High-Throughput, No-Wash Cellular Assays to Monitor Histone H3 Modifications

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1 Introduction

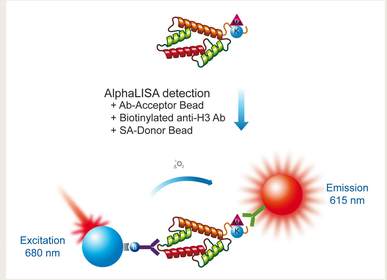
Histone proteins are an integral part of DNA packaging into chromatin, a dynamic process partly regulated by post-translation modification on histone N-terminal tails. Aberrant histone acetylation (ac) or methylation (me) levels is associated with a number of diseases. For example histone deacetylases (HDACs and sirtuins) regulate expression of many genes involved in neurodegeneration and can be aberrantly expressed in different tumors. In addition, deregulations of the methyl marks H3K4me3 and H3K27me3 are found associated with the development of several types of cancer.

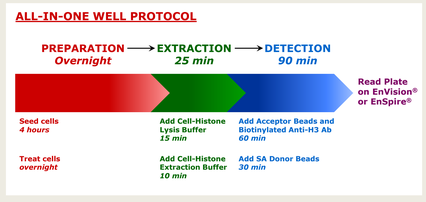
Homogeneous and highly specific AlphaLISA® assays were developed to monitor the histone marks H3K4me2, H3K9ac, H3K27ac and H3K27me3 in high-throughput *in cyto* assays using an all-in-one well histone extraction protocol. These assays, which require only the addition of lysis, histone extraction and detection buffers, were validated using adherent (HeLa and HEK-293) as well as suspension (Jurkat) cell lines.

All four marks could be detected in cell titration experiments, ranging from 100 to 15,000 cells per well in a 384-well plate. In addition, increases in three different marks levels (H3K4me2, H3K9ac and H3K27ac) were monitored following overnight treatment of cells with the promiscuous histone deacetylase inhibitors sodium butyrate and trichostatin A. These increases were corroborated by Western blot analysis using the same antibodies as in the AlphaLISA detection assay. Measurement of H3K27me3 levels were performed in two B cell lymphoma suspension cell lines: OCI-LY-19 and SU-DHL-6. OCI-LY-19 express wild type EZH2 methyltransferase while SU-DHL-6 cells bear a heterozygous mutation on EZH2 that alters its substrate selectivity, resulting in increased H3K27me3 levels. These novel cell-based assays showed suitability for HTS protocols as demonstrated by Z′ factors superior to 0.6 with all four marks measured.

2 Assay Principles

The AlphaLISA technology allows performing no-wash homogeneous proximity immunoassays using Alpha Donor and AlphaLISA Acceptor beads. Cells (10 µL) are seeded in 384-well plates and treated overnight with 5 µL HDAC inhibitor or another epigenetic modulator. After treatment, histones are extracted directly in the culture wells by adding 5 µL Lysis buffer and 10 µL Extraction buffer. Histones bearing the mark of interest are detected by the addition of 10 µL biotinylated anti-Histone H3 (C-terminus) antibody and AlphaLISA Acceptor beads conjugated to an antibody (Ab) specific to the mark. The biotinylated antibody is then captured by the addition of 10 µL Streptavidin (SA) Donor beads, bringing the two beads into proximity. Upon laser irradiation of the Donor beads at 680 nm, short-lived singlet oxygen molecules produced by the Donor beads can reach the Acceptor beads in proximity to generate an amplified chemiluminescent signal measured at 615 nm.





