Miniaturization of an HTRF methyltransferase assay that detects histone modifying activity

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- **Assay miniaturization enables high throughput screening for compounds that impact epigenetics**
- **Excellent data quality (Z´> 0.88) was achieved in just 2 µl assay volume**

Introduction

Histone modifying enzymes are important epigenetic targets in drug discovery and cancer research. Some of the enzymes of interest are methyltransferases. Their activity leads to methylated histones, which affect the regulation of gene transcription. The methyl group is often provided by SAM (S-adenosyl-L-methionine). SET7/9 is a methyltransferase that uses SAM as a cofactor in order to methylate histone H3. In this way SET7/9 is able to modulate p53 activity in a human cancer cell line, indicating the importance of that enzyme in the process of human tumor formation.
In this application note we show the use of the EPIgeneous Methyltransferase Assay kit from Cisbio. The homogeneous approach of the HTRF technique enables the use of the kit for high throughput screening purposes. To limit the associated costs miniaturizing to significantly lower assay volumes was done. This requires very precise instrumentation and was performed with the help of the Echo liquid handler from Labcyte that uses acoustic dispensing (down to 2.5 nl) to accurately transfer kit reagents, enzyme, substrate, and compounds.

Assay Principle

The assay consists of two steps. In the enzymatic step the substrate is incubated with the enzyme in presence of compounds. Cofactor SAM is added to start the reaction. The result will be a methylated substrate while SAM is converted into SAH (S-adenosyl-L-homocysteine). In the second step detection reagents are added that contain an antibody specific to SAH. The antibody is labeled with Lumi4-Tb cryptate. To this d2-coupled SAH is added that competes with the SAH formed during the reaction for the antibody binding sites (Fig. 1).

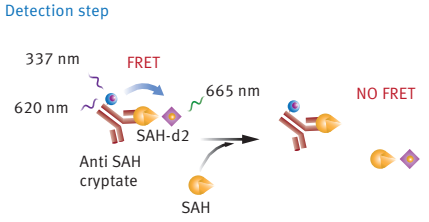

Fig. 1: Epigeneous Methyltransferase Assay Kit (Detection Step). If there is no SAH converted from SAM during the enzymatic reaction Anti-SAH will bind to SAH-d2 leading to a large HTRF signal. If SAH is present in the well after the enzymatic reaction is finished, SAH will compete with SAH-d2 on the binding sites of the antibody leading to a decrease in TR-FRET signal.

Materials & Methods

- EPIgeneous Methyltransferase Assay Kit from Cisbio
- 384-well white, low volume assay plates from Greiner
- 1536-well white assay plates from Corning
- SET7/9 from BPS Bioscience
- Biotinylated histone H3 (1-21) from AnaSpec
- (R)-PFI 2 hydrochloride from Tocris Biosciences
- SAM and SAH from Sigma-Aldrich
- Echo® liquid handler from Labcyte
- PHERAstar® *FS* microplate reader from BMG LABTECH

Reagent preparation
SAM was dissolved in 5 mM H_2SO_4/10% ethanol (v/v) in water, to make a 30 mM solution. Assay buffer consisted of 50 mM TRIS-HCL pH 8.8, 10 mM NaCl, 1 mM DTT, 0.01% Tween-20. HTRF detection buffer one and two were ready to use after thawing at room temperature.

Standard curves
For pretest purposes SAM/SAH standard curves were created. These standard curves mimic the methyl transfer in the enzymatic reaction. 16 standards were prepared in assay buffer, starting with 1 µM SAH (1:2 dilution). Triplicates of each dilution were transferred into 384-well or 1536-well microplates. Detection reagents were added and after 60 min of incubation the plates were read in the PHERAstar *FS*.

Enzyme titration
Half-log dilution SET7/9 titration curve was prepared in Echo-qualified, 384-well source plates. 400 nl of the different enzyme concentrations were transferred to 384-well assay plates in triplicates. 200 nl assay buffer, 200 nL 800 nM biotinylated histone and 200 nl 1 µM SAM were added, plates sealed and incubated at room temperature for 60 min. After incubation, detection buffer one was added to stop the enzymatic reaction. After further 10 minutes of incubation, anti-SAH lumi-Tb cryptate and SAH-d2 prepared in detection buffer two were added. After the final 60 min incubation step the microplate was read in the PHERAstar *FS*.

