Development of High-Throughput Assays to Study Histone H3K4 Methyltransferases & H3K9 Methyl- and Acetyltransferases

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1 Abstract

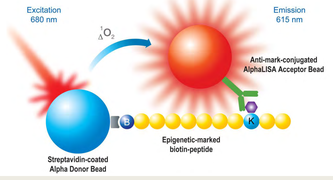
Post-translational modifications of histone proteins play an important part in a wide array of cellular processes including regulation of gene transcription, DNA repair, cell cycle, and metabolism control. For instance, transcriptional activation is associated with acetylation of Histone H3 on residues K9 and K14, and methylation on K4. On the other hand, gene repression has been linked to H3K9, H3K27, and H4K20 methylation. In this regard, the activity of several histone modifying enzymes (e.g., acetyl and methyltransferases, deacetylases, demethylases) have been linked to diseases such as cancer and neurological disorders. Thus, the development of simple and reliable assays for these enzymes could facilitate the identification of new modulatory compounds, eventually leading to the development of clinically relevant drugs.

In this work, we describe the development of enzymatic assays to perform screenings for modulators of H3K9 acetyltransferases, and H3K4 and H3K9 methyltransferases, using homogeneous proximity assays based on two non-radioactive technologies: LANCE® *Ultra* time-resolved fluorescence energy transfer and AlphaLISA® bead-based chemiluminescent assays. To this end, we used as substrate a biotinylated peptide derived from the N-terminus of Histone H3 (residues 1-21) to assay acetyltransferase p300 and methyltransferases G9a and SET7/9. In all cases, we found conditions highly suitable for screening: enzyme concentrations between 0.05 to 5 nM and substrate concentrations between 50 and 500 nM. Acetyl-CoA or S-adenosylmethionine (SAM) concentrations were in the 0.1-20 µM range, enabling sensitive screenings for compounds competing with these cofactors. Entire assays could be carried out in less than 4 hours, with order-of-potency for known inhibitors (anacardic acid, sinefungin, SAH) in good correlation with published literature.

The results presented herein demonstrate how to optimally assess p300, G9a, and SET7/9 activities *in vitro* using a biotinylated H3-derived peptide in non-radioactive, homogeneous assay formats. This could enable simple and fast screenings of compound libraries, facilitating the discovery of novel modulators of histone modifying enzymes.

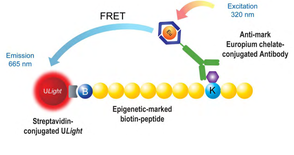
2 Assay Principle

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.

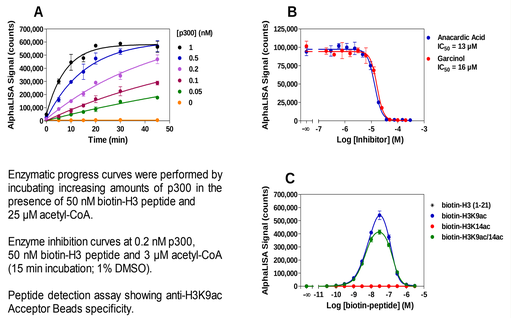
LANCE *Ultra*



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 AlphaLISA Assays

p300 acetyltransferase



A. Enzymatic progress curves were performed by incubating increasing amounts of p300 in the presence of 50 nM biotin-H3 peptide and 25 µM acetyl-CoA.

B. Enzyme inhibition curves at 0.2 nM p300, 50 nM biotin-H3 peptide and 3 µM acetyl-CoA (15 min incubation; 1% DMSO).

C. Peptide detection assay showing anti-H3K9ac Acceptor Beads specificity.

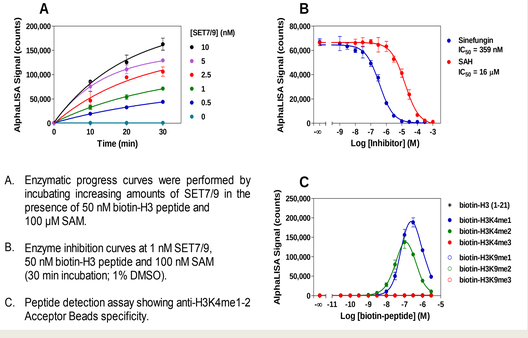
G9a methyltransferase

A. Enzymatic progress curves were performed by incubating increasing amounts of G9a in the presence of 100 nM biotin-H3 peptide and 100 µM SAM. Signal decrease observed at longer reaction times is due to the generation of H3K9me3 peptide, which is not detected by the Acceptor beads.

