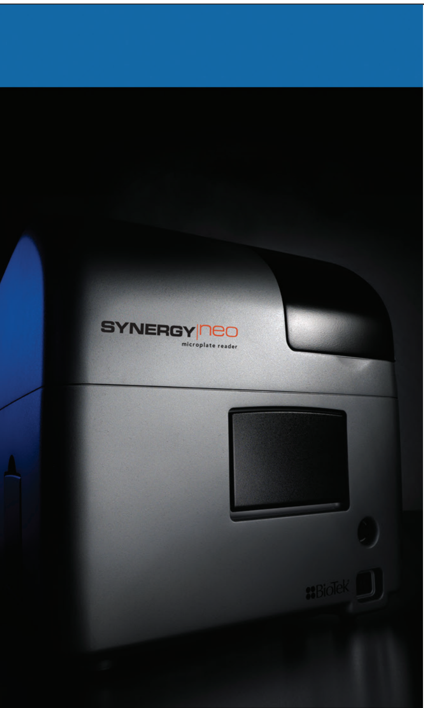
Application Note

Epigenetic Methyltransferase Activity

Automation of a HTRF® Universal Methyltransferase Assay

Peter J. Brescia and Peter Banks, Applications Department, BioTek Instruments, Inc., Winooski, VT
Thomas Roux and François Degorce, Cisbio Bioassays, Codolet, France



Key Words:

Epigenetics

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G9a

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BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Phone: 888-451-5171
Outside the USA: 802-655-4740
Email: **customercare@biotek.com**
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Introduction

The study of epigenetic mechanisms affecting a wide range of cellular process has seen dramatic growth over the past several years. Methyltransferases (MTs) have proven to target a wide variety of substrates including histones, oligonucleosomes, DNA, RNA, small molecules and receptors. Many of these are potential therapeutic targets and are heavily investigated in both fundamental research as well as drug discovery efforts. Given the ubiquitous and diverse nature of both enzyme and substrate an easy to use, universal assay method suitable for high-throughput screening (HTS) is highly desirable. MTs catalyze the transfer of a donor methyl group to amino groups on the target molecule such as nitrogenous DNA bases or peptide N-termini and amino acid side chains. The cofactor S-adenosyl-L-methionine (SAM) is the primary methyl donor providing a reactive methyl group bound to sulfur. The conversion of SAM to S-adenosyl-L-homocysteine (SAH) can then be used as a measurement of MT activity (Figure 1).

Here we demonstrate automation of the assay in a 384-well format suitable for HTS and characterization of inhibitors of methyltransferase/substrate or methyltransferase/cofactor interactions.

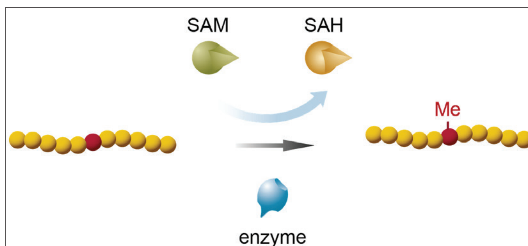

Figure 1. **Methyltransferase enzymatic reaction.** Methyltransferase activity catalyzes methyl transfer from SAM cofactor to target molecules playing roles in regulation of a wide variety of cellular functions. Substrate and enzyme are incubated in the presence of compound and SAM is added to initiate the reaction leading to substrate methylation.

A high-throughput screening (HTS) multi-mode reader was used in conjunction with a homogeneous time-resolved fluorescent energy transfer (HTRF) assay amenable to rapid characterization of MT activity as well as small molecule inhibition in a high throughput format (Figure 2). The methyltransferase activity is assessed in a competitive assay consisting of an anti-SAH antibody labeled with a terbium cryptate and a SAH-d2 tracer. The SAH released during the enzymatic reaction competes with the SAH-d2 tracer leading to a decrease in HTRF signal. Demonstration of the assay includes analysis of assay performance under a variety of buffer conditions suitable for primary screening against both DOT1L and G9a methyltransferases. A combination of liquid handling instruments was used to generate standard curves, automate titration of enzyme, as well as substrate and reagent additions to the assay plate.

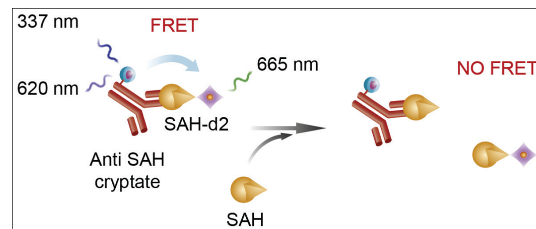

Figure 2. **Assay schematic for the EPIgeneous Methyltransferase Assay Kit.** Upon excitation, the terbium cryptate can transfer its energy to an SAH-d2 conjugate molecule, provided the SAH-d2 is in close proximity to the terbium fluorophore. The presence of released SAH product displaces SAH-d2 resulting in a loss of HTRF signal.