Inhibitor dose response curve
A 12-point half-log dose response series of Set7/9 inhibitor (R)-PFI 2 hydrochloride was prepared in DMSO, starting at the highest concentration of 100 µM. Inhibitor was transferred into 384-well and 1536-well plates followed by assay buffer, 40 nM enzyme, 800 nM biotinylated histone, and 1 µM SAM. All reaction and incubation steps were done as described for the enzyme titration. After final incubation step the microplates were measured in the PHERAstar *FS*.

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All transfer and pipetting steps were done using the Echo liquid handler.

PHERAstar FS instrument settings

Detection mode:	TRF, endpoint
Optic Module:	HTRF 337 665 620
Integration start time:	60 µs
Integration time:	400 µs

Results & Discussion

In Fig. 2 SAM/SAH standard curves are shown that were measured either in 384-well or in 1536-well format. The results indicate that the assay is applicable in different plate formats. It is also shown that a final volume of as little as 1.25 µl can be used.

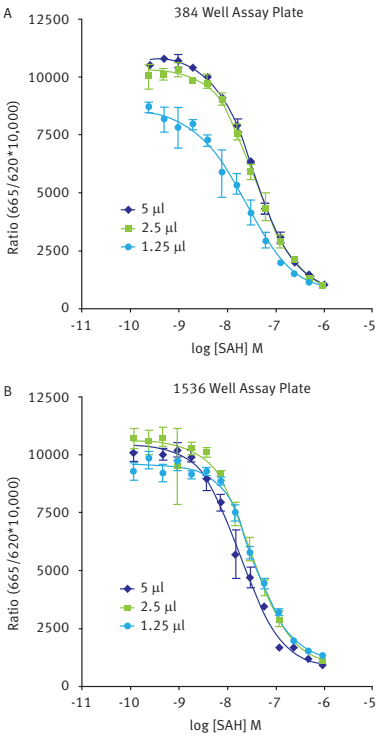

Fig. 2: SAM/SAH standard curves in 384-well **(A)** and 1536-well **(B)** assay plates using different volumes.

A SET7/9 enzyme titration was done in order to find optimal enzyme concentrations. Fig. 3 shows the titration result. From this an enzyme concentration of 40 nM was determined to be optimal to use for subsequent experiments.

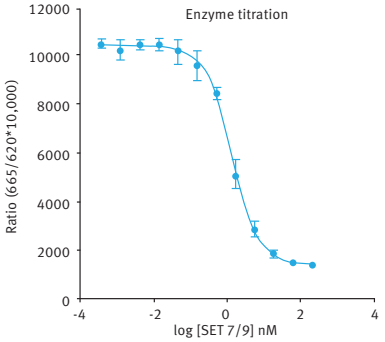

Fig. 3: SET7/9 enzyme titration in the enzymatic reaction volume of 1 µl.

For the SET7/9 inhibitor IC_{50} values could be calculated from the inhibitor dose response curve (Fig. 4).

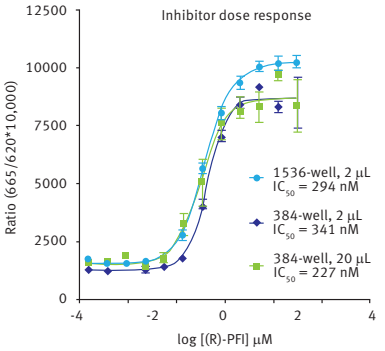

Fig. 4: (R)-PFI 2 hydrochloride inhibitor titration with SET7/9 enzyme.

Miniaturization of the assay was very successful indicated by excellent data quality. The Z´ factors for the miniaturized assays were > 0.88 and agreed very well to the assay parameters obtained for the standard 20 µl assay (Table 1).

Table 1: Comparison of Z´ factors and signal to background (SIB).

Assay format	S/B	Z´factor
384-well (20 µl)	6.4	0.83
384-well (2 µl)	7.9	0.88
1536-well (2 µl)	7.3	0.93

Conclusion

The SET7/9 methyltransferase assay could be miniaturized 10 fold leading to significant cost savings on reagents, enzymes, substrates, and compounds.