B. Enzyme inhibition curves at 0.05 nM G9a, 100 nM biotin-H3 peptide and 15 µM SAM (30 min incubation; 2% DMSO).

C. Peptide detection assay showing anti-H3K9me2 Acceptor Beads specificity.

SET7/9 methyltransferase



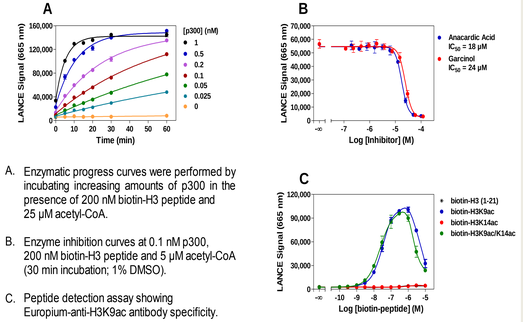
A. Enzymatic progress curves were performed by incubating increasing amounts of SET7/9 in the presence of 50 nM biotin-H3 peptide and 100 µM SAM.

B. Enzyme inhibition curves at 1 nM SET7/9, 50 nM biotin-H3 peptide and 100 nM SAM (30 min incubation; 1% DMSO).

C. Peptide detection assay showing anti-H3K4me1-2 Acceptor Beads specificity.

4 LANCE *Ultra* Assays

p300 acetyltransferase



A. Enzymatic progress curves were performed by incubating increasing amounts of p300 in the presence of 200 nM biotin-H3 peptide and 25 µM acetyl-CoA.

B. Enzyme inhibition curves at 0.1 nM p300, 200 nM biotin-H3 peptide and 5 µM acetyl-CoA (30 min incubation; 1% DMSO).

C. Peptide detection assay showing Europium-anti-H3K9ac antibody specificity.

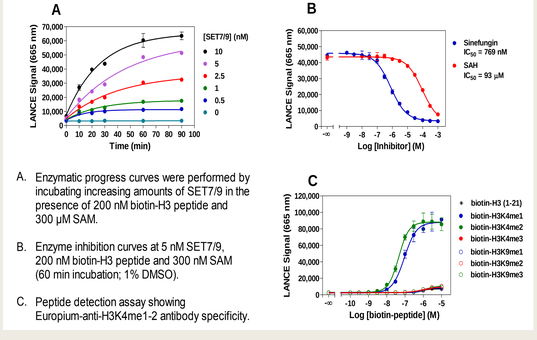
G9a methyltransferase

A. Enzymatic progress curves were performed by incubating increasing amounts of G9a in the presence of 500 nM biotin-H3 peptide and 300 µM SAM. Signal decrease observed at longer reaction times is due to the generation of H3K9me3 peptide, which is not detected by the Eu-Antibody.

B. Enzyme inhibition curves at 0.15 nM G9a, 500 nM biotin-H3 peptide and 20 µM SAM (30 min incubation; 2% DMSO).

C. Peptide detection assay showing Europium-anti-H3K9me2 antibody specificity.

SET7/9 methyltransferase



A. Enzymatic progress curves were performed by incubating increasing amounts of SET7/9 in the presence of 200 nM biotin-H3 peptide and 300 µM SAM.

B. Enzyme inhibition curves at 5 nM SET7/9, 200 nM biotin-H3 peptide and 300 nM SAM (60 min incubation; 1% DMSO).

C. Peptide detection assay showing Europium-anti-H3K4me1-2 antibody specificity.

5 Materials

Common materials (PKI cat#)

- AlphaLISA 5X Epigenetics Buffer 1 Kit (AL008)
- Alpha Streptavidin Donor Beads (6760002)
- LANCE Detection Buffer 10X (CR97-100)
- U*Light*™-Streptavidin (TRF0102)
- White Optiplate™-384 microplates (6007299)
- TopSeal™-A film (6005185)
- EnVision® Multilabel Plate Reader
- Histone H3 (1-21) peptide, biotinylated (AnaSpec #61702)

p300 assay

- Anti-H3K9ac Acceptor Beads (PKI #AL114)
- Eu-anti-H3K9ac antibody (PKI #TRF0400)
- p300 (Enzo #BML-SE451)
- Acetyl-CoA (Sigma #A2056)
- Trichostatin A (TSA) (Sigma #T8552)
- Anacardic acid (Calbiochem #172050)
- Garcinol (Sigma #G5173)
- Biotin-peptides (Anaspec): H3K9ac #64361, H3K14ac #64362, H3K9ac/K14ac #64363.
- Enzymatic assay buffer: 50 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 0.01% Tween-20, 0.01% BSA, 330 nM TSA

