TECHNICAL NOTE

AlphaLISA #23

AlphaLISA Di-Methyl-Histone H3 Lysine 79 (H3K79me2) Cellular Detection Kit

AlphaLISA®

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- AL748HV: 100 assay points (96-well format, half-area plates)
- AL748C: 500 assay points (384-well format)
- AL748F: 5,000 assay points (384-well format)

This AlphaLISA® immunodetection assay monitors changes in the levels of di-methylated histone H3 lysine 79 (H3K79me2) in cellular extracts.

AlphaLISA Assays

The AlphaLISA technology allows performing no-wash homogeneous proximity immunoassays using Alpha Donor and AlphaLISA Acceptor beads. In this technical note, we present an optimized assay for measuring changes in the levels of H3K79me2 after treatment of cells with histone demethylase (HDM) inhibitors 5-carboxy-8-hydroxyquinoline (IOX1) and deferoxamine (DFX), as well as with the methyltransferase (HMT) inhibitor 3-deazaneplanocin A (DZNep). Following a homogeneous histone extraction protocol, the mark of interest is detected by the addition of AlphaLISA Acceptor beads conjugated to a biotinylated anti-Histone H3 (C-terminus) and of a biotinylated anti-H3K79me2 antibody (Ab). The biotinylated antibody is then captured by 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 at 615 nm.

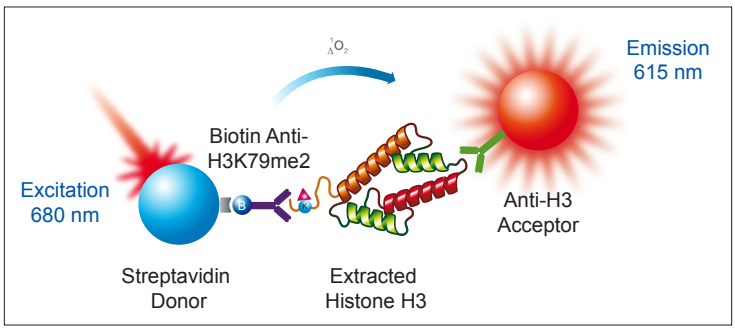

Figure 1. Schematic representation of the AlphaLISA cellular assay for the detection of the histone H3 di-methylated at lysine 79.

Detection of Histone H3 Di-Methylated on Lysine 79 in Cellular Extracts:

Reagents needed for the assay:

AlphaLISA Di-Methyl-Histone H3 Lysine 79 (H3K79me2) Cellular Detection Kit	PerkinElmer # AL748
AlphaLISA Unmodified Histone H3 Lysine 4 (H3K4) Cellular Detection Kit	PerkinElmer # AL719
HeLa cells	ATCC # CCL-2.2™
KYSE-150 cells	DSMZ # ACC 375
MCF7 cells	ATCC # HTB-22
White opaque CulturPlate™-384	PerkinElmer # 6007680
TopSeal™-A film	PerkinElmer # 6050195
Deferoxamine mesylate salt (DFX)	Sigma # D9533
3-Deazaneplanocin A (DZNep)	Cayman Chemical # 13828
5-Carboxy-8-hydroxyquinoline (IOX1)	Sigma # SML0067
PolyScreen® PVDF Hybridization Transfer Membrane for mini-gels	PerkinElmer # NEF 1003001PK
Western Lightning™ ECL Pro	PerkinElmer # NEL 120001EA
Anti-Mouse IgG (Goat), HRP-Labeled	PerkinElmer # NEF 822001EA
Anti-Rabbit IgG (Goat), HRP-Labeled	PerkinElmer # NEF 812001EA

Cell-plating medium:
HeLa and MCF7: MEM/EBSS (HyClone # SH30024) supplemented with 10% FBS.
KYSE-150 HAM's F12 (HyClone # SH30026) supplemented with 10% FBS.

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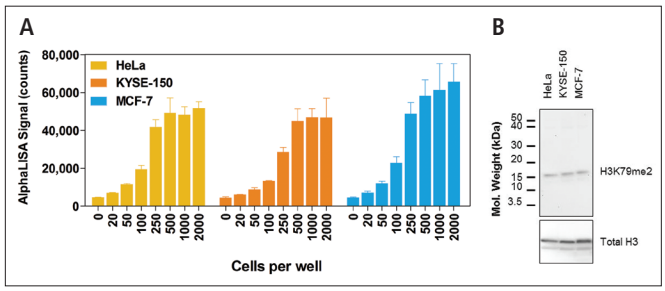
Standard Protocol

