# Assessing Epigenetic Enzyme Activity using HTRF® Epigenetic Assays from Cisbio with the PHERAstar *FS* from BMG LABTECH

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- HTRF assay to detect histone methyltransferase G9a activity can be used to screen for inhibitors in 384-well format
- PHERAstar *FS* one flash fly mode improves read times which will increase throughput

## Introduction

Epigenetics is an emerging arena of science in which changes in the regulation of gene activity and expression are not dependent on the sequence of genes. Instead the changes in expression are the result of DNA methylation or modification of histones. These modifications are essential to the normal developmental process and the results can be devastating when the epigenetic process is disrupted leading to cancer and autoimmune disease. The realization of the importance of epigenetic regulation has led to the search for drugs which are capable of targeting the enzymes that add or remove epigenetic modifications. In order to perform screening to identify compounds which affect epigenetic modifying enzymes it is necessary to establish a reliable assay which is capable of monitoring the activity of these enzymes. Cisbio has made available a number of assays that have been verified to detect the activity of the enzymes involved in histone modifications. These enzymes fall into two broad classes:

Writers: enzymes that add methyl or acetyl groups to histones
Erasers: enzymes that remove methyl or acetyl groups from histones

Here we describe the verification of the performance of a Cisbio HTRF® based assay using the PHERAstar *FS* from BMG LABTECH. This assay allows users to detect the activity of G9a, a histone methyltransferase that methylates histone H3 on lysine 9 (H3K9). G9a mediated H3K9 methylation has been shown to be involved in transcriptional repression of developmental genes which is essential for early embryogenesis[1]. In addition G9a has been shown to have an oncogenic function based on its ability to sustain cancer cell proliferation[2]. Therefore, G9a is an excellent example of the type of epigenetic target that is of high importance in drug development.

## Assay Principle

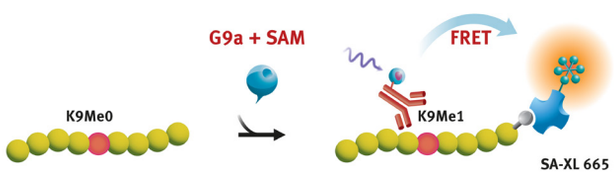


**Fig. 1:** HTRF® G9a Histone H3K9 mono-Methylation Assay Principle

The assay is a Histone H3K9 monomethylation assay that uses a biotinylated peptide that comprises residues 1-21 of histone H3 in which lysine 9 is un-methylated as a substrate (Figure 1).

The assay is performed without washing in a single well using an enzymatic step followed by a detection step. In the enzymatic step substrate, enzyme G9a, and S-(5'-Adenosyl) L-methionine chloride (SAM) are combined. During incubation, active enzyme will transfer a methyl group to the lysine residue resulting in methylated substrate. The amount of modified substrate is detected via addition of a $Eu^{3+}$-cryptate labeled anti- H3K9 me1 detection antibody and XL665-conjugated streptavidin. The antibody will bind specifically to the methylated substrate and fluorescence resonance energy transfer will be observed between HTRF donor (antibody) and acceptor (XL665).

## Materials and Methods

- ☐ H3K9 mono-methylation detection reagents and protocol (Cisbio)
- ☐ 384 well, small volume, white microplates (Greiner)
- ☐ G9a (Reaction Biology Corp.)
- ☐ H3K9 peptide substrate (AnaSpec)
- ☐ SAM & SAH (Sigma)
- ☐ UNC0646 & BIX01294 (R&D Systems)
- ☐ PHERAstar *FS* (BMG LABTECH – Figure 4)

To perform the enzymatic step working solutions were prepared in enzymatic buffer (50 mM Tris-HCl, pH 8.8, 10 mM NaCl, 4 mM DTT, 0.01% Tween 20). The reaction was assembled in a 384 well small volume plate in the following order:

· 4 µl inhibitor or enzymatic buffer
· 2 µl G9a (incubate for 5 minutes at room temperature)
· 4 µl substrate/SAM mix (seal plate and incubate at room temperature)
· 10 µl of detection mixture

After addition of the detection mixture the plate is sealed and incubated at room temperature for at least 1 hour. After 1 hour signal is stable for an extended period. Detection was performed with the PHERAstar *FS* using the following parameters:

*Instrument Settings*

| | |
|---|---|
| Measurement Method: | Time resolved fluorescence (dual emission), Endpoint mode, Top optics |
| Light source: | Laser |
| Optic module: | HTRF (Ex 337, EmA 665, EmB 620) |
| Number of fl ashes: | 1-30 |
| Integration start/time (µs): | 60/400 |
| Focal height: | Adjusted prior to measurement |

*Data analysis*

The HTRF ratio (Signal at 665nm / Signal at 620nm; the result is multiplied with 10,000) is automatically calculated by the MARS Data Analysis Software.

