Development of Homogeneous Proximity Assays for JMJD2A/2C Histone Demethylases

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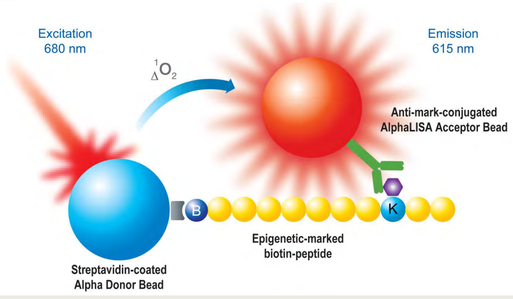
1 Summary

In eukaryotes, the covalent modification of histones has a crucial role in chromatin architecture and plays an important part in a plethora of cellular processes, from chromatin remodeling and transcriptional regulation, to DNA repair and cell cycle control. While histone acetylation is generally associated to an open chromatin state and transcriptional activation, methylation of histones has been related to either activating or repressive functions. It is then clear that precise regulation of these events is critical for appropriate cellular development and function. In this regard, the abnormal level of several histone-modifying activities has been linked to pathological states, such as cancer or neurological disorders. Therefore, developing fast and robust assays for studying these enzymes could significantly accelerate research and the discovery process of clinically relevant compounds.

Here, we detail the development of enzymatic assays for histone H3K9 demethylases using two homogeneous non-radioactive technologies: time-resolved Förster resonance energy transfer, and a bead-based chemiluminescent proximity assay. To this end, a peptide substrate derived from the N-terminus of histone H3 (trimethylated on Lys 9) was used to analyze the catalytic activity of JMJD2A and JMJD2C demethylases. Optimized conditions were highly suitable for screening, with typical enzyme concentrations ranging from 1 to 10 nM, and methyl-peptide substrate between 100 and 300 nM. Required concentrations of alpha-ketoglutarate (2OG) ranged from 1 to 5 µM, allowing sensitive detection of compounds which may compete with this cofactor. Complete assays could be carried out in less than 4 hours, and were further validated with the use of a known inhibitor, 2,4-PDCA. In brief, results shown herein will enable simple, robust, non-radioactive screenings of compound libraries for modulators of histone H3K9 demethylases.

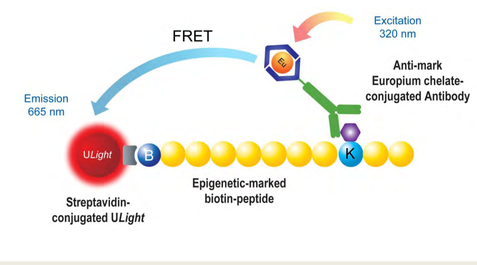
2 Detection Principle

AlphaLISA®



The antibody-conjugated Acceptor bead binds specifically to the modified histone H3 peptide, while the biotin group attached to it brings the Streptavidin Donor bead into proximity, allowing generation of AlphaLISA signal.

LANCE® *Ultra*



The Europium-labeled antibody specifically recognizes the modified histone H3 peptide, while the U*Light*™-Streptavidin binds to the biotin group attached to it, allowing TR-FRET to occur.

3 Methods

Standard protocol for enzymatic reactions :

1. 2.5 µL of corresponding assay buffer ± inhibitor (4X)
2. 5 µL of enzyme (2X)
3. 2.5 µL biotinylated H3K9me3 peptide/2OG/Fe(II)/ascorbate mix *(4X)
4. Incubate at 23°C for the indicated time
5. Proceed to detection of reaction products in AlphaLISA or LANCE *Ultra* (i.e., step #2 of either detection protocol)

* Final concentrations in the 10 µL enzymatic reaction were: 5 µM and 100 µM, for Fe(II) and ascorbate, respectively, and 100 nM or 300 nM of biotinylated H3K9me3 peptide substrate, respectively, for the JMJD2A or JMJD2C assay.

