AF9 (YEATS Domain) and Acyl-Modified Histone Binding Assay: AlphaScreen technology

A protein interaction assay to enable the identification of novel AF9 inhibitors

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Overview: YEATS Domain Epigenetic Reader Proteins

Epigenetic reader proteins have gained widespread interest in the scientific community for their essential roles in cellular function and the potential of targeted drug development to treat diseases such as cancer. Recently, a novel family of reader proteins was identified that recognizes acetylated lysine (Kac) residues on histone tails through a highly conserved YEATS domain. In contrast to the widely studied Kac bromodomain reader, the YEATS domain also shows affinity for extended acyl modifications, particularly lysine crotonylation (Kcr) [1]. There are four YEATS domain containing proteins in humans: AF9, ENL, GAS41, and YEATS2. Due to the emerging role of these YEATS:acyl-histone interactions in cellular transcription and human disease [2], there is a compelling need to identify small molecule inhibitors of this novel class of epigenetic readers.

AF9-YEATS Domain

AF9 is one of the most frequently observed partners in MLL protein fusions that characterize mixed lineage leukemia (MLL), an aggressive pediatric blood cancer [2,3]. It is a component of multiple epigenetic complexes, including the Super Elongation Complex (SEC) and the DOT1L histone methyltransferase complex [4]. AF9 was initially reported to mediate transcriptional control by binding acetylated histones, particularly histone H3 acetylated at lysine 9 (H3K9ac) and H3K27ac [4]. However, it was subsequently found that the AF9-YEATS domain also binds histone lysine crotonyl marks to stimulate inflammatory gene expression [5].

A biochemical assay to quantify AF9 YEATS:acyl-histone interactions

This assay utilizes Perkin Elmer's AlphaScreen® Technology, enabling a quantitative assessment of the AF9 YEATS domain interaction with acetylated or crotonylated histone peptides (**Figure 1A**). The homogenous "mix-and-measure" nature, absence of wash steps, ability to miniaturize and automate the assay in multi-well plates, and robust nature of the approach make it ideal for high-throughput screening (HTS) applications.

AF9 exhibits strong binding to H3K9ac, H3K9cr, H3K27ac and H3K27cr (**Figure 1B**), offering a variety of acyl-histone peptides that can be used to develop a HTS assay. Further, AF9 demonstrated a binding preference for histone crotonylation over acetylation at the H3K27 site (**Figure 1B**). Here, we show representative assay development data for AF9 binding to H3K27cr peptides (**Figures 2-4**).

