EPIgeneous™ Methyltransferase assay: a new HTRF Universal SAH detection assay to assess methyltransferase activity

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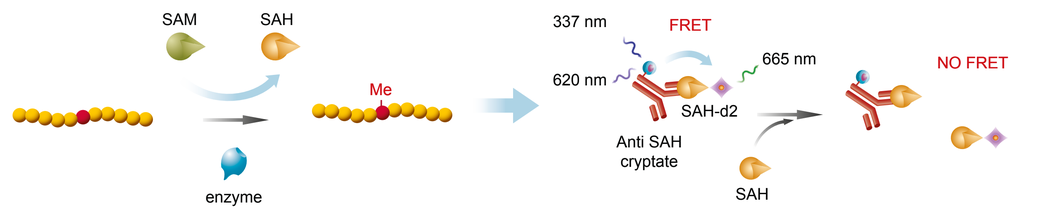
INTRODUCTION

Epigenetics is one of the fastest growing fields in research because of their potential to identify new drugs for therapeutic targets and especially in oncology. A key focus of epigenetics research is on methyltransferases because of their abundance and ability to methylate histones and various other substrates. Cisbio has developped a universal methyltransferase mix and read assay using HTRF technology that provides the sensitivity and flexibility of substrates. This assay, which directly quantifies S-5'-adenosyl-L-homocysteine (SAH), has been successfully validated on a variety of enzymes and substrates (see table). This study presents the principle, protocol and specifications of the assay as well as assay performances and optimization on an enzyme of interest: DOT1L with nucleosome as the substrate.

Enzymes and related substrates validated

METHYLTRANSFERASES	SUBSTRATES
G9a, EZH2 complex, SET7/9	H3 (1-21) or (1-50) peptide
PRMT1, SET8	H4 (1-25) peptide
DOT1L, SETD2, MLL1 complex, NSD1, NSD2	Oligonucleosome
SET7/9	p53
DNMT1	DNA (poly(dI-dC))
hN7, WNV, NSP14, NSP10-16	RNA
COMT	Dopamine
G9a	Recombinant full H3

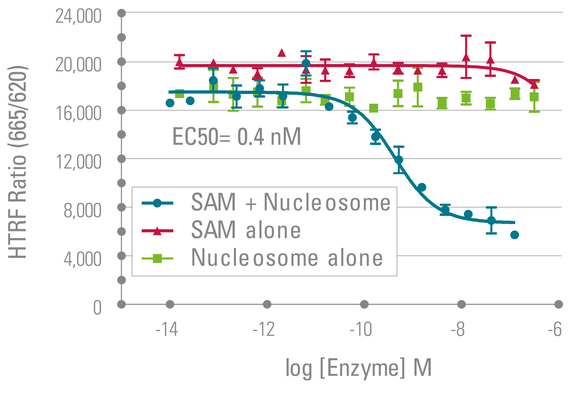
ASSAY PRINCIPLE AND REAGENTS



The assay consists of an enzymatic and detection step. The methyltransferase activity is assessed by measuring the conversion of SAM (S-5'adenosyl-L-methionine) to SAH. In order to directly measure SAH release, an anti SAH antibody labeled with terbium cryptate and a SAH-d2 tracer are used. The SAH released by the enzymatic reaction competes with the SAH-d2 labeled leading to a decrease of the HTRF signal. Oligonucleosome and all methyltransferase enzymes, but those for RNA and dopamine substrates, were provided by Reaction Biology Corp.

DOT1L / OLIGONUCLEOSOME ASSAY

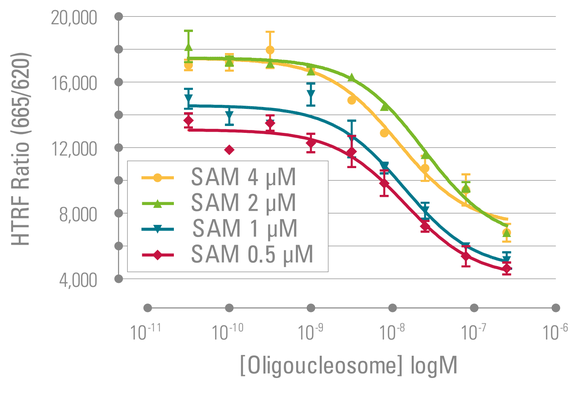
1. DOT1L titration



Detection of DOT1L specific activity and identification of optimal enzyme concentration.

