AptaFluor™ SAH: A Homogenous, Universal Methyltransferase Assay Based on a Microbial Riboswitch

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Overview

Epigenetic regulation affects diverse diseases, and high throughput screening for histone methyltransferase (HMT) inhibitors is an area of intense activity. HMTs produce many different methylated products, and assay methods that detect *S*-adenosylhomocysteine (SAH) – the invariant product of *S*-adenosylmethionine (SAM)-dependent methylation reactions - 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 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 sensors that transduce binding of SAH into positive fluorescence polarization (FP) and time resolved Förster resonance energy transfer (TR-FRET) signals. Surprisingly, we found that splitting the riboswitch into two halves, such that SAH binding induces assembly of a trimeric complex, improved the sensitivity, selectivity and stability of the signaling. The split riboswitch configuration, called the AptaFluor SAH 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. We used the AptaFluor SAH Methyltransferase Assay to detect several purified HMTs at concentrations well below the sensitivity limit for current assays using diverse acceptor substrates ranging from peptides to intact nucleosomes. The potency of HMT inhibitors tested in dose response mode with the assay was consistent with literature values. The stability of the nucleic acid based sensor allowed the use of denaturing detergent as a highly effective quench agent. The AptaFluor SAH Methyltransferase Assay leverages the outstanding molecular recognition and facile engineering of aptamers for robust, universal detection of HMTs at physiological SAM concentrations.

AptaFluor™ SAH Methyltransferase Assay:

Direct, Homogenous SAH Detection with a TR-FRET Readout

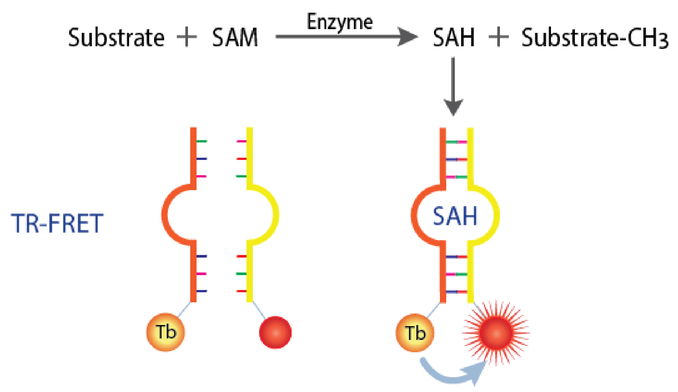
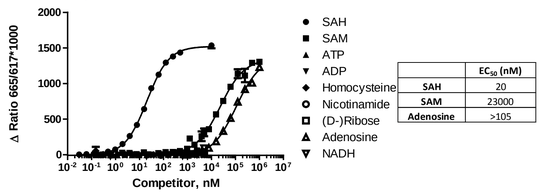


Figure 1. AptaFluor SAH Methyltransferase Assay Principle: SAH-driven assembly of a split aptamer allows FRET between a Terbium chelate donor and a DyLight 650 acceptor.

Leverages the Exquisite Selectivity and Sensitivity of the SAH Riboswitch

A



	EC_{50} (nM)
SAH	20
SAM	23000
Adenosine	>105

B

nM SAH	Z'
2500.00	0.89
1250.00	0.91
625.00	0.92
312.50	0.91
156.25	0.86
78.13	0.91
39.06	0.83
19.53	0.80
9.77	0.87
4.88	0.81
2.44	0.68
1.22	0.51
0.61	0.05

Figure 2. Selectivity for SAH. **A.** 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. **B.** Excellent Z` and lower limit of detection obtained using the AptaFluor assay.

Ultra-Sensitive SAH Detection Allows the Use of Physiological SAM Concentrations

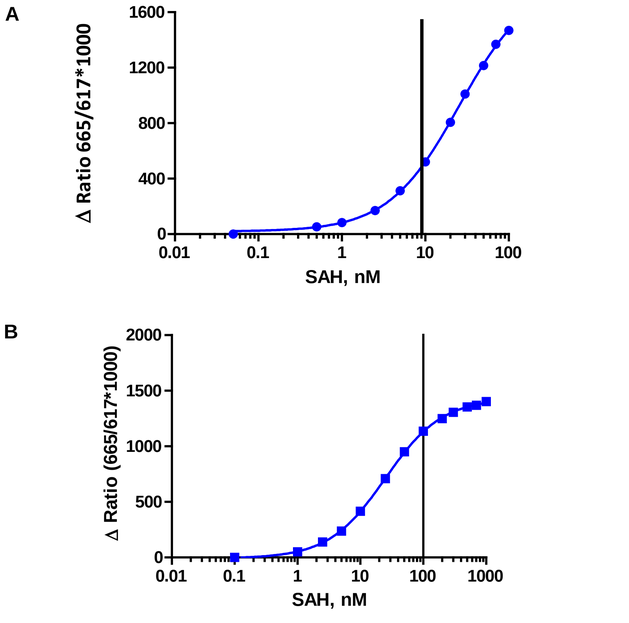


Figure 3. Standard Curves Mimicking Enzymatic Conversion of SAM to SAH. A. Starting at 100 nM, the SAM concentration was reduced and SAH was increased proportionately, n = 12. Z` = 0.83 @ 10% conversion (10 nM SAH/90 nM SAM). **B**. Starting at 1000 nM, the SAM concentration was reduced and SAH was increased proportionately, n = 12. Z` = 0.92 @ 10% conversion (100 nM SAH/900 nM SAM).