Notes:

- JMJD2A AlphaLISA enzymatic assays were stopped by the addition of Acceptor beads prepared in Epigenetics Buffer 1.
- JMJD2C LANCE *Ultra* enzymatic assays were stopped by the addition of the Eu-antibody/U*Light*-SA mix containing 1 mM EDTA.

AlphaLISA Detection Protocol :

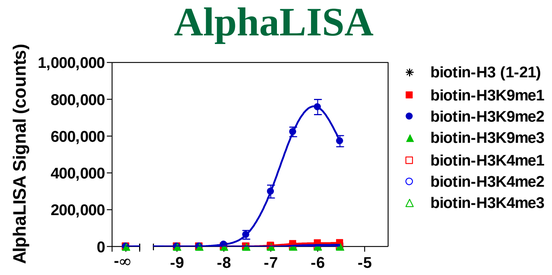
1. 10 µL containing the enzymatic reaction (for antibody specificity analysis, this volume was replaced by the biotin-peptides diluted in 50 mM Tris-HCl pH 8.0)
2. 5 µL Acceptor Beads 5X (final 20 µg/mL), diluted in AlphaLISA 1X Epigenetics Buffer 1
3. Incubate at 23°C for 60 min
4. 10 µL Streptavidin Donor Beads 2.5X (final 20 µg/mL), diluted in AlphaLISA 1X Epigenetics Buffer 1
5. Incubate at 23°C for 30 min
6. Read signal on EnVision® reader using Alpha mode

LANCE *Ultra* Detection Protocol :

1. 10 µL containing the enzymatic reaction (for antibody specificity analysis, this volume was replaced by the biotin-peptides diluted in 50 mM Tris-HCl pH 8.0)
2. 10 µL Eu-antibody/U*Light*-SA/EDTA 2X mix (final 2 nM/50 nM/1 mM), in LANCE 1X Detection Buffer
3. Incubate at 23°C for 60 min
4. Read signal on EnVision reader using LANCE TR-FRET mode

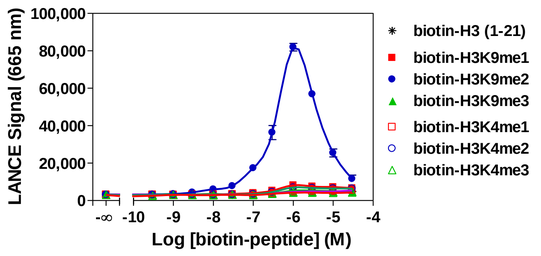
4 Anti-H3K9me2 Antibody Specificity

AlphaLISA



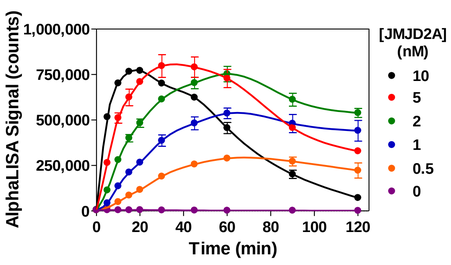
Peptide detection assay employing anti-H3K9me2 Acceptor beads. Titration curves were performed using biotinylated peptides bearing different methylation marks, as indicated. Note that only di-methylated lysine 9 is detected by the anti-H3K9me2 Acceptor beads.

LANCE *Ultra*

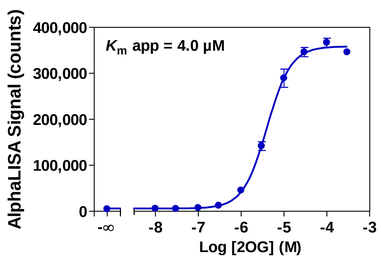


Peptide detection assay employing Europium-labeled anti-H3K9me2 antibody. Titration curves were performed using biotinylated peptides bearing different methylation marks, as indicated. Note that only di-methylated lysine 9 is detected by the Eu-anti-H3K9me2 antibody.