Human recombinant DOT1L was serially diluted to the indicated concentrations and the assay carried out with 10ng/µl (= 77 nM) oligonucleosome as substrate and 2 µM SAM for 2 h at 30°C. The negative controls (no SAM or no nucleosome) show the measurement of the enzymatic specific activity. A DOT1L concentration of 4.5 nM (EC80) was selected for further experiments. This concentration lead to 10% conversion of SAM into SAH

2. Oligonucleosome and SAM titrations

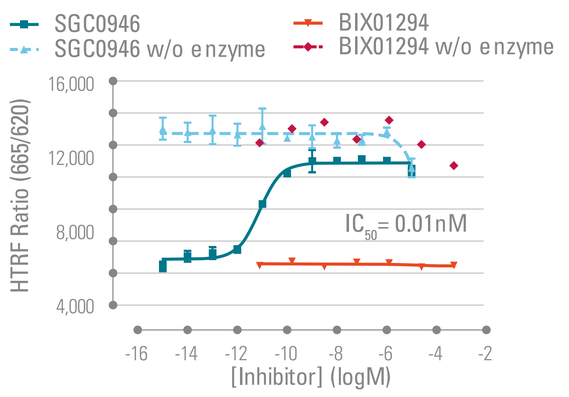


Determination of optimal substrate and SAM concentrations.

Oligonucleosome was titrated with several concentrations of SAM. DOT1L is used at 4.5 nM and incubated with SAM and substrate 2 h at 30°C.

0.5 µM of SAM, a concentration below reported Km of 0.67 µM (1), is selected for subsequent experiments. For oligonucleosome, 77 nM (EC80) is selected for further tests.

3. Inhibitor titration

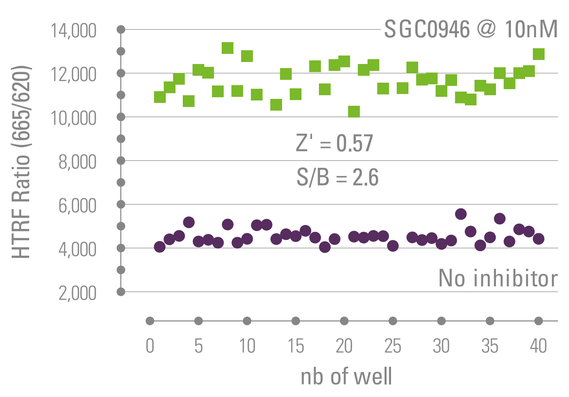


EPIgeneous™ methyltransferase assay validated by measuring activity of SGC0946 inhibitor.

This assay was performed using 0.5 µM SAM (EC40), 77 nM oligonucleosome (EC80) and 4.5 nM DOT1L (EC80). The enzymatic reaction was stopped with the detection reagents after a 2 h incubation at 30°C.

- IC50 of SGC0946 is in good agreement with the literature (2).
- As expected, BIX01294 which is a G9a selective inhibitor does not inhibit DOT1L.
- Controls of inhibitors without enzyme show that they do not affect the detection reagents.

4. Z' factor

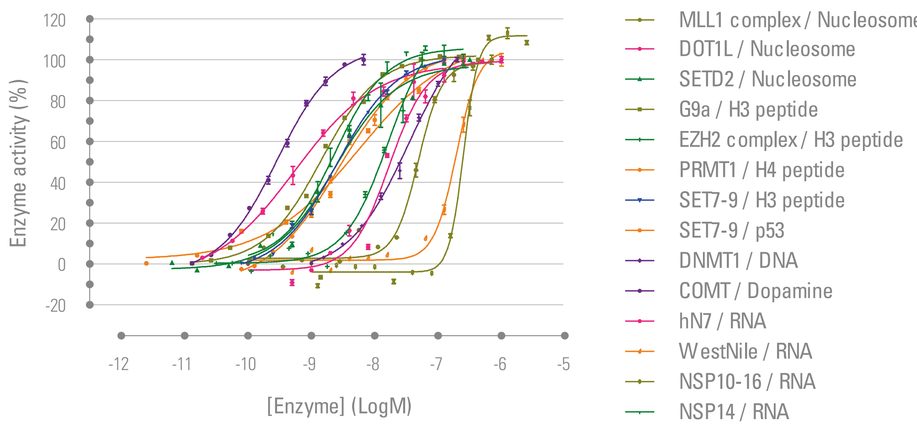


Assay robustness demonstrated through Z' factor determination.

