

A Homogenous, Universal Enzymatic Assay for Histone Methyltransferases 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 – are therefore advantageous 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. There is a significant sensitivity challenge as well because HMTs are slow enzymes and use low levels of SAM. Currently there are no SAH assays with sufficient selectivity and sensitivity to allow universal detection HMTs under typical screening conditions, and researchers are resorting to alternative approaches that require intensive assay development and/or are less amenable to HTS. 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 showed that binding of SAH to the riboswitch can be transduced into 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. We used the split aptamer assays to detect SAH produced by purified HMTs at low nanomolar levels – several-fold below the sensitivity limit for current assays – using diverse acceptor substrates ranging from peptides to intact nucleosomes. These riboswitch-based SAH sensors provide the basis for sensitive, robust, universal detection of HMTs in an HTS-compatible format.

AptaFluor™ SAH Methyltransferase Assay:

Direct, Homogenous SAH Detection with a TR-FRET Readout

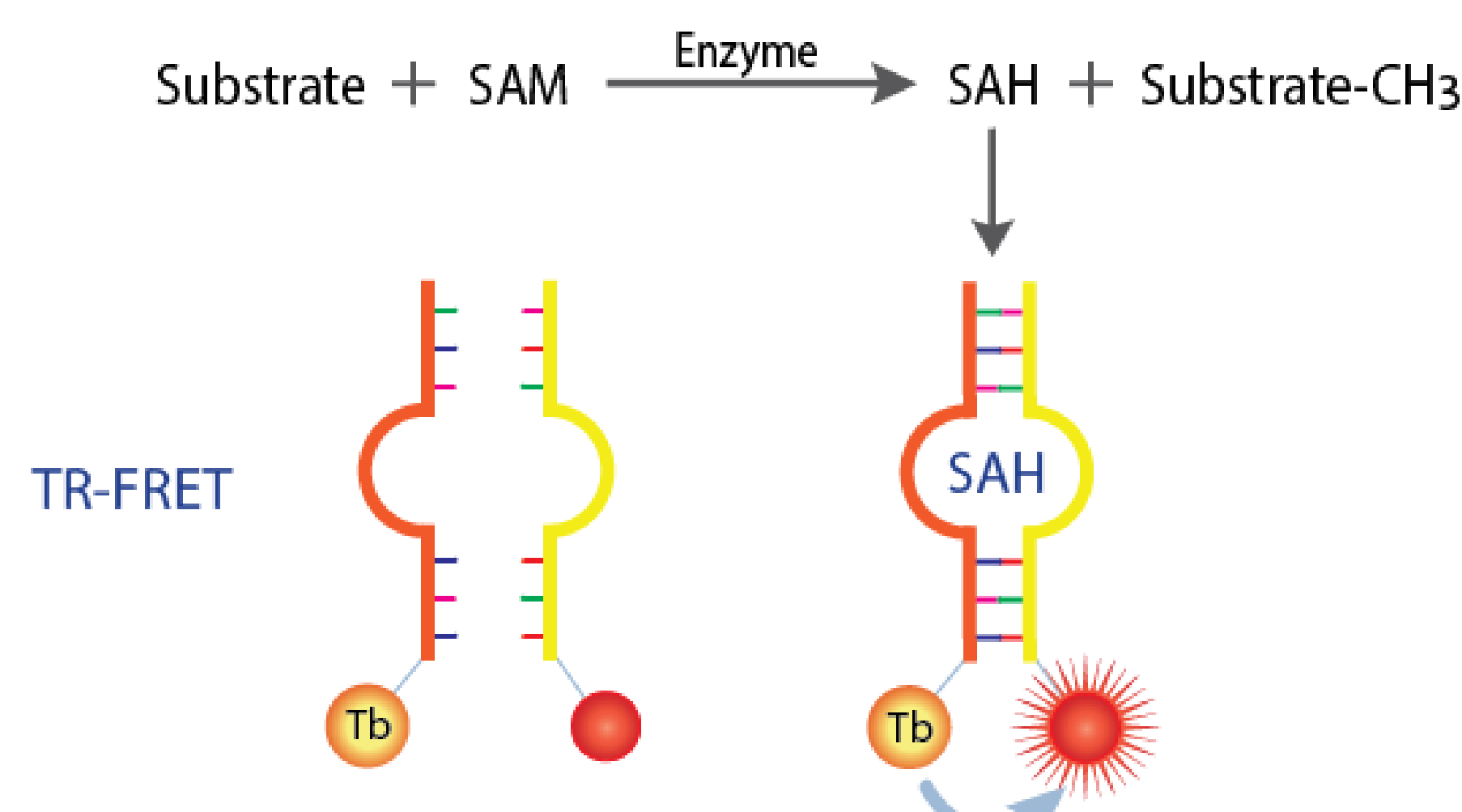


Figure 1. AptaFluor™ SAH Methyltransferase Assay Principle: SAH-driven assembly of a split aptamer allows FRET between a Tb chelate donor and a DyLight 650 acceptor.