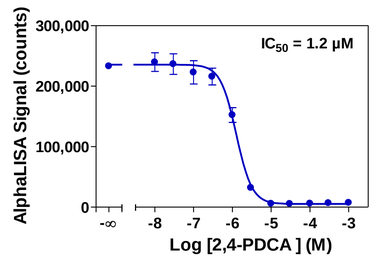
5 JMJD2A AlphaLISA assay



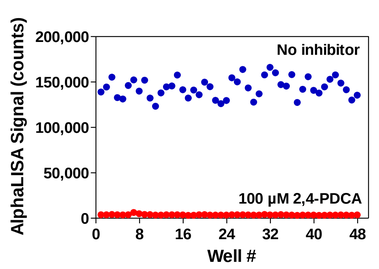
Enzymatic Progress Curves. Assays were performed by incubating increasing concentrations of JMJD2A with the biotinylated H3K9me3 peptide substrate, plus 50 µM 2OG. Signal decrease observed at higher enzyme concentration or reaction time is due to the generation of mono-methylated and unmethylated lysine 9 peptides.



2OG Titration.
2OG was added to 1 nM JMJD2A and the biotinylated H3K9me3 peptide substrate, and reactions were incubated for 30 min.



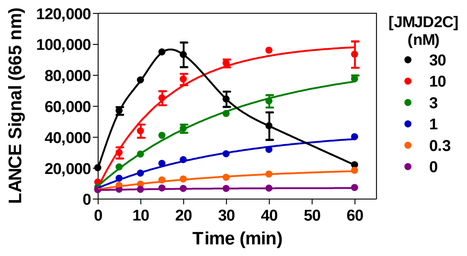
Enzyme Inhibition.
2,4-PDCA, at different concentrations, was pre-incubated with 1 nM JMJD2A for 10 min. Enzymatic reactions were initiated by the addition of the biotinylated H3K9me3 peptide substrate plus 5 µM 2OG, and incubated for 30 min. Enzymatic reactions contain 2% DMSO.



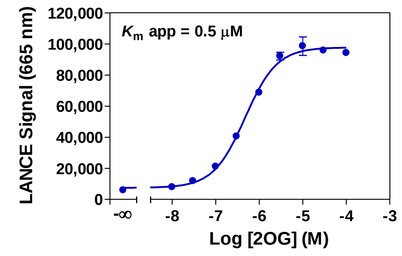
Z' = 0.76
S/B = 43

Z' factor Determination.
JMJD2A (1 nM) was pre-incubated with or without 100 µM 2,4-PDCA for 10 min. Enzymatic reactions were initiated by the addition of the biotinylated H3K9me3 peptide substrate plus 5 µM 2OG, and incubated for 30 min. Enzymatic reactions contain 2% DMSO.

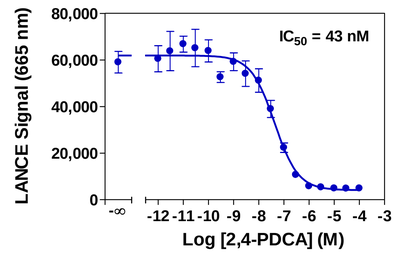
6 JMJD2C LANCE *Ultra* assay



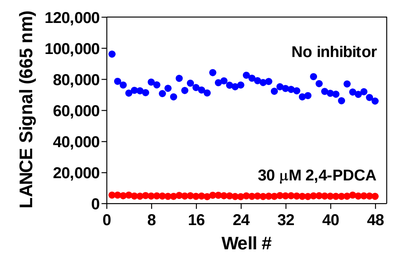
Enzymatic Progress Curves. Experiments were performed by incubating increasing concentrations of JMJD2C with the biotinylated H3K9me3 peptide substrate plus 50 µM 2OG. Signal decrease observed at higher enzyme concentration or reaction time is due to the generation of mono-methylated and unmethylated lysine 9 peptides.



2OG Titration.
2OG was added to 10 nM JMJD2C and the biotinylated H3K9me3 peptide substrate, and reactions were incubated for 30 min.



