AlphaNuc™ SETD8 HMT Assay

A nucleosome-based approach for the discovery of SETD8 inhibitors

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Overview

AlphaNuc is a novel assay platform combining EpiCypher's industry-leading recombinant nucleosomes with the AlphaScreen detection system to quantitate histone modifying enzyme activity. Here we show the development of a robust, non-radioactive assay utilizing histone methyltransferase SETD8 and recombinant nucleosomes, representing their physiologically relevant substrate. This assay has a high Z' value and is suitable for adaptation to high-throughput screening.

Introduction

Histone post-translational modifications (PTMs) act as vital signaling integrators to regulate chromatin structure and function [1]. Monomethylation at lysine 20 on histone H4 (H4K20me1) stimulates transcriptional activation and plays a critical role in regulating transcription during cell division [2]. In addition, H4K20me1 may regulate the DNA double-strand break response through the recruitment of effectors, such as 53BP1. This modification is catalyzed by a single histone methyltransferase (HMT), SETD8 (also known as SET8, PR-Set7 and KMT5A), which is aberrantly activated in many types of cancer, including breast, bladder, small cell lung carcinoma, chronic myelogenous leukemia, and pancreatic carcinoma. Reduced SETD8 expression using shRNA has been shown to reduce breast cancer susceptibility, making it a compelling target for therapeutic inhibitor development [3].

EpiCypher has developed an optimized high-throughput SETD8 inhibitor assay using our AlphaNuc platform. This technology utilizes PerkinElmer's AlphaLISA™ system to deliver a 'no-wash' assay compatible with recombinant nucleosome (rNuc) substrates, representing the endogenous target of SETD8.

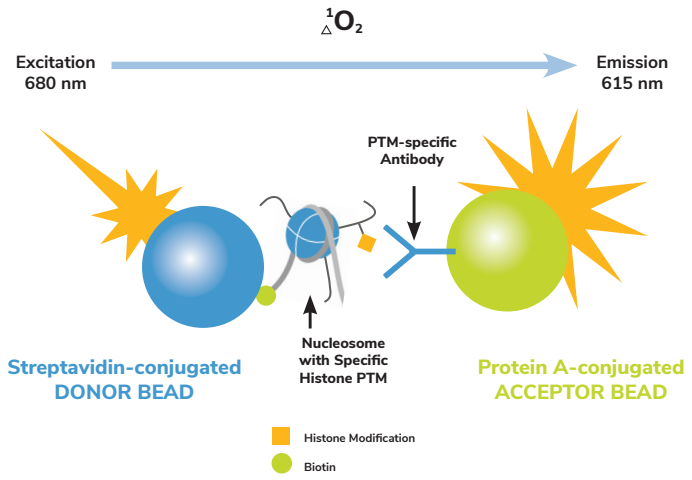

FIGURE 1: ALPHANUC ASSAY PRINCIPLE. *SETD8 methylates H4K20 on the recombinant nucleosome substrate. Streptavidin-conjugated Donor beads capture the biotinylated nucleosome DNA. Protein A-conjugated AlphaLISA Acceptor beads are used to capture the primary antibody that recognizes H4K20me1. Donor and Acceptor beads are brought into close proximity through antibody binding of the histone PTM. Excitation of the Donor beads (680 nm) provokes the release of a singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm.*

Consider the data: Nucleosomes are superior substrates for biochemical assay development

Nucleosomes are the fundamental and repeating units of chromatin, consisting of ~147 base pairs of DNA integrally wrapped around a histone octamer. Nucleosomes represent the in vivo substrate for many histone modifying enzymes. Currently available biochemical assays for studying the activities of histone modifying enzymes utilize synthetic histone peptide or recombinant histone protein substrates.

However, a subset of relevant HMTs prefer nucleosome substrates for activity in vitro and in vivo. Recent work with SETD8 demonstrates a >10,000-fold decrease in Km of the SAM cofactor and IC50 of the Sinefungin inhibitor when recombinant nucleosomes (EpiCypher, Catalog No. 16-0006) were used in place of peptide substrates. Moreover, 40 compounds were identified that only inhibit SETD8 when nucleosomes were used as substrates [4].

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Continued from page 1

Reagents and Materials Required for the Assay

- GST-SETD8, Recombinant Human (EpiCypher Catalog No. 15-1005)
- Mononucleosomes, Recombinant Human Biotinylated (EpiCypher Catalog No. 16-0006)
- S-adenosylmethionine (SAM) (New England Biolabs Catalog No. B9003S)
- Anti-H4K20me1 (Cell Signaling Technology Catalog No. 9724)
- AlphaScreen Streptavidin Donor Beads (PerkinElmer Catalog No. 6760002)
- AlphaScreen Protein A Acceptor Beads (PerkinElmer Catalog No. 6760137)
- AlphaPlate-384™, Light gray (PerkinElmer Catalog No. 6005350)
- TopSeal™-A PLUS (PerkinElmer Catalog No. 6050185)
- Sinefungin (Enzo Life Sciences Catalog No. ALX-380-070)
- HMT buffer: 50 mM Tris-HCl, pH8.8, 5 mM MgCl2, 1 mM DTT, 0.01% Tween-20, 0.1% BSA
- Detection buffer: (**HMT buffer** without DTT) 50 mM Tris-HCl, pH8.8, 5 mM MgCl2, 0.01% Tween-20, 0.1% BSA