Leverages the Exquisite Selectivity of the SAH Riboswitch

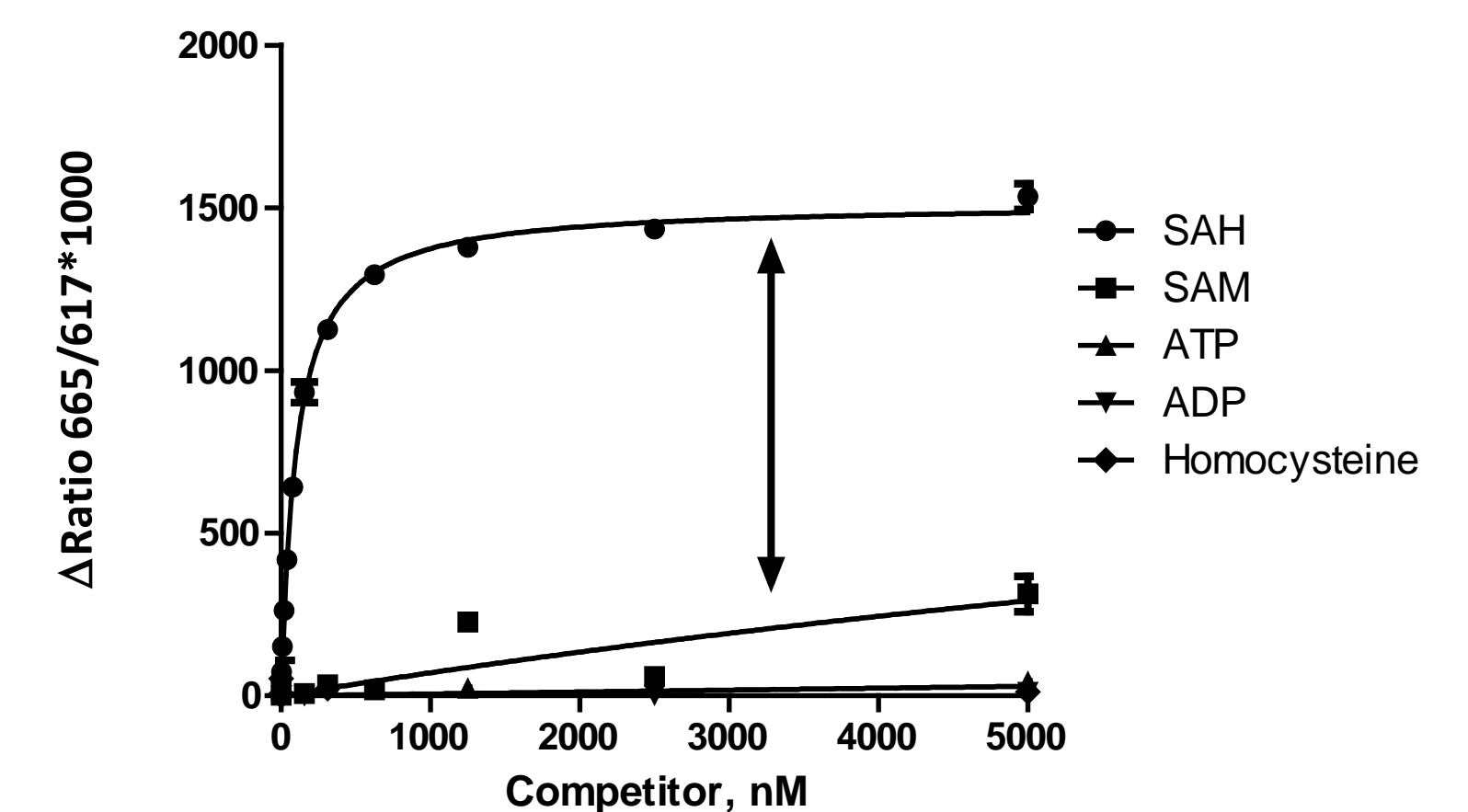


Figure 2. Selectivity for SAH. The modified SAH riboswitch discriminates between SAH and related nucleotides, including SAM, which differs by a single methyl group. This makes unambiguous detection of SAH possible in the presence of excess SAM.