3 Materials

Cell lines
- HeLa (# CCL-2.2™) and Jurkat (# TIB-152™) from ATCC
- HEK 293FT cell line (# R700-07) from Life Technologies Corporation
- B cell lymphoma OCI-LY-19 (# ACC 528) and SU-DHL-6 (# ACC 572) from DSMZ - GmbH

AlphaLISA assay detection reagents, consumables and instrument
- CulturPlate-384™, white opaque with lid (PerkinElmer, Inc., # 6007680)
- AlphaLISA Epigenetic Cellular Detection Kits contain: AlphaLISA Cell-Histone™ Lysis buffer, AlphaLISA Cell-Histone Extraction buffer, AlphaLISA Cell-Histone Detection buffer (10X), AlphaLISA anti-mark Acceptor beads, Alpha SA Donor beads and AlphaLISA Biotinylated Anti-Histone H3 (C-terminus) Antibody.
  - AL714: Acetyl-Histone H3 Lysine 9 (H3K9ac)
  - AL716: Di-Methyl-Histone H3 Lysine 4 (H3K4me2)
  - AL720: Acetyl-Histone H3 Lysine 27 (H3K27ac)
  - AL722: Tri-Methyl-Histone H3 Lysine 27 (H3K27me3)
- TopSeal™-A Adhesive Sealing films (# 6050195) and EnVision® Multilabel Alpha Reader were from PerkinElmer, Inc.

Modified Histone-H3 Peptides Histone H3-derived peptides bearing various epigenetic marks are all available from AnaSpec, Inc, except H3K4ac (1-21) peptide which was custom synthesized.

Inhibitors or modulators HDAC inhibitors (HDACi) Trichostatin A (TSA, cat# T8552), and sodium butyrate (NaB, cat# B5887) were from Sigma-Aldrich, Inc.

Western blot reagents
- Secondary antibodies: Anti-Rabbit IgG (Goat), Alkaline Phosphatase Conjugate (#NEF814001EA), Anti-Mouse IgG (Goat), Alkaline Phosphatase Conjugate (#NEF824001EA) were from PerkinElmer, Inc.
- Detection reagent: Western Lightning™ CDP-Star® with Nitro-Block II™ Enhancer (PerkinElmer, Inc, # NEL616001KT)

4 Methods

Detection of specific mark levels and their modulation Mark-specific AlphaLISA detection was performed on cells seeded in 384-well CulturPlates at densities ranging from 100 to 15,000 cells per well. Cells were either treated overnight with 20 mM NaB or left untreated.

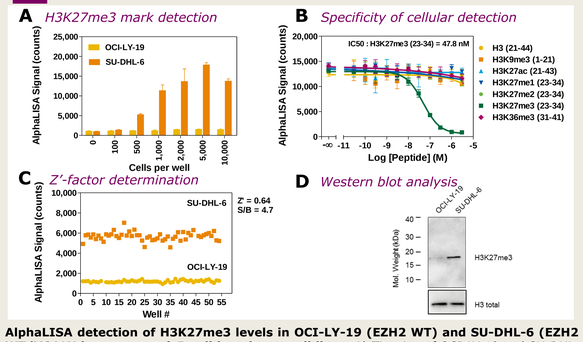
Specificity of cellular detection Specificity of mark-specific cellular detection was determined by competing the Acceptor bead capture of specific histone mark using short histone H3-derived peptides bearing various epigenetic marks. HeLa cells treated overnight with 20 mM NaB were used for determining specificity of H3K4me2, H3K9ac and K3K27ac assays. SU-DHL-6 cells were used for H3K27me3. Peptides diluted serially were added to the wells just before the addition of the AlphaLISA detection reagents.

Z′-factor determination For H3K4me2, H3K9ac and H3K27ac, Z′-factor values[1] were determined by comparing the AlphaLISA signal of HeLa cells untreated and treated with 20 mM NaB. For H3K27me3, AlphaLISA signals of OCI-LY-19 and SU-DHL-6 cell lines were compared. Cells were incubated overnight in 48 wells/condition in medium containing 0.5% DMSO.

Inhibition curves Inhibition of HDAC activity was determined using an overnight treatment of HeLa cells with increasing concentrations of two non-selective HDAC inhibitors, TSA and NaB, in medium containing 0.5% DMSO.