Outstanding Reagent and Signal Stability Provide Flexibility for Automated HTS

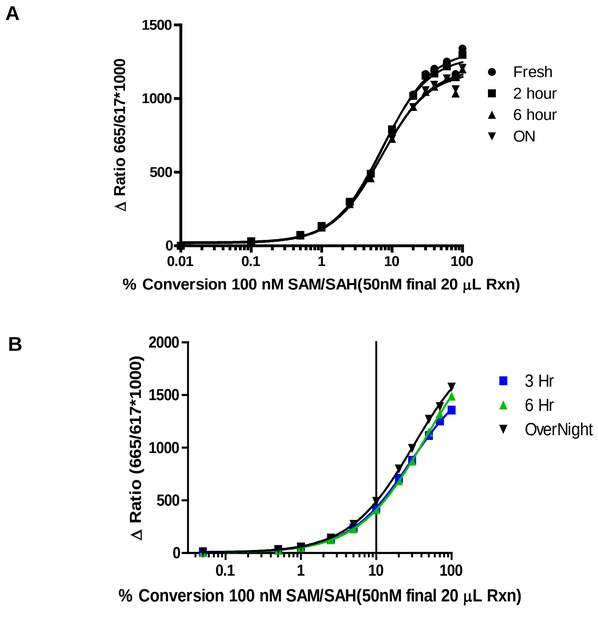
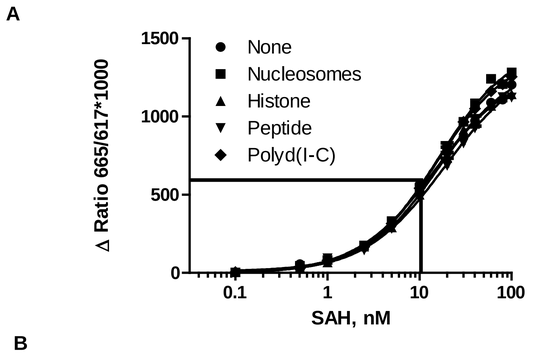


Figure 4. Reagent and Signal Stability. A. Reagent stability was assessed using 100 nM SAM/SAH standard curves after the detection reagents were left at room temp for the indicated times; n=6. **B.** Signal stability (at room temperature) was assessed by reading plates at the indicated times after addition of detection reagents to plates; n = 6.

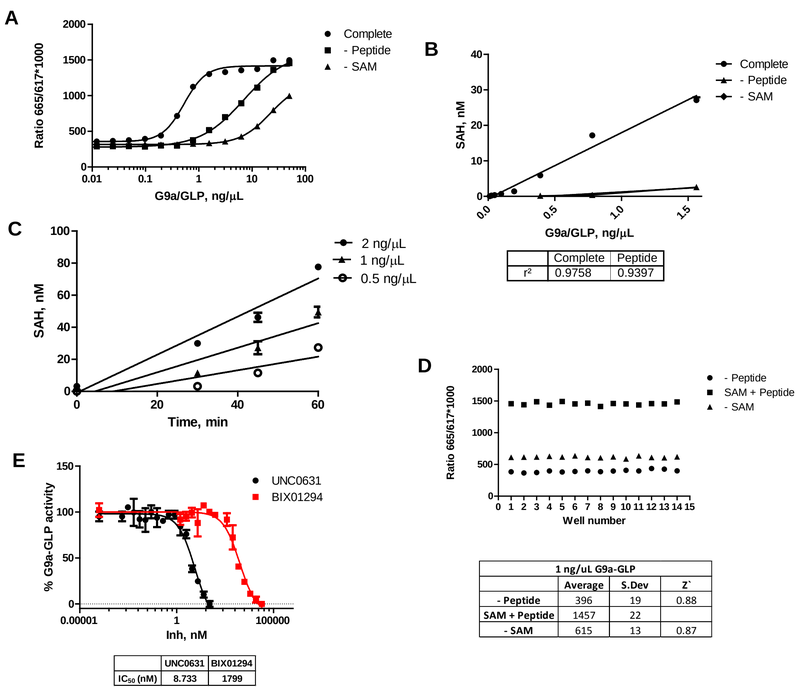
Compatible with Diverse Methyltransferase Acceptor Substrates



	SAM Conc. (nM)	Acceptor substrate	EC_{80} (ng/µL)
PRMT4	200	H3.3 Protein	9.574
NSD2	500	Nucleosomes	7.399
G9a	200	H3(1-21) Peptide	0.8678
EZH2	500	H3.3 Protein	20.44
Dot1L	200	Nucleosomes	3.545

Figure 5. Toleration of MT Acceptor Substrates. A. 100 nM SAH-SAM standard curves were set up in the presence of nucleosomes (10 ng/µL) , histone H3-3 (3ng/µL), Histone H3 (1-21) peptide (10 µM) and Poly d (I-C) (2.5 mU/µL); control wells lacked a MT substrate. **B.** Table showing the validation of AptaFluor SAH Methyltransferase assay with different Methyltransferases and their respective substrates at the SAM concentrations shown above.

