

AlphaNuc™ NSD2 HMT Assay

A nucleosome-based approach for the discovery of NSD2 inhibitors

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Overview

AlphaNuc is a novel assay platform combining EpiCypher's industry-leading recombinant nucleosomes with the AlphaScreen detection system from PerkinElmer to detect histone modifying enzyme activity on nucleosomal substrates. Nucleosomes are the physiologically relevant substrate for many histone modifying enzymes. Here we show the development of a robust, non-radioactive HMT assay utilizing recombinant nucleosomes and the histone methyltransferase NSD2. This assay has a high Z' value and is suitable for adaptation to high-throughput screening.

Introduction

The histone lysine methyltransferase (HMT) NSD2 (Nuclear SET Domain-Containing Protein 2, also named MMSET and WHSC1) catalyzes dimethylation of histone H3 at lysine 36 (H3K36me2) and is implicated in development and progression of the incurable hematologic malignancy multiple myeloma. NSD2 – via H3K36me2 catalysis – drives tumorigenesis by acting as an epigenetic mutagen that re-programs the epigenome of cells, leading to activation of normally silenced oncogenes such as Myc [1]. Recent studies have indicated the specific down regulation of NSD2 expression can dramatically attenuate the tumorigenicity of myeloma cells in culture and in animal xenograft tumor models [1, 2]. Further, the ability of NSD2 to transform cells and support myeloma cell growth in tumor models requires its catalytic activity [1], making the catalytic domain of NSD2 a compelling target for therapeutic inhibitor development. Below is an optimized high-throughput NSD2 inhibitor assay using EpiCypher's AlphaNuc platform. This technology utilizes Perkin Elmer's AlphaLISA™ system to deliver a 'no-wash' assay compatible with recombinant nucleosome (rNuc) substrates, the endogenous target of NSD2.

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. Nucleosome substrates represent the *in vivo* template for any histone targeting enzyme. Currently available biochemical

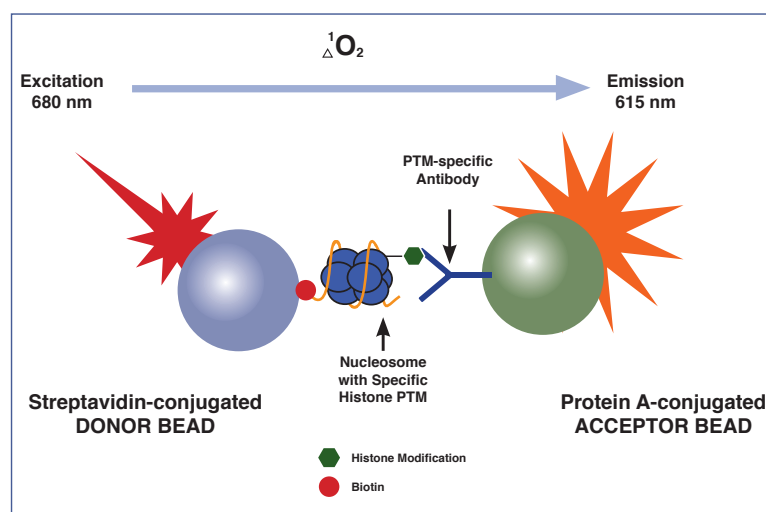


Figure 1: AlphaNuc Assay Principle. NSD2 methylates lysine 36 on histone H3 on the nucleosome substrate. Streptavidin-coated Donor beads capture the biotinylated nucleosome DNA. Protein A-conjugated AlphaLISA™ Acceptor beads are used to capture primary antibody that recognizes H3K36me2. Donor and Acceptor beads come into proximity through antibody binding of the histone modification. 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.

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assays for studying the activities of histone-modifying enzymes are highly dependent on synthetic histone peptides or recombinant histone protein substrates. However, it is known that a subset of biologically and clinically relevant HMTs (including NSD2) prefers nucleosome substrates for activity *in vitro* and *in vivo* [3].