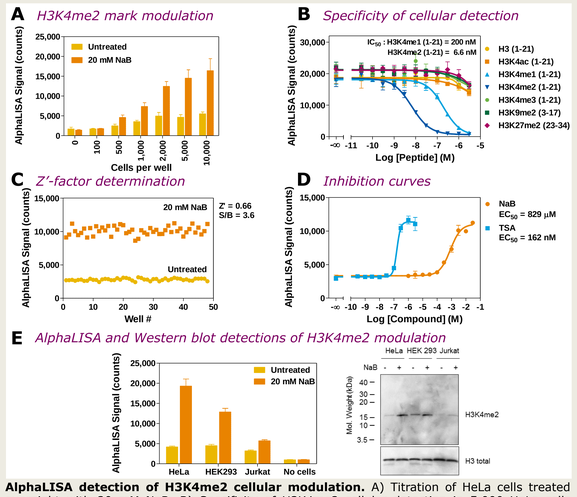
AlphaLISA and Western blot parallel detection of specific histone marks H3K4me2, H3K9ac and H3K27ac mark modulation by 20 mM NaB in HeLa, HEK 293FT and Jurkat cells, and H3K27me3 mark level in OCI-LY-19 and SU-DHL-6 cells, were analyzed in parallel by AlphaLISA detection and Western blotting. For AlphaLISA assays, samples were normalized using 2,000 cells per well. For Western blot analysis, cellular extracts were normalized for total histone H3. For Western blot, cell lysates were separated by SDS-PAGE on a 10%-20% gradient gel. Following transfer to nitrocellulose, the mark of interest was detected using the same antibody present on the Acceptor beads. For total H3, an antibody recognizing a histone H3 C-terminal epitope was used.

5 H3K27me3 Cellular Detection



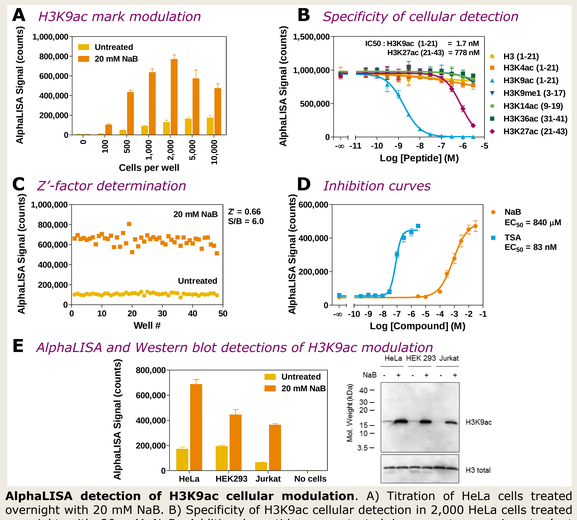
AlphaLISA detection of H3K27me3 levels in OCI-LY-19 (EZH2 WT) and SU-DHL-6 (EZH2 WT/Y641N heterozygote) B cell lymphoma cell lines. A) Titration of OCI-LY-19 and SU-DHL-6 cells. B) Specificity of H3K27me3 cellular detection in 5,000 SU-DHL-6 cells. Additional peptides were tested in separate experiments (not shown). Only the H3K27me3 peptide competed with high affinity for the interaction between the Acceptor beads and histone proteins. C) Z′-factor value determination using 5,000 OCI-LY-19 and 5,000 SU-DHL-6 cells/well. D) Western blot analysis corroborates the AlphaLISA detection of H3K27me3 in OCI-LY-19 and SU-DHL-6 cells.