Ultrasensitive SAH Detection Allows the Use of Physiological SAM Concentrations

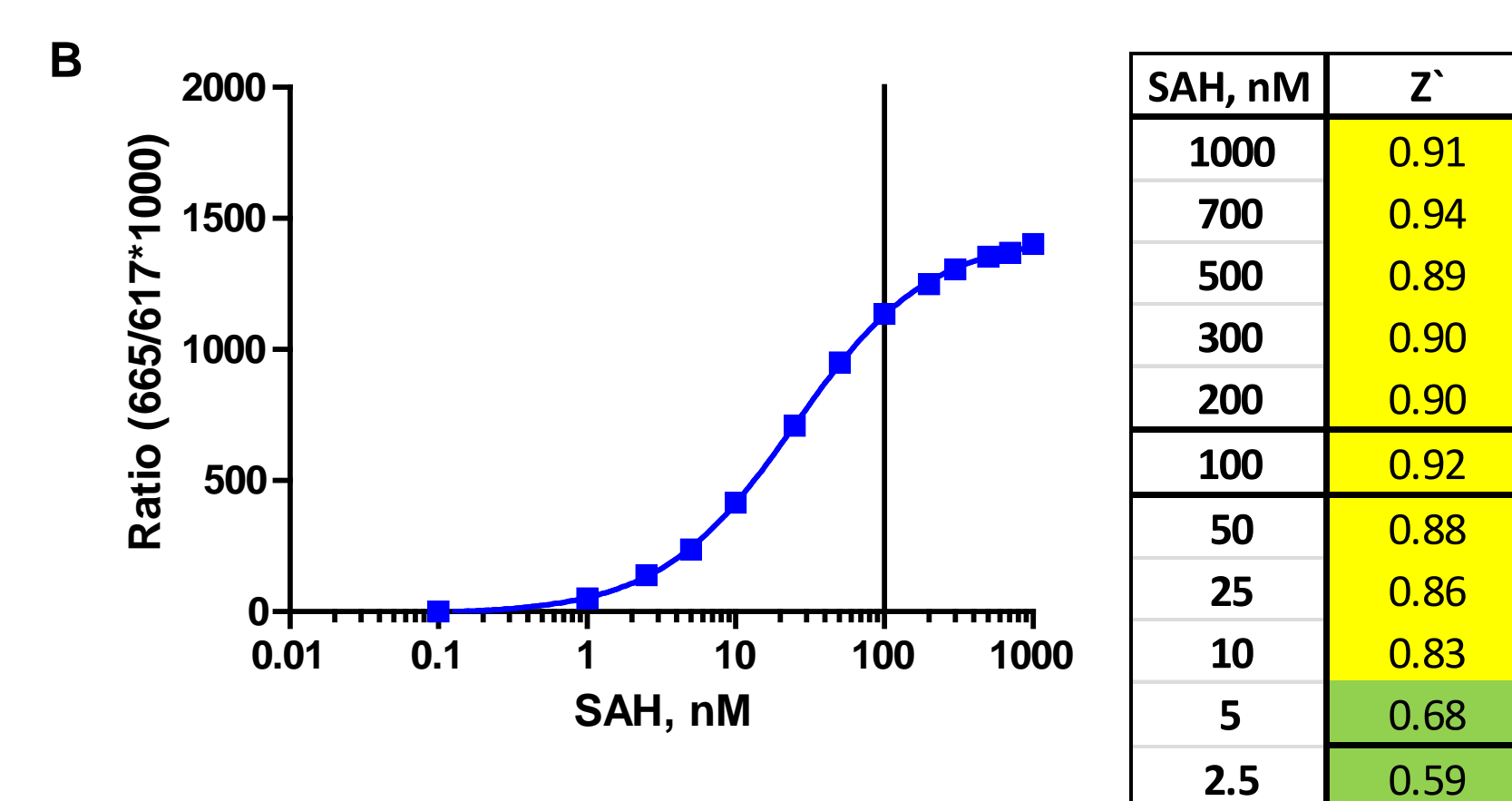
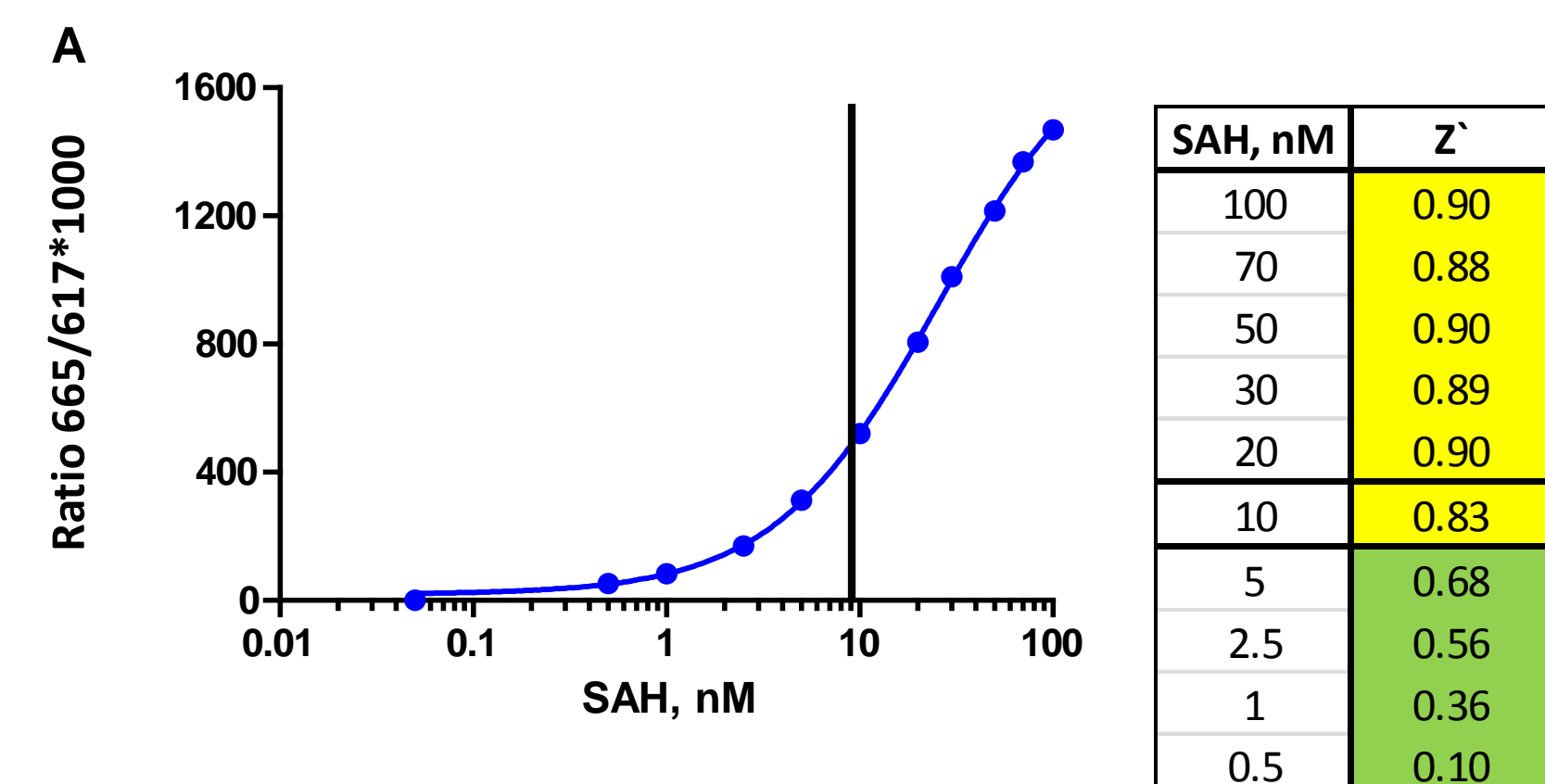


