TECHNICAL NOTE #104

AlphaNuc™ NSD3 HMT Assay

A nucleosome-based approach for the discovery of NSD3 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 assay utilizing recombinant nucleosomes and the histone methyltransferase NSD3. This assay has a high Z' value and is suitable for adaptation to high-throughput screening.

Introduction

The histone lysine methyltransferase (HMT) NSD3 (also named WHSC1L1) catalyzes mono- and dimethylation of histone H3 at lysine 36 (H3K36me1 and -2) and is amplified in several cancers including breast, AML, and myelodysplastic syndrome [1]. Below is an optimized high-throughput NSD3 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 NSD3.

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 many histone targeting enzymes. Currently available biochemical 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 NSD3) prefers nucleosome substrates for activity *in vitro* and *in vivo* [2].

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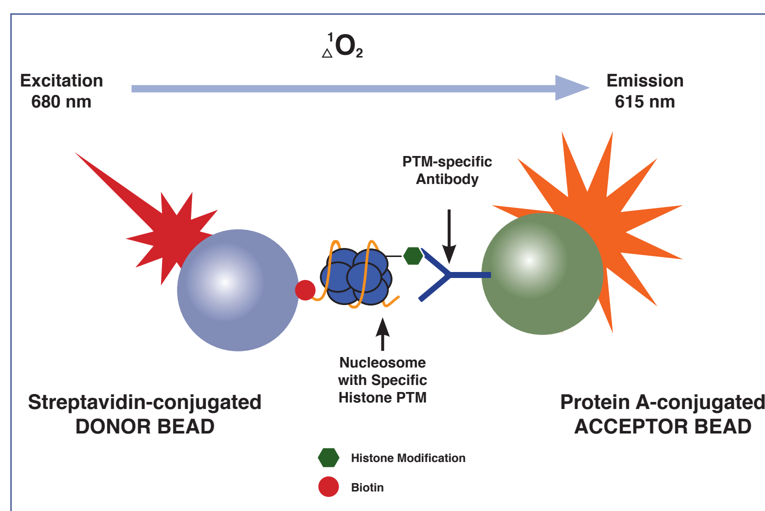

Figure 1: AlphaNuc Assay Principle. An AlphaLISA™-based assay has been developed to quantitate H3K36me2 levels that result from NSD3 activity toward recombinant nucleosomes. A Streptavidin-coated Donor Bead captures the nucleosome HMT substrate via its biotinylated DNA. When the Donor Bead is excited at 680 nm, a short-lived singlet oxygen molecule is released. This causes 615 nm emission from the Protein A-conjugated Acceptor Bead only when the associated anti-H3K36me2 is bound to its PTM target, thereby bringing the beads in close proximity. The intensity of 615 nm emission can therefore be used to quantiftate H3K36me2 levels resulting from NSD3 activity.

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Reagents and Materials Required for the Assay

- NSD3 Catalytic Domain, Recombinant Human (EpiCypher Catalog No. 15-1009)
- 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 $MgCl_2$, 1 mM DTT, 0.01% Tween-20, 0.1% BSA
- Detection buffer: (HMT buffer without DTT) 50 mM Tris-HCl, pH8.8, 5 mM $MgCl_2$, 0.01% Tween-20, 0.1% BSA

Standard Protocol

Note: Use **HMT buffer** to prepare HMT reaction solutions just before use

- 2X inhibitor mix
- 4X NSD3 mix: 400 nM (1X = 100 nM)
- 4X rNuc mix: rNuc - 40 nM; SAM - 400 µM

AlphaNuc Inhibitor Assay Procedure

Note: centrifuge the AlphaPlate (~200 rcf for 1 sec) after the addition of each reagent to maximize reproducibility

A. HMT reaction (total volume: 15 µl) – 1 hour and 10 min

1. Add 7.5 µl of **2X inhibitor mix** to the wells of the AlphaPlate-384
2. Add 3.75 µl of **4X NSD3**
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 1 hour

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 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 and 30 min

9. Prepare 5X Donor beads at 100 µg/ml in detection 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 and incubate in the dark at RT for 1 hour and 30 min
12. Read signal in AlphaScreen 384 mode with the Envison Multilabel Plate Reader

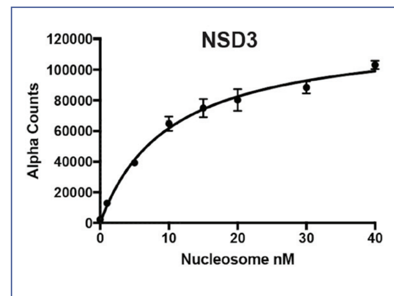

Figure 2: Substrate Titration. HMT reactions were performed by incubating NSD3 (100 nM) with increasing amounts of human recombinant nucleosomes (rNuc, 1 nM to 40 nM) and SAM (100 µM). A mixture of Acceptor beads and anti-H3K36me2 was added and incubated for 60 minutes. Donor beads were added and signal was read after 90 min. For subsequent IC_{50} and Z' factor assays, 10 nM of rNuc was selected.

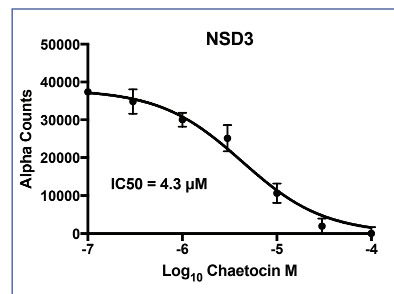

Figure 3: Inhibition Assay Validation. HMT inhibitor Chaetocin (100 nM to 100 µM) was pre-incubated with NSD3 (100 nM) for 10 minutes. Enzymatic reactions were initiated by adding 10 nM rNuc and 100 µM SAM to the reaction cocktail. Enzymatic reactions and subsequent sample processing were performed as in Figure 2.

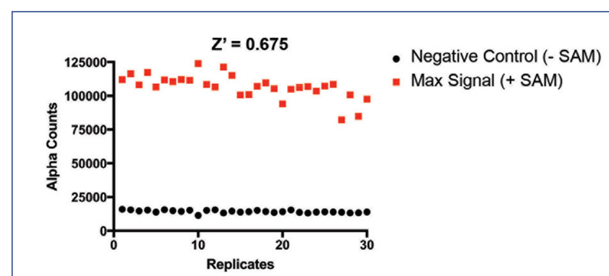

Figure 4: Z' Factor Determination. NSD3 (100 nM) and rNuc (10 nM) were incubated with (Max Signal) or without (Negative Control) SAM. Enzymatic reactions and subsequent sample processing were performed as in Figure 3. Z' factor was calculated as previously [3]. Z' Scores > 0.5 are suitable for high-throughput screening.

References

1. Wagner, E.J. and P.B. Carpenter, Understanding the language of Lys36 methylation at histone H3. *Nat Rev Mol Cell Biol*, 2012. **13**(2):115-26.
2. Allali-Hassani, A. et al. A Basic Post-SET Extension of NSDs Is Essential for Nucleosome Binding In Vitro. J Biomol Screen, 2014. 19(6): 928-935.
3. Zhang, J.H., T.D. Chung, and K.R. Oldenburg, A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen*, 1999. **4**(2): 67-73.

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