Materials and Methods

Materials

EPIgeneous™ Methyltransferase Assay Kit (Item No. 62AHPEB), was a gift of Cisbio US, Bedford, MA, USA). DOT1L (Cat. No. HMT-35-130) methyltransferase, G9a (Cat. No. HMT-11-102) methyltransferase and Nucleosomes (HeLa Oligo) (Cat. No. HMT-11-101) substrate were purchased from Reaction Biology (Malvern, PA, USA). Histone H3 (1-21), Biotinylated (Cat. No. 61702) substrate was purchased from AnaSpec, Inc. (Fremont, CA, USA). SGC 0946 (Cat. No. 4541) and UNC 0646 (Cat. No. 4342) methyltransferases inhibitors were purchased from Tocris Biosciences/R&D

Systems (Minneapolis, MN, USA). Assay plates used for all experiments were 384-well, white, low-volume Greiner plates (Cat. No. M3561040, Sigma-Aldrich Corp., St. Louis, MO, USA).

Methods

Data was analyzed using Gen5 Data Analysis software (BioTek Instruments, Inc. (Winooski, VT), Microsoft® Excel® (Microsoft, Redmond, WA), and GraphPad Prism® (GraphPad Software, LaJolla, CA, USA).

Reagent Preparation

Standard curves were prepared in various typical MT enzyme buffers[1]. Enzymes were prepared in enzyme reaction buffers specific to each MT at 2.5x the final concentration (f.c.) as previously described[1]. Compounds, substrate and SAM cofactor were prepared at 5x f.c. in the appropriate enzymatic buffer. Detection Buffer One was ready to use requiring thawing at RT, due to the presence of DMSO, prior to use. SAH-d2 was prepared in Detection Buffer Two. The concentration of SAH-d2 was prepared in accordance with the manufacturers' recommendations dependent on the SAM concentration used for each MT reaction. Anti-SAH-Tb cryptate Ab. was prepared by diluting 50-fold in Detection Buffer Two. Inhibitors were prepared at 5x f.c. in the appropriate enzyme reaction buffer as were SAM/SAH standards (Table 1). The assay was performed as depicted in the workflow diagram below (Figure 3).

Buffer	Composition
Buffer 5 G9a	50 mM Tris-HCl pH 8.8, 10 mM NaCl, 1 mM DTT, 0.01% Tween20
Buffer 6 DOT1L	50 mM Tris-HCl pH 8.5, 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 0.01% Tween20

Table 1. Methyltransferase Enzymatic Buffers.

Liquid Handling

A Precision™ Microplate Pipetting System was used to serial titrate enzyme and inhibitors across a 96-well plate, as well as mix and transfer various concentrations of SAM/SAH for development of standard curves. Prepared enzyme and inhibitor titrations and SAM/SAH standard curves were transferred from 96-well reagent plates to 384-well assay plates manually. A MultiFlo™ Microplate Dispenser was used to dispense EPIgeneous Methyltransferase reagents to the 384-well assay plates.

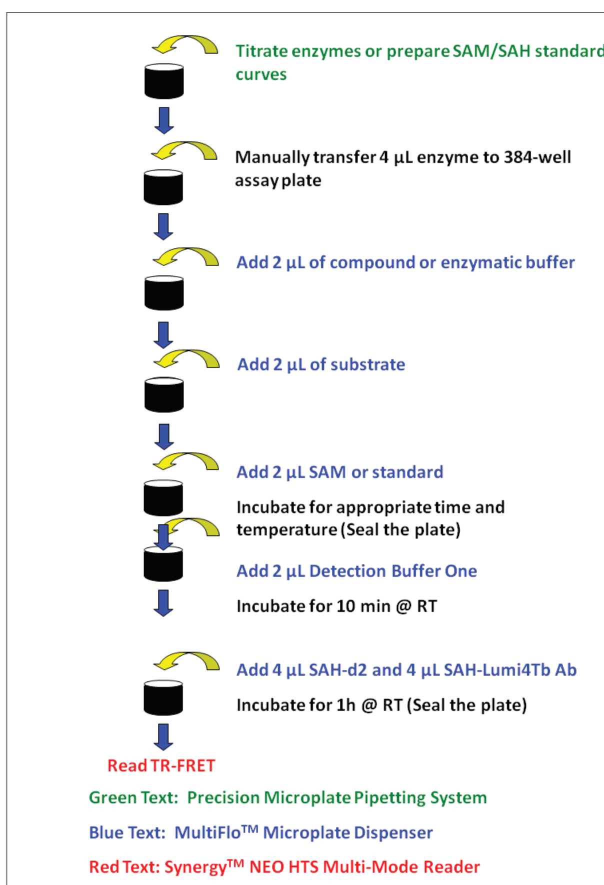

Figure 3. Automated Assay Procedure. Titration and preparation of standard curves was accomplished by Precision. Inhibitor, substrates, and assay specific reagents dispensed using MultiFlo. Detection of 620 and 665 nm signals accomplished using the Synergy Neo.