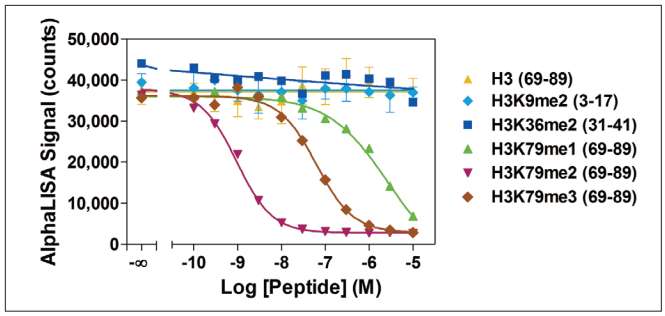
- Distribute 10 µL of cells in the wells of a CulturPlate-384 microtiter plate.
- Fill outer and unfilled wells with sterile water or PBS.
- Incubate adherent cells for 3-4 h at 37 °C in a 5% CO_2 atmosphere to allow cell adhesion. Skip this step for cells growing in suspension.
- Add 5 µL of culture medium or modulator prepared at 3X its final concentration in medium.
- Incubate cells for 16-72 h at 37 °C in a 5% CO_2 atmosphere. For longer incubation, a medium change is recommended.
- Add 5 µL of Cell-Histone™ Lysis buffer.
- Incubate 15 min at room temperature.
- Add 10 µL of Cell-Histone Extraction buffer.
- Incubate 10 min at room temperature.
- Dilute the 10X Cell-Histone Detection buffer to 1X with water.
- Prepare a 5X mix of anti-Histone H3 (C-ter) Acceptor beads at 200 µg/mL and biotinylated anti-Histone H3K79me2 at 15 nM in 1X Cell-Histone Detection buffer.
- Add 10 µL of the 5X mix of Acceptor beads/biotin anti-H3K79me2 antibody (final concentration 40 µg/mL and 3 nM, respectively).
- Cover with TopSeal-A film and incubate for 60 min at 23 °C.
- Prepare in subdued light a 5X solution of SA Donor beads at 100 µg/mL in 1X Cell-Histone Detection buffer.
- Add 10 µL Donor beads (final concentration 20 µg/mL).
- Cover with TopSeal-A film and incubate for 30 min at 23 °C in the dark.
- Read signal in Alpha mode with the EnVision® or EnSpire® Multilabel plate reader. Plates can be read after overnight (O/N) incubation to maximize signal intensity.

Experiment 1: Detection of Histone Mark



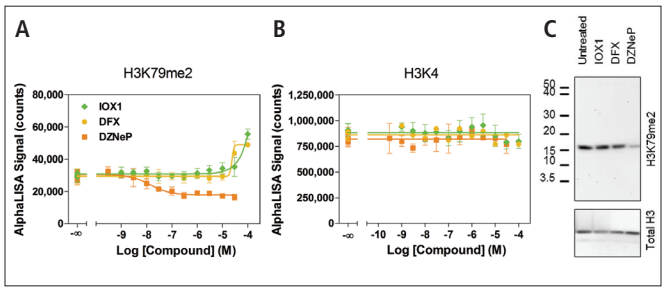
Detection of H3K79me2 levels in HeLa, KYSE-150 and MCF7 cells. A) AlphaLISA detection of H3K79me2. Serial dilutions of cells corresponding to densities ranging from 20 to 2,000 cells per well were performed in CulturPlate-384 microplate. Cell-Histone Lysis and Extraction buffers were sequentially added prior to detection reagents. B) Western blot detection of H3K79me2 and total histone H3 levels. Cell lysates normalized to total histone H3 levels were separated by SDS-PAGE on a 10%-20% gradient gel. Following transfer to PolyScreen membrane, histone H3 proteins di-methylated at lysine 79 were detected using the same antibody present on the biotinylated antibody. For total histone H3, an antibody recognizing a histone H3 C-terminal epitope was used. Western blots were revealed using HRP-labeled anti-species secondary antibodies and Western Lightning ECL Pro substrate.

Experiment 2: Specificity of Cellular Detection



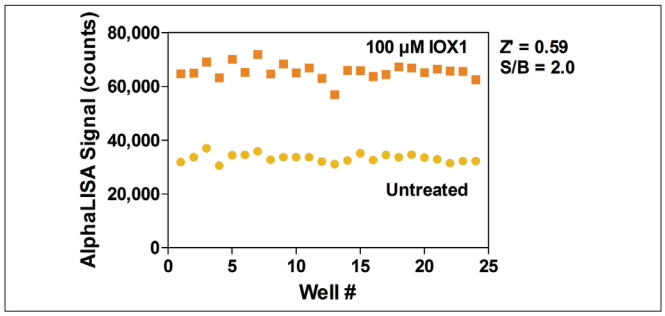
HeLa cells were seeded at a density of 500 cells/well. Serial dilutions of histone H3-derived peptides bearing various epigenetic marks were added to the wells at concentrations ranging from 100 pM to 10 µM immediately before the addition of the AlphaLISA detection reagents. Additional peptides tested but not shown include H3K4me2 and H3K27me2. The H3K79me2 peptide diplayed the highest affinity and competed with the interaction between the biotinylated antibody and histone proteins with an IC50 value of 0.95 nM. H3K79me3 and H3K79me1 were less potent with IC50 values of 60 nM and 2.7 µM, respectively.

Experiment 3: Compound-Induced Modulation



A) and B) AlphaLISA assays. HeLa cells were seeded at a density of 1,000 cells per well and treated for 48 h with the broadly-selective HMT inhibitor DZNep (from 300 pM to 30 µM), the histone demehtylase inhibitors DFX (from 1 nM to 100 µM) or IOX1 (from 1 nM to 100 µM), in medium containing 0.3% DMSO. A) Detection of the H3K79me2 mark. B) Detection of the unmodified H3K4 mark used to assess total histone H3 levels. C) Western blot analysis of H3K79me2 and total histone H3 levels in HeLa cells treated for 48 h with DZNep (30 µM), DFX (30 µM), IOX1 (30 µM) or 0.3% DMSO control vehicle (Untreated). A decrease in the H3K79me2 mark level was observed in the AlphaLISA assay and Western blot after treating cells with DZNep and an increase of the Alpha signal was observed after treating cells with IOX1 and DFX at 100 µM. However, no significant change was observed after treatment with only 30 µM of the two compounds in either the AlphaLISA assay or Western blot.

Experiment 4: Z'-factor Determination



HeLa cells were seeded at a density of 1,000 cells per well and treated 48 h with 100 µM IOX1 in medium containing 0.2% DMSO. The Z'-factor value compares IOX1-treated and untreated cells.

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