This assay was performed using 0.5 µM SAM (EC40), 77 nM oligonucleosome (EC80) and 4.5 nM DOT1L (EC80).

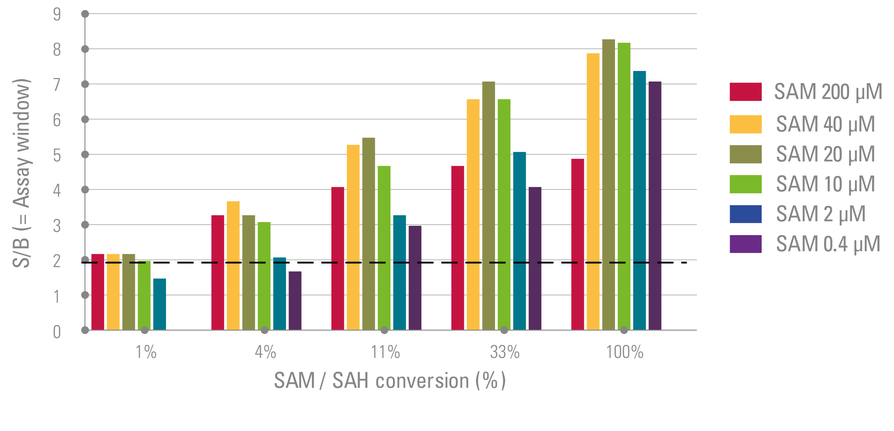
The Z' factor was obtained with biological balanced conditions and underlines the robustness of the assay and its suitability for HTS in biological relevant conditions.

VALIDATED METHYLTRANSFERASES



The HTRF EPIgeneous™ Methyltransferase assay has been validated with a large set of enzymes and different substrates, as listed in the introduction table. The graph above summarizes the activities detected for a selection of enzyme/substrate combinations. Enzymatic reactions were carried out at RT or 30°C for 1 to 2 h with 0.5 to 10 µM of SAM and related substrates according to enzymes tested. Reactions were stopped with detection reagents addition in 384 low volume plate (20 µL) and then read on Pherastar FS (flashlamp) after 1 h incubation.

ASSAY SENSITIVITY



The graph represents the assay windows obtained with a range of SAM concentrations and at different conversion percentages of SAM into SAH. The assay has been optimized to be suitable for a large range of SAM concentrations in the enzymatic step (0.4 – 200 µM). With these concentrations of SAM, the assay is able to assess the enzymatic activity with 4 to 100% turn over of the enzyme (hence 4 to 100% conversion of SAM into SAH).

CONCLUSION

We have developped a universal methyltransferase mix and read assay using HTRF technology that provides:

- The flexibility of substrates. The assay is validated with a large set of methyltransferase sub families so far: PKMT and PRMT on histone peptides, nucleosomes or other proteins (p53) ; DNA MT ; RNA MT and COMT with dopamine.
- Non radioactive assay with high sensitivity by measuring the release of SAH. Avoid false positives and counter screening due to coupling enzymes and indirect measurement format.
- Flexible enzymatic assay conditions: large SAM concentration range compatibility (0.4 – 200 µM)
- Enough sensitivity to work in biological relevant conditions (avoiding enzyme saturation) as shown with DOT1L / nucleosome assay example.

This study demonstrates that all enzymes and substrates developed by Reaction Biology Corp. are fully compatible with this new Universal SAH assay, and enable the optimization of robust assay formats for the study and the screening of methyltransferase targets.

REFERENCES

(1) Richon et al. Chem Biol Drug Des, 2011 &Yao et al. J Am Chem Soc, 2011

(2) Yu et al. Nature commun., 2012

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