Evaluation of EZH2 wild type and mutant Y641F assay using a HTRF® kit

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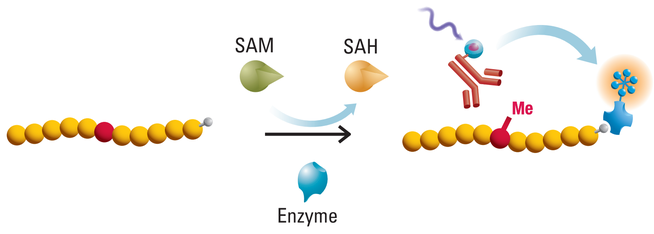
Introduction

EZH2 is one of the most studied targets in Epigenetics. It is targeted for the treatment of certain non-Hodgkin's lymphomas and breast cancer subtypes.
Cisbio Bioassays has generated a panel of reagent tools using HTRF® technology between europium cryptate (donor) and XL665 (acceptor) to investigate epigenetic targets. In this study, the methylation state of H3(1-50)K27me3 was monitored by a HTRF kit that combines a specific anti-trimethyl histone H3K27 antibody labeled with europium cryptate and streptavidin-XL665 (SA-XL665). This assay was optimized using enzyme concentration, reaction time, and SAM Km. Moreover, IC_{50} of several known inhibitors and Z' factor for high-throughput screening were analyzed. The results from this study demonstrates Cisbio's HTRF assays are suitable for screening inhibitors of EZH2.

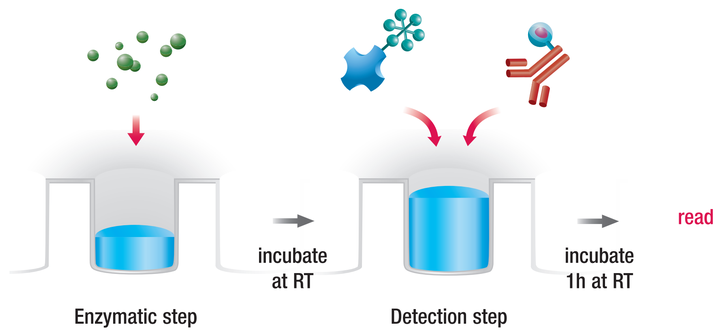
Assay Principle



The histone methyltransferase (HMT) EZH2 wild type and Y641F mutant assay consists of enzymatic and detection steps. The methylated substrate is detected by anti-H3K27me3-K (donor) and streptavidin-XL665 (acceptor).
The donor and acceptor bind to the methylated substrate creating a sandwhich assay. When the donor is excited, it will generate a fluorescence signal at 620nm, meanwhile some of its energy will transfer to acceptor, thus the acceptor will generate a fluorescence signal at 665nm. The final HTRF ratio (665nm/620nm) would be high if the enzyme has good activity. If the compound added has inhibited the enzyme, then the substrate methylation would be inhibited, and the HTRF ratio would decrease.

Enzymatic step (10 µl)
4 µl enzyme
+ 2 µl compounds
(or enzymatic buffer)
+ 2 µl SAM
+ 2 µl substrate

Detection step (20µl)
+ 5 µl Ab-K
+ 5 µl Streptavidin-XL665

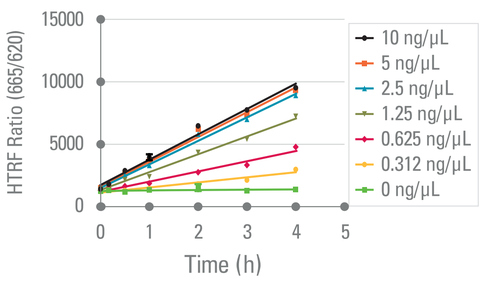


EZH2 wild type and Y641F assay

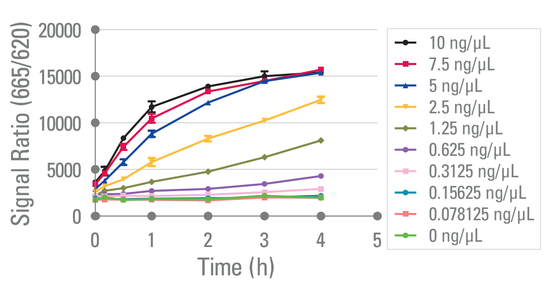
1. Enzyme titration and time course

Demonstration of optimal enzyme concentration and enzymatic reaction time.
Human recombinant EZH2 wild type and Y641F was serially diluted from 10 ng/µL to 0 ng/µL. H3(1-50)-biotin and H3(1-50)K27me1-biotin were used at 500 nM as substrate for EZH2 wild type and Y641F respectively. Enzyme and substrate were incubated with 100 µM SAM for different time (from 0 hour to 5 hour) at RT.
One hour after detection reagent anti-H3K27me3-K and SA-XL665 were added to the assay wells, plate was read on a plate reader.
A 4h reaction time with enzyme concentration of 2.5 ng/µL was selected for further experiments of EZH2 wild type.
The same condition was selected for EZH2 Y641F in further experiments.