Microplate reader settings

Following incubation the fluorescent signals were read on the Synergy™ Neo using 330/80 nm excitation and 620/10nm and 665/8nm emission filters, along with a 650 nm cutoff dichroic mirror with matched dual PMT technology. The above parameters are summarized below in table 2.

Data reduction

HTRF ratio values are reported as the ratio of acceptor fluorophore intensity/donor fluorophore intensity (665/620 nm)*10,000. The HTRF ratio was plotted versus log percent (%) SAM conversion.

Synergy™ Neo Read Parameters	
Filter Sets	
Mode	Dual PMT
Excitation	330 nm
Dual Emission	620/665 nm
Gain (Side/Top PMTs)	Auto
Read Speed	
Read Speed	Normal
Delay after Plate Movement	0
Measurement per Data Point	25
TRF Parameters	
Delay	150 μsec
Data Collection Time	500 μsec
Read Height	
Auto-Adjust Determined	8.5 mm
Light Source	
Source	Xenon Flash
Lamp Energy	Low

Table 2. Synergy Neo HTRF Read Parameters.

Results

Standard curves

SAM/SAH standard curves were prepared in enzymatic buffers typical of those used for methyltransferases reaction chemistry to demonstrate assay compatibility and robustness of automated methods. The standard curves mimic SAM-to-SAH conversion. A 9-point standard curve, with a zero point, was prepared in distinct enzyme buffers in a 96-well assay plate by preparing and mixing 50 µL of a 1:3 serial dilution of either 0.5 or 1 µM SAH calibrator with 100 µL 0.5 or 1 µM SAM, respectively. Four replicates of each dilution were transferred to a 384-well assay plate. The assay plate was prepared and incubated as described above. Additional reagents were added as depicted above and incubated for the appropriate time. Following the one hour incubation period the plate was read on the reader and the HTRF ratio was plotted versus log SAM concentration (Figure 4).

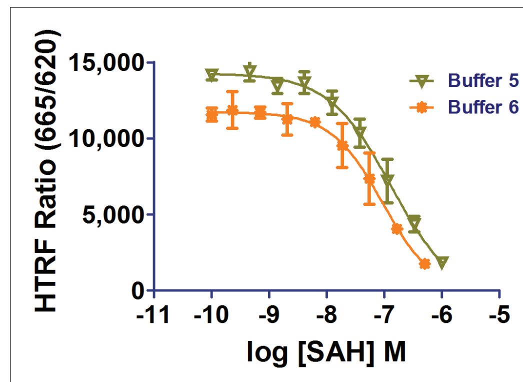

Figure 4. SAM/SAH standard curves. Standard curves were generated in enzymatic buffers to mimic methyltransferase activity resulting in SAH production. Various concentration of SAH calibrator was mixed with SAM while maintaining a constant total concentration ([SAM] + [SAH]) throughout.

Enzyme Titration

An 8-point 1:10 serial dilution series, with a zero point, was prepared in a 96-well plate for each enzyme tested. Each dilution point was transferred to the assay plate in quadruplicate. Reactions were allowed to proceed as previously described. Reactions were stopped by the addition of Detection Buffer One, incubated for 10 min., followed by the addition of the remaining detection reagents. The assay measurements were taken following the final incubation period and plotted as log enzyme concentration vs. HTRF ratio (Figure 5).