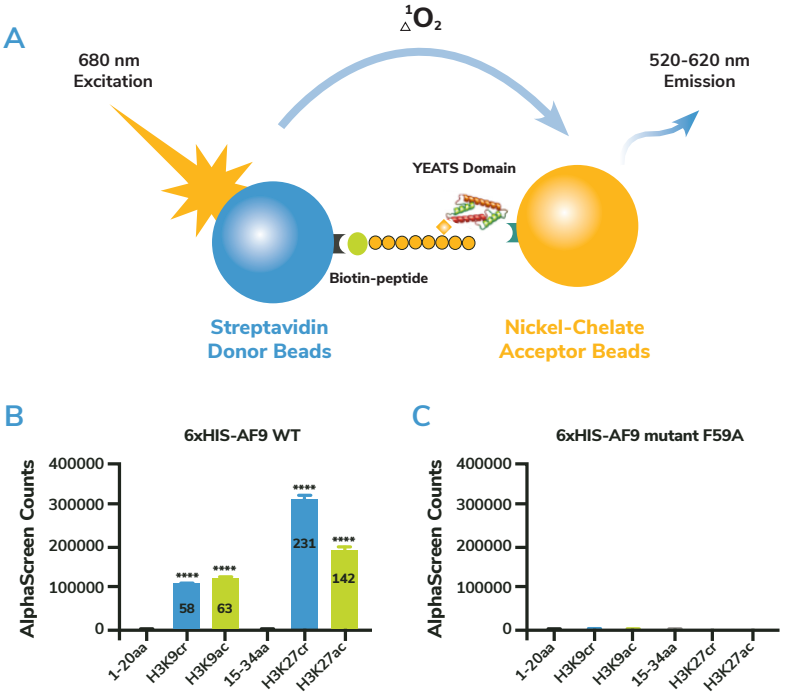

FIGURE 1: ASSAY PRINCIPLE. *An Amplified Luminescent Proximity Homogeneous Assay (Alpha) can be used to quantify histone binding to the YEATS reader domain. (A) A Streptavidin Donor Bead captures the biotinylated histone peptide and the 6xHIS-tagged YEATS protein is bound by the Nickel-chelate Acceptor Beads. When the Donor Bead is laser excited at 680 nm, a short-lived singlet oxygen molecule is released. When the Acceptor and Donor beads are brought within proximity by AF9 binding to peptide, the singlet oxygen causes emission from the Acceptor Beads. (B) This assay was optimized to quantify the interaction of the AF9 YEATS domain with acetylated (ac) or crotonylated (cr) histone peptides. AF9 binding to H3K9ac, H3K9cr, and H3K27cr, H3K27ac histone peptides is enriched over unmodified H3 peptide. Values within the bars indicate Z' values compared to unmodified H3 (****p<0.0001 compared to unmodified H3 using One-way ANOVA with Bonferroni post-test, n=9). (C) Point mutation of a conserved aromatic residue in the AF9 YEATS domain (F59A) previously identified to abolish binding to acyl lysines [4, 5] also abolishes the AlphaScreen signal.*

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TECHNICAL NOTE #108

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Reagents and Materials Required for the Assay:

NOTE: Store protein aliquots @ -80°C, avoid repeated freeze/thaw

REAGENTS/MATERIALS	VENDOR	CAT#
6xHIS AF9 YEATS Domain (Wild type)	EpiCypher®	15-0071
6xHIS AF9 YEATS domain (F59A)	EpiCypher®	15-0072
Biotin H3 1-20aa	EpiCypher®	12-0001
Biotin H3K9ac 1-20aa	EpiCypher®	12-0003
Biotin H3K9cr 1-20aa	EpiCypher®	12-0099
Biotin H3 15-34aa	EpiCypher®	12-0016
Biotin H3K27ac 15-34aa	EpiCypher®	12-0042
Biotin H3K27cr 15-34aa	EpiCypher®	12-0100
non-Biotin H3 1-20aa	EpiCypher®	12-8001
non-Biotin H3K9ac 1-20aa	EpiCypher®	12-8103
non-Biotin H3K9cr 1-20aa	EpiCypher®	12-8099
non-Biotin H3 15-34aa	EpiCypher®	12-8104
non-Biotin H3K27ac 15-34aa	EpiCypher®	12-8042
non-Biotin H3K27cr 15-34aa	EpiCypher®	12-8100
Nickel-chelate AlphaScreen Acceptor beads	PerkinElmer	6760619
Streptavidin AlphaScreen Donor beads	PerkinElmer	6760002
AlphaPlate-384™, Light Gray	PerkinElmer	6005350
TopSeal™-A Plus Film	PerkinElmer	6050185

Assay Buffer

50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% BSA;

add 0.1% NP-40 fresh *DO NOT ADD A REDUCING AGENT

Standard Protocol

NOTE: Briefly centrifuge plate (≤ 800 xg for ~10 seconds) after addition of each reagent to maximize reproducibility

**AlphaScreen Donor beads are light sensitive and should be handled only in subdued lighting.

1. Add 4 µL of 5X test inhibitors, competitor peptides, or vehicle control
2. Add 4 µL of 5X 6xHIS-AF9 protein (60 nM final)
3. Incubate 15 minutes at RT
4. Add 4 µL of 5X Biotin-H3 peptides (80 nM final)
5. Incubate 30 minutes at RT
6. Add 8 µL 2.5X Nickel-chealate Acceptor Beads (20 µg/mL final) and Streptavidin Donor Beads (20 µg/mL final) in the DARK**
7. Incubate 60 minutes at RT
8. Read using an Alpha Plate Reader: Ex 680 nm, Em 520-620 nm