Standard Protocol

N.B. Use HMT buffer to prepare HMT reaction solutions just before use

- 2X inhibitor
- 4X NSD2: 400 nM (1X = 100 nM)
- 4X rNuc mix: rNuc - 40 nM; SAM - 400 µM

AlphaNuc Inhibitor Assay Procedure

For best results users should centrifuge the plate briefly (~200 rcf for 1 sec) after the addition of each reagent

A. HMT reaction (total volume: 15 µl) – 20 min

1. Add 7.5 µl of 2X inhibitor to the wells of the AlphaPlate-384
2. Add 3.75 µl of 4X SETD8
3. Incubate 10 min at RT for inhibitor assay
4. Add 3.75 µl of 4X rNuc mix
5. Cover plate with TopSeal-A PLUS and incubate 10 min at RT

B. Protein A Acceptor beads/antibody incubation – 1 hour

6. Prepare 5X Acceptor beads / antibody mix in Detection buffer: dilute Protein A Acceptor beads 1 to 50 (100 µg/ml) and add H4K20me1 antibody diluting 1 to 100
7. Add 5 µl of 5X Acceptor beads / antibody mix to each well to make final concentration of 20 µg/ml of Acceptor beads and 1:500 dilution of anti-H4K20me1
8. Cover plate with TopSeal-A PLUS and incubate for 1 hour at RT

C. Streptavidin-conjugated Donor beads incubation – 30 min

9. Prepare 5X Donor beads at 100 µg/ml in Detection buffer in subdued light just before use
10. Add 5 µl of 5X Donor beads mix to each well (final concentration 20 µg/ml)
11. Cover plate with TopSeal-A PLUS and incubate in the dark at RT for 30 min
12. Read signal in AlphaScreen 384 mode with the Envison Multilabel Plate Reader

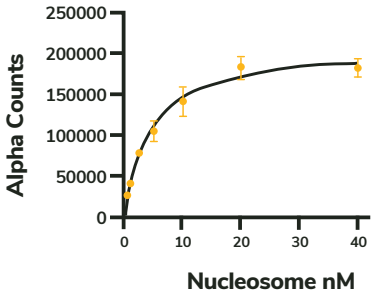

FIGURE 2: SUBSTRATE TITRATION. *HMT reactions were performed by incubating SETD8 (1 nM) with increasing amounts of recombinant human nucleosomes (rNuc, 0.5 nM to 40 nM) and SAM (100 µM). A mixture of Acceptor beads and anti-H4K20me1 was added and incubated for 60 minutes. Donor beads were finally added and signal was read after 30 minutes. For subsequent IC50 and Z' factor determination assays, 10 nM rNuc was used.*

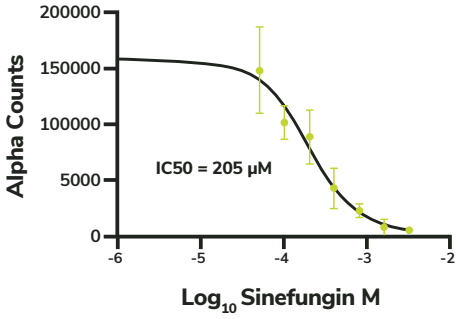

FIGURE 3: INHIBITOR ASSAY VALIDATION. *HMT reactions were performed by incubating the HMT inhibitor Sinefungin (1 µM to 3.2 mM) with SETD8 (1 nM). Enzymatic reactions were initiated by adding rNuc (10 nM) and SAM (100 µM) to the reaction cocktail. Sample processing was as in Figure 2. Measured IC50 value is consistent with published results [4].*

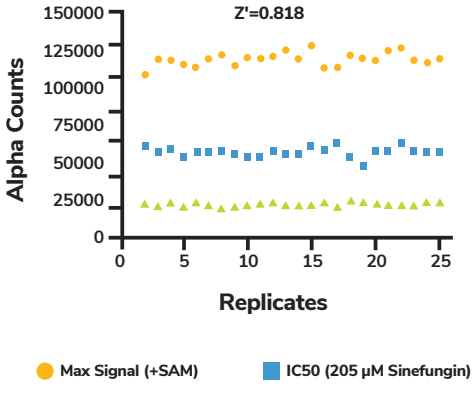

FIGURE 4: Z' FACTOR DETERMINATION. *SETD8 (1 nM) and rNuc (10 nM) were incubated with or without SAM, or in the presence of Sinefungin (230 µM) for IC50 determination. Enzymatic reactions and subsequent sample processing were performed as in Figure 3. Z' factor was calculated as described previously [5].*

References

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