HMT Case Study: G9a/GLP Complex



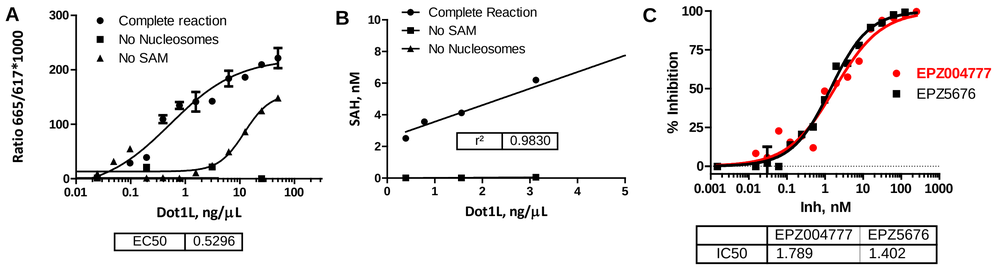
	Complete	Peptide
r^2	0.9758	0.9397

1 ng/uL G9a-GLP			
	Average	S.Dev	Z`
- Peptide	396	19	0.88
SAM + Peptide	1457	22	
- SAM	615	13	0.87

	UNC0631	BIX01294
IC_{50} (nM)	8.733	1799

Figure 6. Case study for G9a/GLP. A. G9a/GLP complex was titrated in the presence of 200 nM SAM and 10 µM H3 (1-21) peptide for 60 minutes at RT to determine an optimal (EC_{80}) concentration of 1 ng/µL. Enzyme reactions were quenched with denaturing detergent. **B.** TR-FRET values were converted to SAH production to assess reaction progress. **C.** Time courses demonstrate linear SAH production with time. **D.** A Z` of 0.8 was obtained (n=16) for an initial velocity G9a/GLP reaction (1 ng/µL, 60 min). **E.** Dose response curves with known inhibitors UNC0631 and BIX01294 showed IC_{50} values of 9 nM and 2 µM, respectively.

HMT Case Study: Dot1L



EC50	0.5296

r^2	0.9830

	EPZ004777	EPZ5676
IC50	1.789	1.402

Figure 7. Case Study for Dot1L. A. Dot1L was titrated in the presence of 200 nM SAM and 10 ng/µL of mono/di nucleosomes to determine an optimal EC_{80} concentration of 6ng/µL. Reactions were incubated at 30C for two hours. **B.** TR-FRET values were converted to SAH production to assess reaction progress. **C.** Dose response curves with two known inhibitors of Dot1L, EPZ004777 and EPZ5676 show an IC_{50} value of 1.7 nM and 1.4 nM respectively.

HMT Case Study: PRMT5

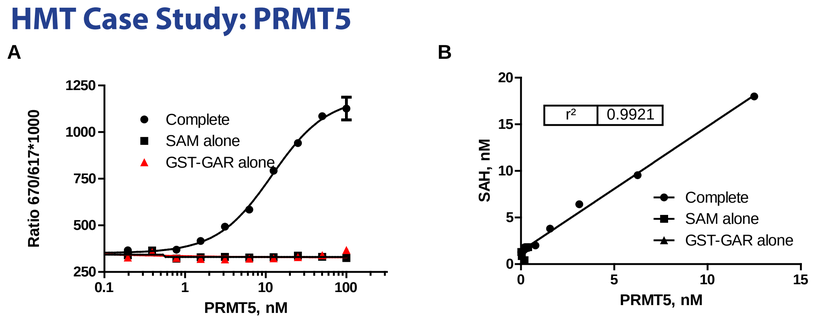


Figure 8. Case Study for PRMT5. A. PRMT5 was titrated in the presence of 200 nM SAM and 25 ng/µL of GST-GAR to determine an optimal EC_{80} concentration of 25 nM. Reactions were incubated at 30°C for two hours. **B.** TR-FRET values were converted to SAH production to assess reaction progress..

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.*
- ***Ultra-Sensitive and Broad Dynamic Range*** *- the assay has an LLD of 1.25 nM SAH and produces outstanding Z' values at 10 nM to 1000 nM SAH.*
- ***Outstanding Reagent and Signal Stability*** *– reagents and signal are both stable for several hours, providing user flexibility.*
- ***Universal*** *– tolerates common HMT acceptor substrates and enables detection of diverse HMTs.*

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