Figure 3. Standard curves mimicking enzymatic conversion of SAM to SAH. A. Starting at 100 nM, the SAM concentration was reduced and SAH was increased proportionately, n = 12. Z' = 0.83 @ 10% conversion (10 nM SAH/90 nM SAM). B. Starting at 1000 nM, the SAM concentration was reduced and SAH was increased proportionately, n = 12. Z' = 0.92 @ 10% conversion (100 nM SAH/900 nM SAM).

Outstanding Reagent and Signal Stability Provide Flexibility for Automated HTS

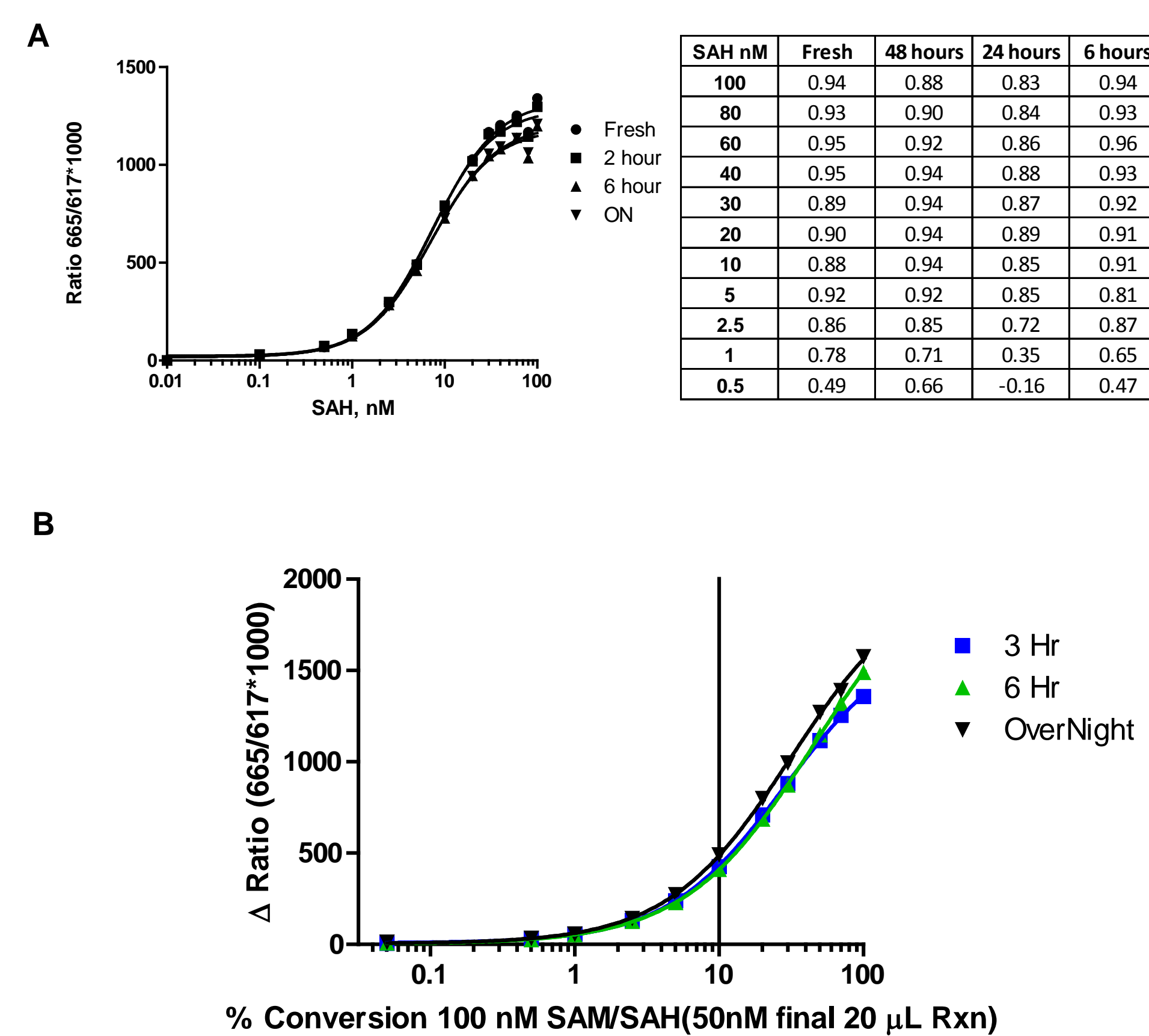


Figure 4. Reagent and Signal Stability. A. Reagent stability was assessed using 100 nM SAM/SAH standard curves after the detection reagents were left at room temp for the indicated times; n=6. B. Signal stability (at room temperature) was assessed by reading plates at the indicated times after addition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