G9a assay

- Anti-H3K9me2 Acceptor Beads (PKI #AL117)
- Eu-anti-H3K9me2 antibody (PKI #TRF0403)
- G9a (BPS Bioscience #51001)
- Poly-L-lysine 0.1% w/v (Sigma #P8920)
- BIX-01294 (Sigma #B9311)
- Enzymatic assay buffer: 50 mM Tris-HCl pH 9.0, 50 mM NaCl, 1 mM DTT, 0.01% Tween-20

SET7/9 assay

- Anti-H3K4me1-2 Acceptor Beads (PKI #AL116)
- Eu-anti-H3K4me1-2 antibody (PKI #TRF0402)
- SET7/9 (Enzo #ALX-201-178-C100)
- Enzymatic assay buffer: 50 mM Tris-HCl pH 8.8, 5 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20

For both G9a & SET7/9 assays

- Biotin-peptides (Anaspec): H3K4me1 #64355, H3K4me2 #64356, H4Kme3 #64357, H3K9me1 #64358, H3K9me2 #64359, H3K9me3 #64360
- S-(5′-Adenosyl)-L-methionine chloride (SAM; Sigma #A7007)
- S-(5′-Adenosyl)-L-homocysteine (SAH; Sigma #A9384)
- Sinefungin (Sigma #S8559)

6 Methods

AlphaLISA Detection Assay

1. 10 µL of biotin-peptide 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 21°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 21°C for 30 min
6. Read signal on EnVision reader (Alpha Mode)

LANCE *Ultra* Detection Assay

1. 10 µL of biotin-peptide in 50 mM Tris-HCl pH 8.0
2. 10 µL Eu-antibody / U*Light*-SA 2X mix (final 2 nM/ 50 nM), diluted in LANCE 1X Detection Buffer
3. Incubate at 21°C for 60 min
4. Read signal on EnVision reader (LANCE TR-FRET mode)

Standard protocol for enzymatic reactions

1. 5 µL of corresponding assay buffer (± 2X inhibitors)
2. 2.5 µL of enzyme (4X)
3. 2.5 µL biotinylated histone H3 peptide/co-substrate mix (4X)
4. Incubate at 21°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)

Notes

- p300 enzymatic assays were stopped by the addition of anacardic acid (50 µM and 30 µM in final volume for AlphaLISA and LANCE *Ultra*, respectively). For AlphaLISA assays, anacardic acid was pre-mixed with the Acceptor beads whereas, for LANCE *Ultra* assays, it was added directly to the well before the addition of the Eu-antibody/U*Light*-SA mix.
- G9a enzymatic AlphaLISA assays were stopped by the addition of the Acceptor beads diluted in AlphaLISA 1X Epigenetics Buffer 1. G9a enzymatic LANCE *Ultra* assays were stopped by adding the Eu-antibody/U*Light*-SA mix diluted in LANCE 1X Detection Buffer supplemented with 0.0002% poly-L-lysine (final 0.0001% in 20 µL).
- SET7/9 enzymatic assays (Alpha and LANCE *Ultra*) were found to be stopped by the addition of the detection reagents diluted in the corresponding detection buffers.

7 Summary

- Non-rad, homogeneous immunoassays for monitoring histone H3 modification *in vitro* were developed for AlphaLISA and LANCE *Ultra* platforms.
- All assays employ an H3-derived biotin-peptide substrate, paired with modification-specific, antibody conjugates.
- H3K9 acetylation, and H3K4 and H3K9 methylation assays were successfully optimized in all-in-one-well format, and their robustness for high throughput screening (HTS) demonstrated, with Z′-factor values ranging from 0.7 to 0.9 (not shown).
- A comprehensive description of these assays and their optimization is available on our website at www.perkinelmer.com/epigenetics.