Reagents and Materials Required for the Assay

- NSD2 / MMSET Catalytic Domain, Recombinant Human (EpiCypher Catalog No. 15-1002)
- Mononucleosomes, Recombinant Human Biotinylated (EpiCypher Catalog No. 16-0006; Lot #15321007)
- S-adenosylmethionine (SAM) (New England Biolabs Catalog No. B9003S).
- Anti-H3K36me2 (Abcam Catalog No. ab9049)
- AlphaScreen Protein A Acceptor Beads (PerkinElmer Catalog No. 6760137)
- AlphaScreen Streptavidin Donor Beads (PerkinElmer Catalog No. 6760002)
- AlphaPlate-384™, Light gray (PerkinElmer Catalog No. 6005350)
- TopSeal™-A PLUS (PerkinElmer Catalog No. 6050185)
- Chaetocin (Tocris Bioscience Catalog No. 4504). Prepare stock solution at 10 mM in 100% DMSO.
- 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) – 3 hours and 10 min

1. Add 7.5 μl of **2X inhibitor** to the wells of the AlphaPlate-384
2. Add 3.75 μl of **4X NSD2**
3. Incubate for 10 min for inhibitor assay
4. Add 3.75 μl of recombinant nucleosomes **4X rNuc mix**
5. Cover plate with TopSeal-A PLUS and incubate at RT for 3 hours

B. Protein A Acceptor beads/antibody incubation (5 μl) – 1 hour

6. Prepare 5X mix of Acceptor beads / antibody mix in **Detection buffer**: dilute Protein A Acceptor beads to 100 μg/ml and add H3K36me2 antibody to a dilution of 5 μg/ml
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 μg/ml final concentration of anti-H3K36me2
8. Cover plate with TopSeal-A PLUS and incubate at RT for 1 hour

C. Streptavidin-conjugated Donor beads incubation (5 μl) – 1 hr

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** to each well (final concentration at 20 μg/ml)
11. Cover plate with TopSeal-A PLUS; incubate in the dark at RT for 1 hr
12. Read signal in AlphaScreen 384 mode with the EnVision Multilabel Plate Reader

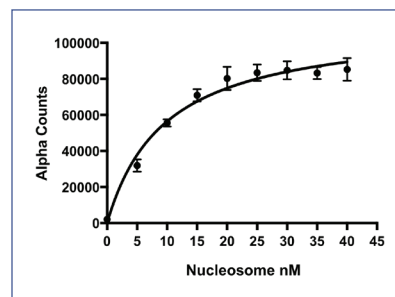


Figure 2: Substrate Titration. HMT reactions were performed by incubating NSD2 (100 nM) with human recombinant nucleosomes (rNuc, 0.5 nM to 40 nM) and SAM (100 μM) for 3 hours. A mixture of Acceptor beads and anti-H3K36me2 was added and incubated for 1 hour. Donor beads were finally added and signal was read after 1 hour. We used 10 nM of rNuc for subsequent IC50 and Z' factor assays.

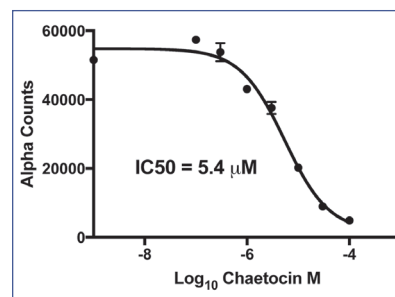


Figure 3: NSD2 inhibition using Chaetocin. For this study, Chaetocin (ranging from 100 nM to 100 μM) was pre-incubated for 10 min with NSD2 (100 nM). Enzymatic reactions were initiated by adding rNuc (10 nM) and SAM (100 μM) to the reaction cocktail. Enzymatic reactions and subsequent sample processing were performed as described in Figure 2.

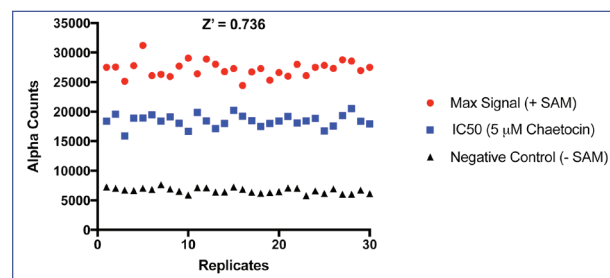


Figure 4: Z' Factor Determination. NSD2 (100 nM) and rNuc (10 nM) were incubated with or without Chaetocin (5 μM). Reactions lacking SAM were used as a negative control. Enzymatic reactions and subsequent sample processing were performed as described in Figure 3. Z' factor was calculated as previously described [4]. Z' Scores greater than 0.5 are suitable for high-throughput screening.

References

1. Kuo, A.J. *et al.* NSD2 links dimethylation of histone H3 at lysine 36 to oncogenic programming. *Mol Cell*, 2011. **44**(4): 609-20.
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