Compatible with Diverse Methyltransferase Acceptor Substrates

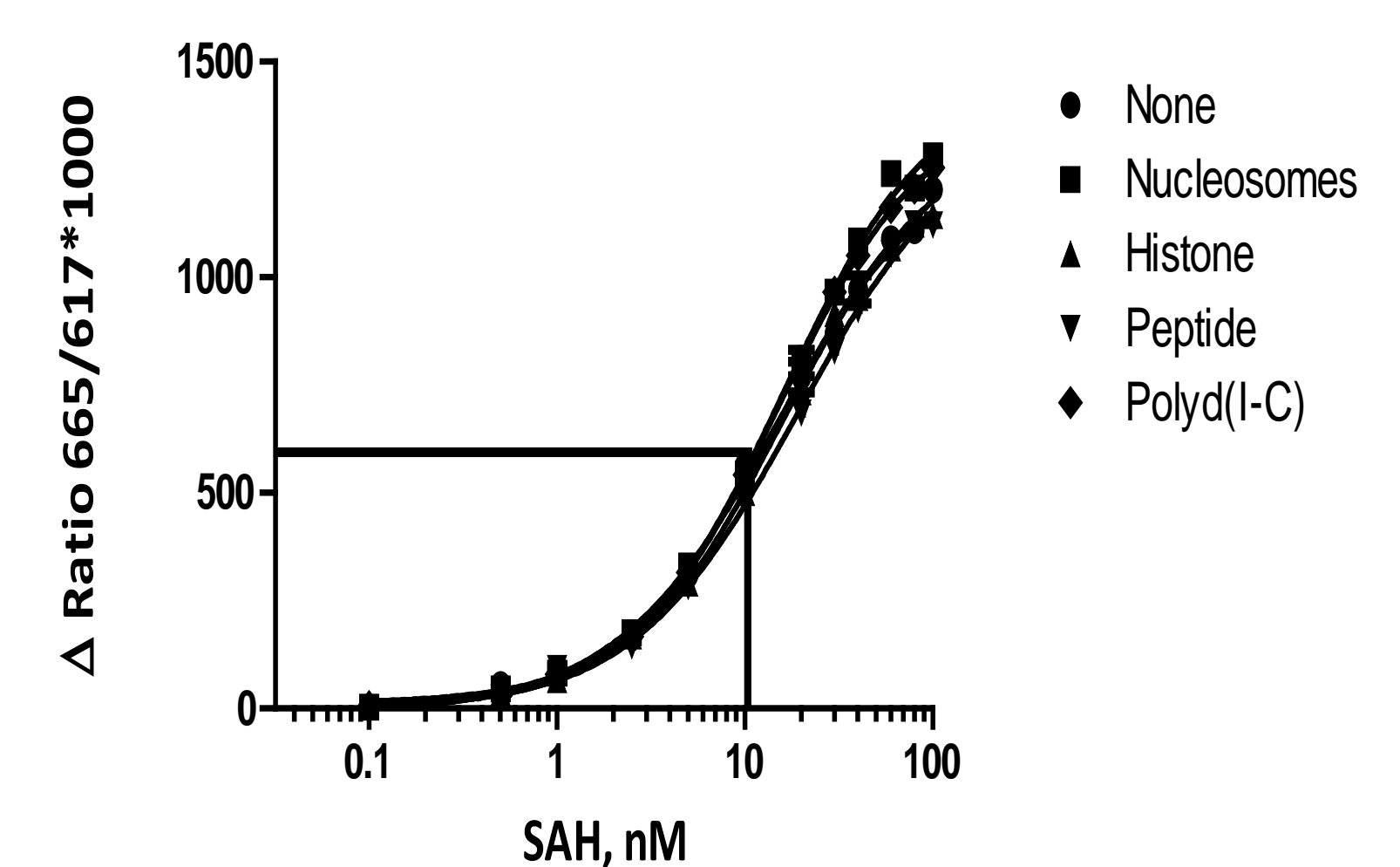


Figure 5. Tolerant of MT acceptor substrates. 100 nM SAH-SAM standard curves were set up in the presence of nucleosomes (10 ng/μL), histone H3-3 (3ng/μL), Histone H3 (1-21) peptide (10 μM) and Poly d (I-C) (2.5 μM/μL); control wells lacked a MT substrate.