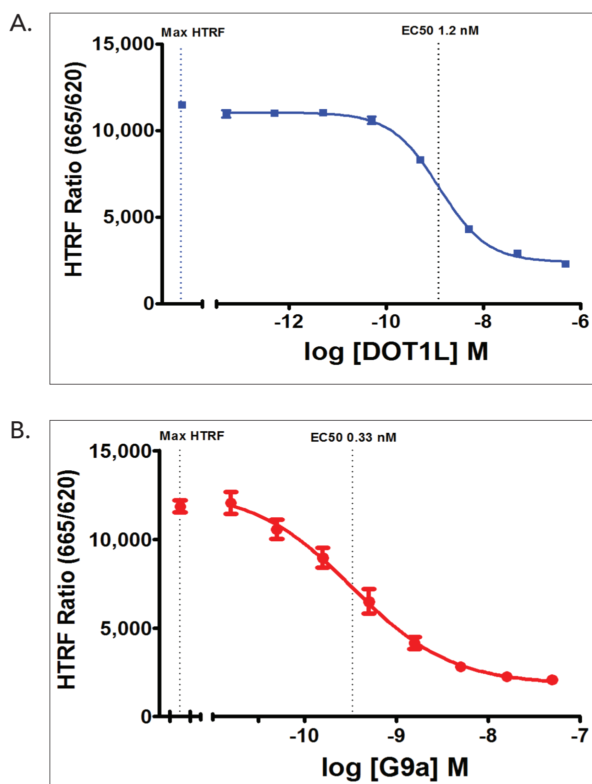

Figure 5. Enzyme titrations. A) DOT1L enzyme titration with oligonucleosome substrate and B) G9a enzyme titration with H3 (1-25) peptide substrate.

The enzyme titration data indicate EC_{50} concentrations of 1.2 and 0.33 nM for DOT1L and G9a, respectively. These values correlate well with previously determined values although the DOT1L appears to be 3-fold less active than previously reported[1].

Inhibitor Titration

An 8-point 1:10 or 1:2 serial dilution series, with a zero point, was prepared for inhibitor UNC0646 or SGC0946, respectively, in a 96-well plate. Each dilution point was transferred to the assay plate in quadruplicate containing EC_{80} concentrations of 5.9 and 1.9 nM for DOT1L and G9a, respectively. Reactions were allowed to proceed as previously described and reagents added as described above. The assay measurements were taken following the final incubation period and plotted as log inhibitor concentration vs. HTRF ratio (Figure 6).

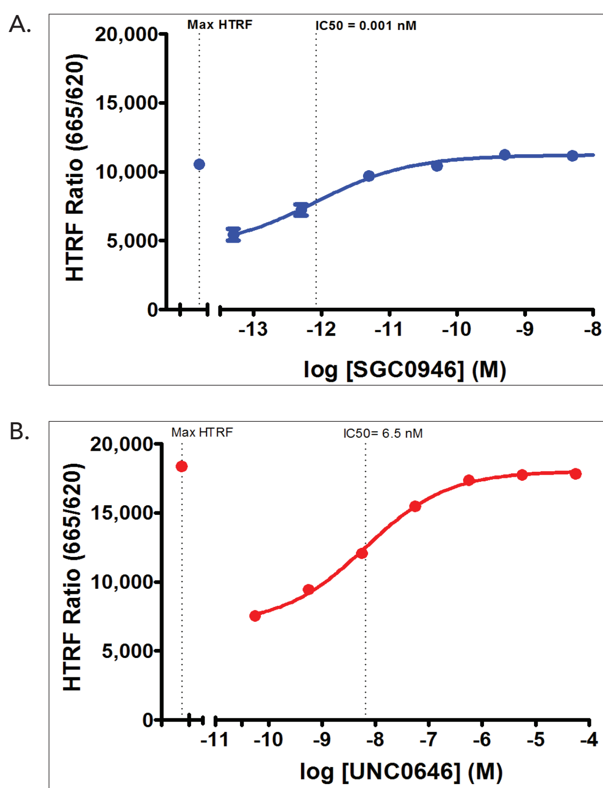

Figure 6. **Inhibitor titrations. A)** SGC0946 inhibitor titration with DOT1L enzyme at EC_{80} concentration of 5.9 nM and **B)** UNC0646 inhibitor titration with G9a enzyme at EC_{80} concentration of 1.9 nM.

The inhibition data indicate IC_{50} concentrations of 0.001 and 6.5 nM for DOT1L and G9a, respectively. The DOT1L inhibitor SGC0646 shows potent inhibition with an IC_{50} of 0.001 nM, a 10-fold increase in potency when compared to previously determined values[1]. The difference may be explained by the slightly lower activity seen with the DOT1L as described above. Inhibition of G9a by UNC0646 with an IC_{50} of 6.5 nM correlates well with previously published data[1].

Conclusion

The EPIgeneous Methyltransferase assay shows good performance in a wide variety of buffer conditions conducive to investigation of a range of methyltransferase enzyme/substrate combinations. The use of liquid handling instrumentation for reagent dispensing and serial dilution and mixing of standards simplify assay processes and primary screening efforts. We have shown that both enzyme characterization and pharmacology data can be easily generated in a format conducive to performing HTS. The use of an HTS microplate reader with dual-detection capabilities dramatically increases throughput capabilities.

References

1. Roux, T, et. al. (2013). *EPIgeneous™ Methyltransferase Assay: Enzymatic Examplifications.* Unpublished data. Cisbio Bioassays, Codelet, France.

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