6 H3K4me2 Cellular Detection



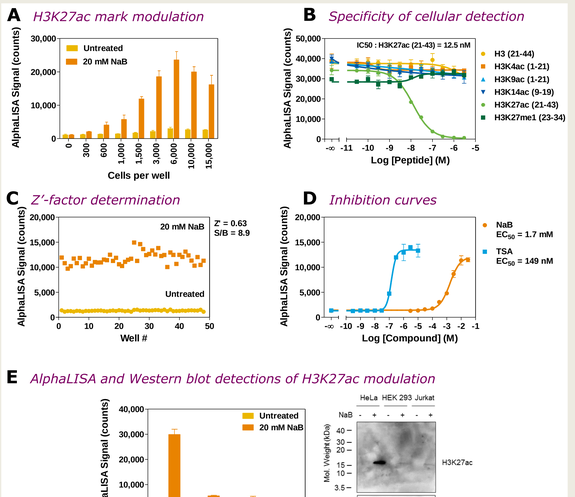
AlphaLISA detection of H3K4me2 cellular modulation. A) Titration of HeLa cells treated overnight with 20 mM NaB. B) Specificity of H3K4me2 cellular detection in 5,000 HeLa cells treated overnight with 20 mM NaB. Additional peptides were tested in separate experiments (not shown). Only the H3K4me2 peptide competed with high affinity for the interaction between the Acceptor beads and histone proteins, while the H3K4me1 peptide showed 30-fold lower affinity. C) Z′-factor value determination using 5,000 untreated and 5,000 NaB-treated (20 mM) HeLa cells/wells incubated overnight. D) Inhibition of HDAC activity using an overnight treatment of 5,000 HeLa cells with TSA and NaB. E) Western blot analysis corroborates the AlphaLISA data for H3K4me2 modulation by 20 mM NaB in 3 cell lines.

7 H3K9ac Cellular Detection



AlphaLISA detection of H3K9ac cellular modulation. A) Titration of HeLa cells treated overnight with 20 mM NaB. B) Specificity of H3K9ac cellular detection in 2,000 HeLa cells treated overnight with 20 mM NaB. Additional peptides were tested in separate experiments (not shown). Only the H3K9ac peptide competed with high affinity for the interaction between the Acceptor beads and histone proteins. C) Z′-factor value determination using 2,000 untreated and 2,000 NaB-treated (20mM) HeLa cells/well incubated overnight. D) Inhibition of HDAC activity using an overnight treatment of 2,000 HeLa cells with TSA and NaB. E) Western blot analysis corroborates the AlphaLISA data for H3K9ac modulation by 20 mM NaB in 3 cell lines.

8 H3K27ac Cellular Detection



AlphaLISA detection of H3K27ac cellular modulation. A) Titration of HeLa cells treated overnight with 20 mM NaB. B) Specificity of H3K27ac cellular detection in 2,000 HeLa cells treated overnight with 20 mM NaB. Additional peptides were tested in separate experiments (not shown). Only the H3K27ac peptide competed with high affinity for the interaction between the Acceptor beads and histone proteins. C) Z′-factor value determination using 2,000 untreated and 2,000 NaB-treated (20mM) HeLa cells/well incubated overnight. D) Inhibition of HDAC activity using an overnight treatment of 2,000 HeLa cells with TSA and NaB. E) Western blot analysis corroborates the AlphaLISA data for H3K27ac modulation by 20 mM NaB in 3 cell lines.

9 Summary

- We developed robust, rapid, and highly-specific all-in-one-well epigenetic assays for monitoring the histone marks H3K4me2, H3K9ac, H3K27ac and H3K27me3 in cells using the homogeneous AlphaLISA technology.
- H3K4me2, H3K9ac and H3K27ac mark modulation was demonstrated after treatment with the non-selective HDAC inhibitors sodium butyrate and trichostatin A in both adherent and suspension cell lines.
- Different levels of the H3K27me3 mark were measured in B lymphoma cell lines OCI-LY-19 and SU-DHL-6, confirming published data.[2]
- AlphaLISA data obtained for each mark were corroborated by Western blot analysis.
- These novel cell-based assays are suitable for HTS protocols, as demonstrated by Z′ factors for all four marks superior to 0.6.
- A comprehensive description of these assays and their optimization is available on our website at: www.perkinelmer.com/epigenetics

REFERENCES
1. *Zhang et al., (1999) Journal of Biomolecular Screening 4: 67–73.*
2. *Sneeringer et al., (2010) Proc Natl Acad Sci U S A. 107(49): 20980-20985.*