HMT Case Study: G9a/GLP Complex

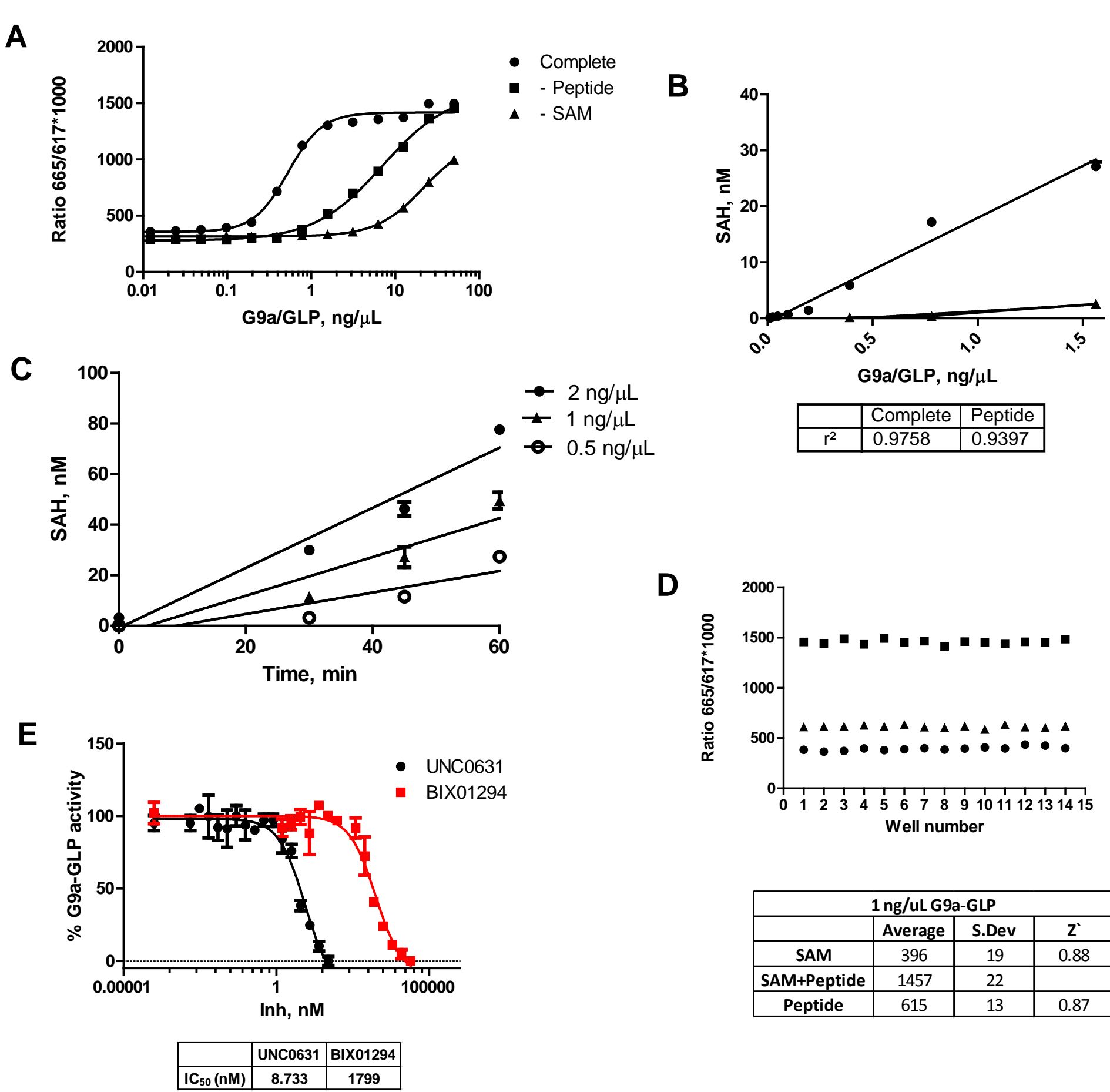


Figure 6. Case study for G9a/GLP. A. G9a/GLP complex was titrated in the presence of 200 nM SAM and 10 μM H3 (1-21) peptide for 60 minutes at RT to determine an optimal (EC₅₀) concentration of 1 ng/μL. Enzyme reactions were quenched with denaturing detergent. B. TR-FRET values were converted to SAH production 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 IC₅₀ values of 9 nM and 2 μM, respectively.

