three assays are all capable of detecting PRMT4 activity at 200 nM and 2 µM SAM.*
- *The greater sensitivity of AptaFluor assay allows the use of lower enzyme concentrations at both low (200 nM) and high (2 µM) SAM.*
- *The Z' value for the AptaFluor assay was considerably higher than observed for the other two assay methods using PRMT4 @ 200 nM SAM.*

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