References

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4. Li Y, Wen H, Xi Y, Tanaka K, Wang H, Peng D, Ren Y, Jin Q, Dent SY, Li W, Li H, Shi X. AF9 YEATS domain links histone acetylation to DOT1L-mediated H3K79 methylation. Cell. 2014 Oct 23;159(3):558-71.
5. Li Y, Sabari BR, Panchenko T, Wen H, Zhao D, Guan H, Wan L, Huang H, Tang Z, Zhao Y, Roeder RG, Shi X, Allis CD, Li H. Molecular Coupling of Histone Crotonylation and Active Transcription by AF9 YEATS Domain. Mol Cell. 2016 Apr 21;62(2):181-93.
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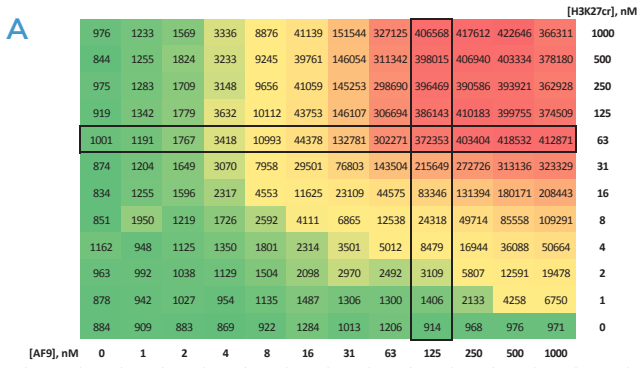

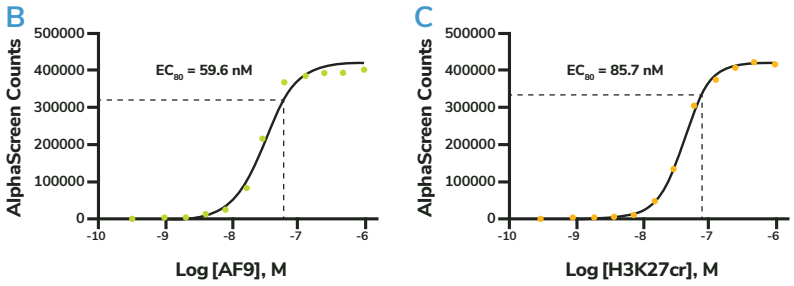

FIGURE 2: PROTEIN TITRATIONS. (A) *6xHIS-AF9 (horizontal axis) was titrated against Biotin-H3K27cr peptide (vertical axis). Titrations of each binding partner from 1000 nM down to 1 nM (1:2 dilutions) determined the optimal concentrations for assay development. AlphaScreen signal is shown and colored by relative intensity. The boxed regions outline the AF9/histone titrations graphed below. Nonlinear regression analysis identified the concentration at which 80% response is achieved (EC80) for AF9 titrated against 63 nM H3K27cr (B) and H3K27cr titrated against 125 nM AF9 (C). Based on these findings, 60 nM AF9 and 80 nM H3K27cr peptide was selected for subsequent experiments.*

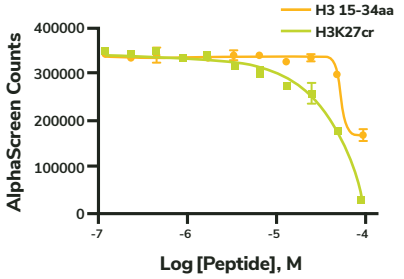

FIGURE 3: COMPETITION ASSAY. *Non-biotinylated histone competitor peptides (12-point dose-response in 1:2 titrations) and 6xHIS AF9 were pre-incubated for 15 minutes. Biotinylated peptides were added and incubated for 30 minutes. Finally, Nickel-chelate Acceptor and Streptavidin Donor beads were added and incubated for 60 minutes. After the incubation, AlphaScreen signal was read with 680 nm excitation, and measuring emission at 570 nm with a 100 nm bandwidth filter. Competition with the non-biotinylated H3K27cr peptide inhibited the AlphaScreen signal in the low micromolar range, consistent with the binding affinity of AF9 to acyl-modified histones [4, 5].*

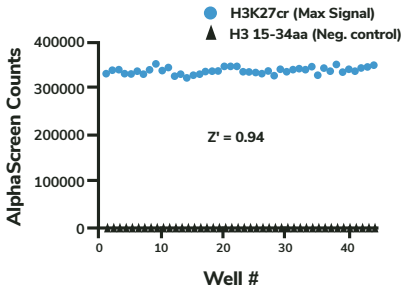

FIGURE 4: Z' FACTOR DETERMINATION. *6xHIS AF9 (60 nM) was incubated with Biotin-unmodified H3 peptide (80 nM) as a negative control. Incubation with Biotin-H3K27cr (80 nM) determined the maximum signal. A Z' factor of 0.94 was calculated as described [6], indicating the assay is suitable for high throughput screening (Z'>0.5).*

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