HMT Case Study: Dot1L

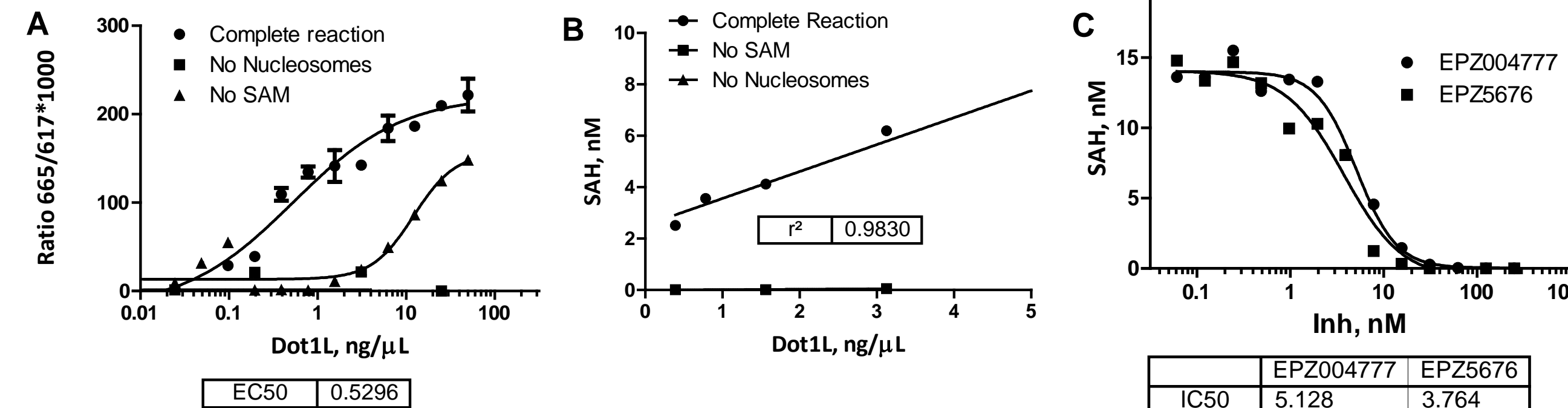


Figure 7. Case study for Dot1L. A. Dot1L was titrated in the presence of 200 nM SAM and 10 ng/μL of mono/di nucleosomes to determine an optimal EC₅₀ concentration of 6ng/μL. Reactions were incubated at 30C for two hours. B. TR-FRET values were converted to SAH production to assess reaction progress. C. Dose response curves with two known inhibitors of Dot1L, EPZ004777 and EPZ5676 show an IC₅₀ value of 5.1 nM and 3.7 nM respectively.

HMT Case Study: EZH2

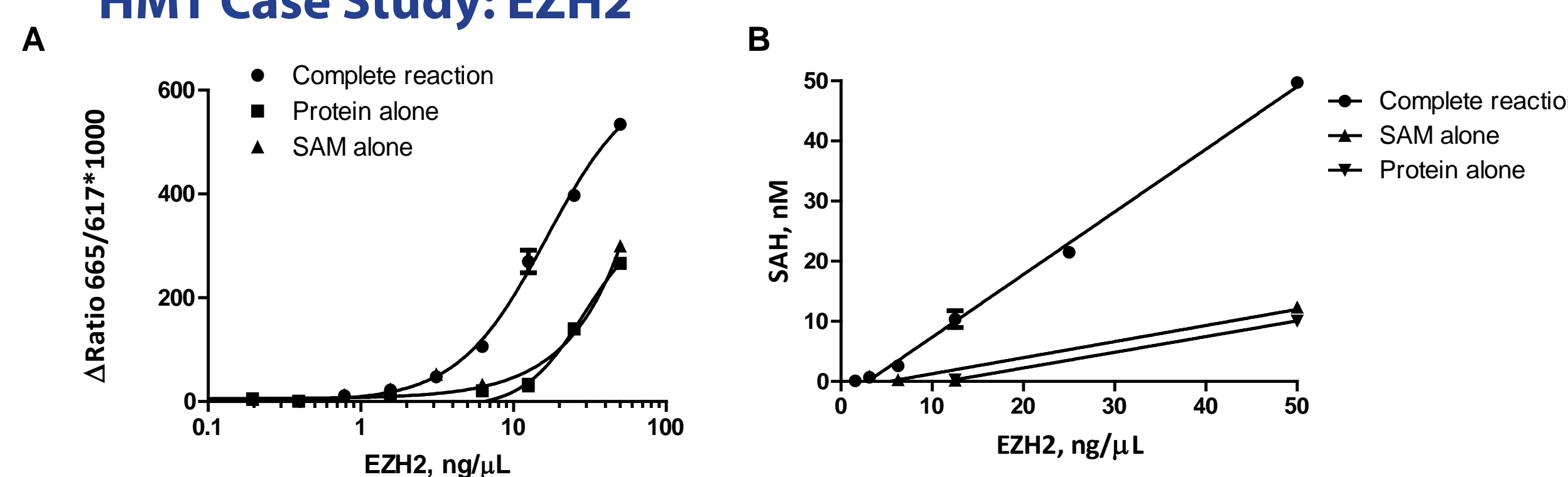


Figure 8. Case study for EZH2. A. EZH2 was titrated in the presence of 500 nM SAM and 3 ng/μL of histones H3-3 to determine an optimal EC₅₀ concentration of 35 ng/μL. Reactions were incubated at 30°C for two hours. B. TR-FRET values were converted to SAH production to assess reaction progress.

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.**
- Ultrasensitive 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 flexibility for automated HTS environments.**
- Universal – tolerates common HMT acceptor substrates and enables detection of diverse HMTs, including Dot1L and EZH2.**