EZH2 wild type



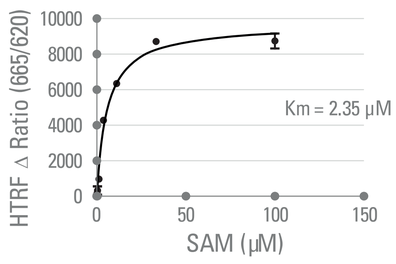
EZH2 Y641F

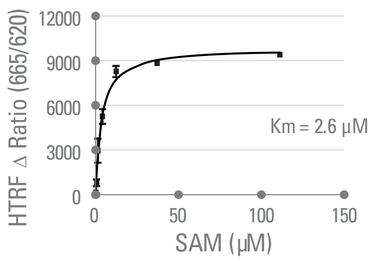


2. SAM titrations

Determination of optimal SAM concentration.
For EZH2 wild type: SAM was serially titrated from 100 µM and incubated with 2.5 ng/µL enzyme and 500 nM H3(1-50)-biotin peptide substrate for 4h at RT.
For EZH2 Y641F: SAM was serially titrated from 110 µM and incubated with 2.5 ng/µL enzyme and 500 nM H3(1-50)K27me1-biotin peptide substrate for 4h at RT.

2.4 µM and 2.6 µM of SAM is selected for subsequent experiments for EZH2 wild type and Y641F separately.

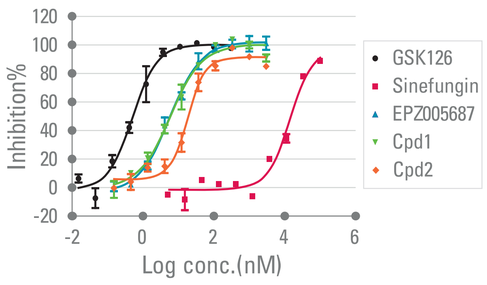




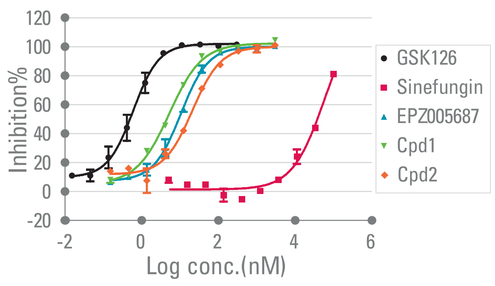
3. Inhibitor titration

Inhibition assay validated by measuring several reference compound.
Compound were serially titrated to the indicated concentrations.
For EZH2 wild type, compound incubate with 2.5 ng/µL enzyme, and assay was initiated by 2.4 µM SAM and 500 nM H3(1-50)-biotin.
For EZH2 Y641F, compound incubate with 2.5 ng/µL enzyme, and assay was initiated by 2.6 µM SAM and 500 nM H3(1-50)K27me1-biotin.

IC50 of EPZ005687 is in good agreement with the literature (1).
IC50 of GSK126 is in good agreement with the literature (2).



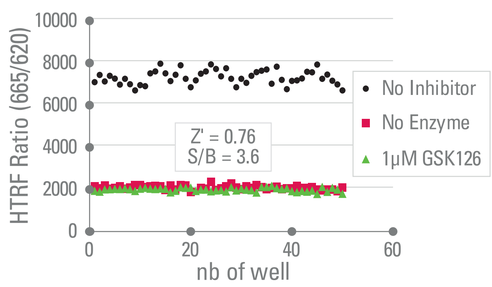
	GSK126	Sinefungin	EPZ005687	Cpd1	Cpd2
IC50	0.5195	14,003	5.927	5.933	18.50

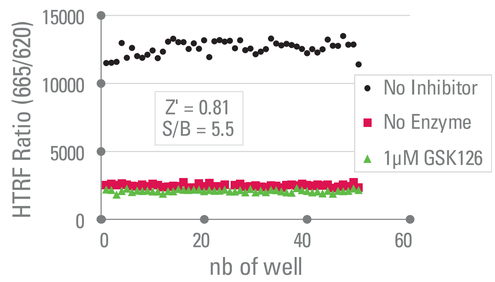


	GSK126	Sinefungin	EPZ005687	Cpd1	Cpd2
IC50	0.6047	58,479	10.55	5.523	21.25

4. Z' factor

Demonstration of assay robustness by Z' factor determination.
Assay for EZH2 wild type was performed using 2.4 µM SAM, 500 nM H3(1-50)-biotin and 2.5 ng/µL enzyme, with or without 1 µM GSK126.
Assay for EZH2 Y641F was performed using 2.6 µM SAM, 500 nM H3(1-50)K27me1-biotin and 2.5 ng/µL enzyme with or without 1 µM GSK126.
The Z' factor was obtained with balanced conditions and underlines the robustness of the assay and its suitability for HTS in biological relevant conditions.





Condition and result summary

		EZH2 WILD TYPE	EZH2 Y641F
Enzymatic step	Substrate	H3(1-50)-biotin	H3(1-50)K27me1-biotin
	Sub conc	1,75 mm	500 nM
	SAM	2.4 µM	2.6 µM
	Enzyme conc	2.5 ng/µL	2.5 ng/µL
	Enzymatic time	4h	4h
Detection step	Detection reagent	anti-H3K27me3-K SA-XL665	anti-H3K27me3-K SA-XL665
	Incubation time	1h	1h
IC50 of Reference	GSK126	0.52 nM	0.60 nM
	Sinefungin	14 003 nM	58 479 nM
	EPZ005687	5.9 nM	11 nM
	Cpd1	5.9 nM	5.3 nM
	Cpd2	19 nM	21 nM
Z'	no Enzyme	0.76	0.81
	1µM GSK126	0.76	0.81

Conclusion

We have evaluated the EZH2 wild type and Y641F assay with the mix and read kit using HTRF technology that provides

- Assay conditions was successfully optimized.
- The IC_{50} of the reference compounds are in good correlation with the literature.
- With a high Z' obtained in balanced conditions, the robustness of the assay indicates the suitability for HTS in biologically relevant conditions.

References

(1) Knutson et al. Nature Chem Biol, 2012
(2) Wigle et Copeland, COCB 2013

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