## Results and Discussion

Previous results indicated optimal performance by using a G9a concentration from 0.125-0.25 nM in 40 to 60 minute reaction time. Therefore the experiments described here employ a G9a concentration of 0.12 nM and a 40 minute reaction time. To verify performance a substrate titration was performed to determine the Km for the peptide in this experimental protocol. The results of this titration experiment are shown in Figure 2.

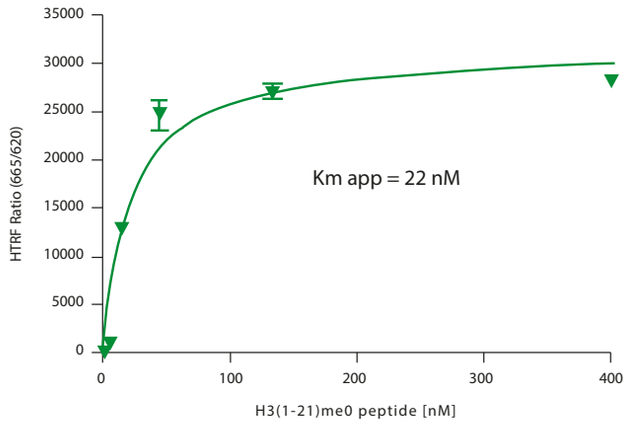


**Fig. 2: Peptide Substrate Titration**
The apparent Km value for the peptide substrate was determined with the enzyme concentrations and reaction time described above and concentration of 15 µM SAM was used. Serial dilutions of the biotinylated H3K9 (1-21) me0 substrate were prepared which span from 400 to 2 nM. The streptavidin XL-655 concentration in the detection reagent was varied based on the peptide concentration to keep a constant ratio of 1:4 (streptavidin XL-655: peptide). An apparent Km value of 22 nM was determined from this experiment using a Michaelis Menten plot.

Final validation of the G9a H3K9 methylation assay was obtained by measuring the activity of known G9a inhibitors (Figure 3)

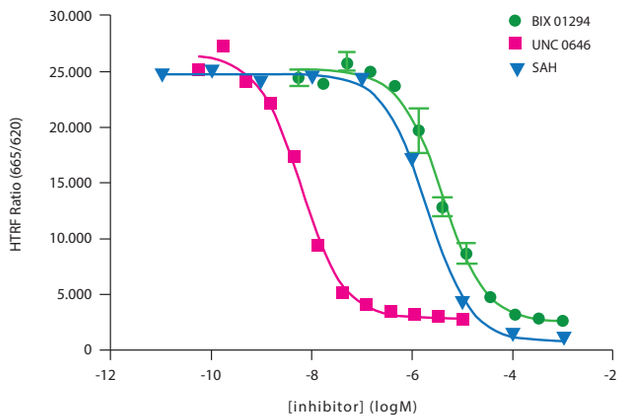


**Fig. 3: Enzyme inhibition curves for three G9a inhibitor compounds**
Inhibitors were serially diluted and pre-incubated with G9a for 5 minutes. The enzymatic reaction was initiated with the addition of 15 µM SAM and 40 nM biotinylated H3 (1-21) me0 peptide.

$IC_{50}$ values were calculated from enzyme inhibition curves similar to those shown in Figure 3. The $IC_{50}$ results, are presented in Table 1. Table 2 compares the read times of the PHERAstar *FS* when flash number is varied.

**Table 1: $IC_{50}$ values for three different G9a inhibitors**

| *Inhibitor* | *$IC_{50}$* |
|---|---|
| ***BIX 01294*** | ***4.1 µM*** |
| ***UNC 0646*** | ***6.2 nM*** |
| ***SAH*** | ***1.9 µM*** |

**Table 2: Read times for varying TRF laser conditions in a 384 well plate**

| *Laser mode:* | *Precise (27 flashes)* | *Rapid (7 flashes)* | *Flying (1 flash)* |
|---|---|---|---|
| ***Read time:*** | ***2m32sec*** | ***48 sec*** | ***21 sec*** |

## Conclusion

- The Cisbio HTRF® G9a Histone H3K9 mono-Methylation Assay provides an excellent platform to screen for inhibitors of these enzymes with a central role in epigenetic regulation
- Assay performance on the PHERAstar *FS* is verified using substrate, cofactor and inhibitor titrations
- One flash fly mode significantly improves read times which will increase throughput
- Excellent performance in one flash fly mode can be attributed to:
  - UV laser as an excellent excitation source
  - HTRF® specific modules which provide true simultaneous dual emission detection
  - Matched PMT's designated for TRF detection


**Fig. 4: The PHERAstar *FS* microplate reader from BMG LABTECH**
Employs advanced features such as a UV-Laser and assay specific modules for optimal HTRF performance

## References


1. Tachibana, M et al.(2002) *Genes & Development* **16**, 1779-1791
2. Ding, J et al. (2013) *Cell Metabolism* **18**, 896-907



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