Enzyme Inhibition.
2,4-PDCA, at different concentrations, was pre-incubated with 10 nM JMJD2C for 10 min. Enzymatic reactions were initiated by the addition of the biotinylated H3K9me3 peptide substrate plus 1 µM 2OG, and incubated for 30 min. Enzymatic reactions contain 2% DMSO.



Z' = 0.76
S/B = 16

Z' factor Determination.
JMJD2C (10 nM) was pre-incubated with or without 30 µM 2,4-PDCA for 10 min. Enzymatic reactions were initiated by the addition of the biotinylated H3K9me3 peptide substrate plus 1 µM 2OG, and incubated for 30 min. Enzymatic reactions contain 2% DMSO.

7 Materials

Common materials:

- White Optiplate™-384 microplates (PerkinElmer #6007299)
- TopSeal™-A film (PerkinElmer #6005185)
- EnVision® Multilabel Plate Reader (PerkinElmer #2103)

AlphaLISA JMJD2A assay:

- Anti-methyl-Histone H3 Lysine 9 (H3K9me2) Acceptor Beads (PerkinElmer #AL117)
- Alpha Streptavidin Donor Beads (PerkinElmer #6760002)
- Recombinant human JMJD2A (BPS Bioscience #50103)
- AlphaLISA® 5X Epigenetics Buffer 1 Kit (PerkinElmer #AL008)
- Enzymatic assay buffer: 50 mM Tris-HCl pH 7.5, 0.01% Tween-20, 0.1 % BSA

LANCE *Ultra* JMJD2C assay:

- Europium-anti-methyl-Histone H3 Lysine 9 (H3K9me2) (PerkinElmer #TRF0403)
- LANCE® *Ultra* U*Light*™-Streptavidin (PerkinElmer #TRF0102)
- Recombinant human JMJD2C (BPS Bioscience #50105)
- 0.5 M EDTA pH 8.0 (Invitrogen #15575-020)
- LANCE® Detection Buffer 10X (PerkinElmer #CR97-100)
- Enzymatic assay buffer: 50 mM Tris-HCl pH 7.5, 0.01% Tween-20, 0.01 % BSA

For peptide detection, and JMJD2A & JMJD2C enzymatic assays:

- Histone H3 (1-21) peptide, biotinylated (AnaSpec #61702)
- Histone H3 (1-21) lysine 4 mono-methylated peptide, biotinylated (AnaSpec #64355)
- Histone H3 (1-21) lysine 4 di-methylated peptide, biotinylated (AnaSpec #64356)
- Histone H3 (1-21) lysine 4 tri-methylated peptide, biotinylated (AnaSpec #64357)
- Histone H3 (1-21) lysine 9 mono-methylated peptide, biotinylated (AnaSpec #64358)
- Histone H3 (1-21) lysine 9 di-methylated peptide, biotinylated (AnaSpec #64359)
- Histone H3 (1-21) lysine 9 tri-methylated peptide, biotinylated (AnaSpec #64360)
- α-Ketoglutaric acid potassium salt (2OG) (Sigma #K2000)
- (+) Sodium L-ascorbate (Sigma #11140)
- Ammonium iron(II) sulfate hexahydrate (Fe(II)) (Sigma #215406)
- 2,4-Pyridinedicarboxylic acid (2,4-PDCA) (Sigma #P63395)

8 Summary

- Homogeneous, non-radioactive demethylase assays targeting histone H3 lysine 9 were developed for AlphaLISA and LANCE *Ultra* platforms in 384-well plate format.
- Both assay platforms employ an H3-derived biotin-peptide trimethylated on lysine 9 as substrate, paired with anti-H3K9me2 specific antibody conjugates.
- Assay robustness for high throughput screening (HTS) was demonstrated, with Z′-factor values >0.75 (overnight reading yielded Z′-factors >0.74).
- A comprehensive description of these assays and their optimization is available online. Please visit our website at www.perkinelmer.com/epigenetics.