

Comparison of endogenous methyl marks on histone H3 in cancer-derived cell lines using AlphaLISA

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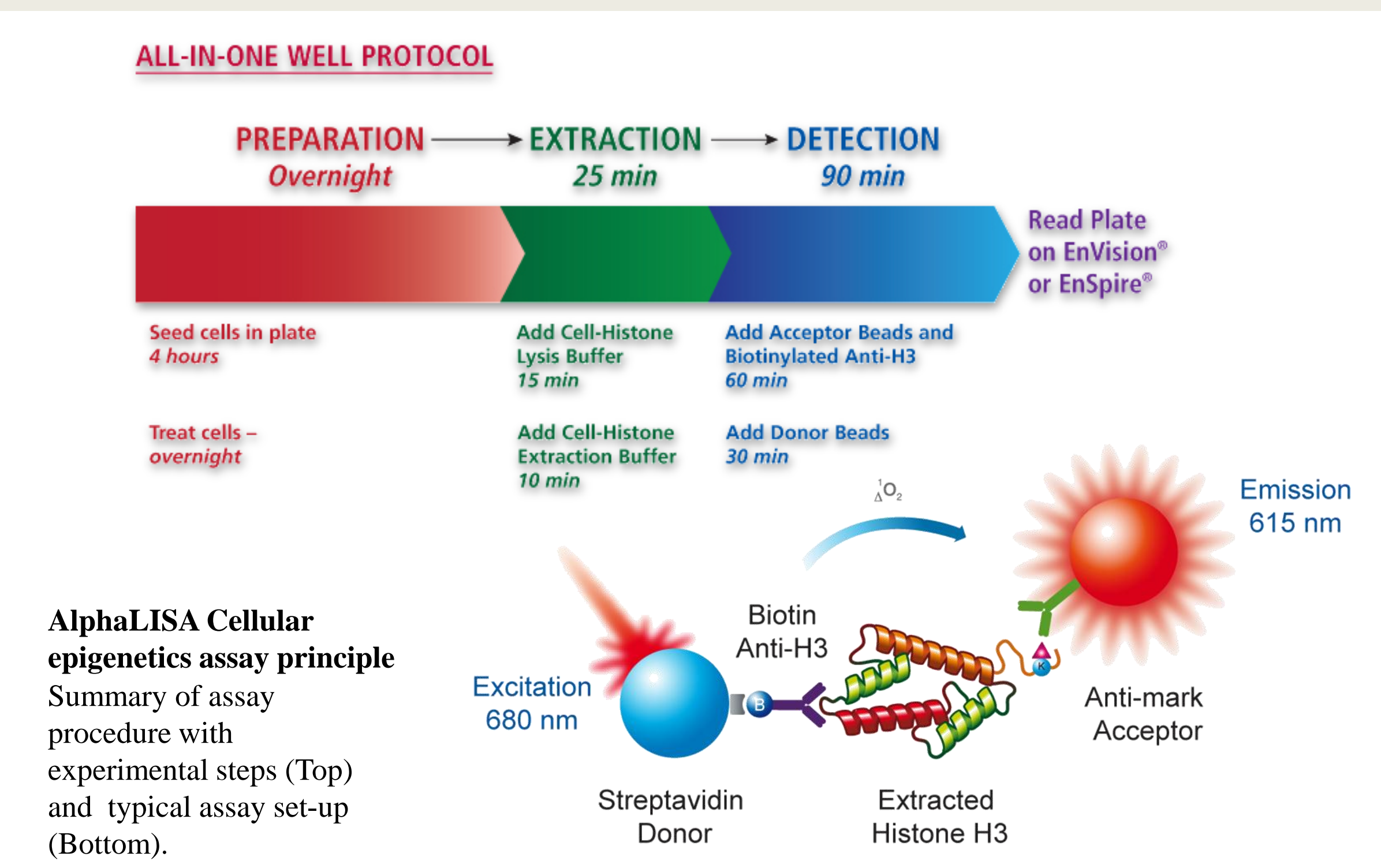
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1 Abstract

To gain further insight on H3 methyl mark regulation, AlphaLISA® cellular assays were developed for the detection of six methyl marks, namely H3K4me2, H3K9me2, H3K27me2, H3K27me3, H3K36me2 and H3K79me2. These bead-based no-wash assays rely on the capture of extracted full length histone H3 proteins by a biotinylated antibody and an Acceptor beads coupled antibody. Each antibody pair includes an anti-histone H3 (C-terminus) antibody and an anti-histone H3 mark antibody. In this study, endogenous levels for the six marks were compared in nine cancer-derived cell lines. The AlphaLISA assays revealed significant differences between cell lines that were all corroborated by Western blot analysis. We then assessed the HTS amenability of the methodology by using as a model system the detection of H3K9me2 levels in HeLa cells. A series of tool compounds expected to either increase or decrease H3K9me2 levels was evaluated. Our data agree with previous reports showing that the G9a methyl transferase inhibitor UNC0638 significantly reduces H3K9me2 levels, whereas treatment with the JMJ demethylase inhibitors deferoxamine and 5-carboxy-8-hydroxyquinoline (IOX1) results in increased H3K9me2 mark levels. Furthermore a Z'-factor determination comparing untreated HeLa with UNC0638-treated cells yielded a value of 0.54, confirming the H3K9me2 assay robustness and suitability for HTS.

The Alpha Technology for cellular histone detection is based on a simple extraction method that does not require tedious acid extraction and centrifugation steps. With its ease of execution and quality of the achieved data, this technology represents a powerful alternative to Western blot analysis and ELISA. These novel tools will certainly assist in providing new biological insights on histone mark regulation, leading to the discovery of new pharmacological leads.

2 Epigenetic AlphaLISA Assay Principles



The AlphaLISA technology is a no-wash proximity immunoassay. After compound treatment, histones are extracted directly in the culture wells by adding Lysis and Extraction buffers. Histone marks are detected by subsequent addition of a biotinylated anti-Histone H3 (C-terminus) antibody and AlphaLISA Acceptor beads conjugated to an antibody (Ab) specific to the mark. 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 in a 520-620 nm window.

3 Materials

Name	Cancer cell type	Provider
HeLa	Cervix epithelial adenocarcinoma	ATCC # CCL-2.2™
KYSE-150	Esophageal squamous cell carcinoma	DSMZ # ACC 375
MCF7	Mammary gland epithelial adenocarcinoma	ATCC # HTB-22
U-2 OS	Epithelial osteosarcoma	ATCC # HTB-96
MDA-MB-231	Mammary gland epithelial adenocarcinoma	ATCC # HTB-26
OCI-LY-19	Diffuse large cell B cell lymphoma	DSMZ # ACC 528
SU-DHL-6	Diffuse large cell B cell lymphoma	DSMZ # ACC 572
Jurkat	Acute T cell leukemia T lymphocyte	ATCC # CRL-2063™
A549	Epithelial lung carcinoma	ATCC # CCL-198

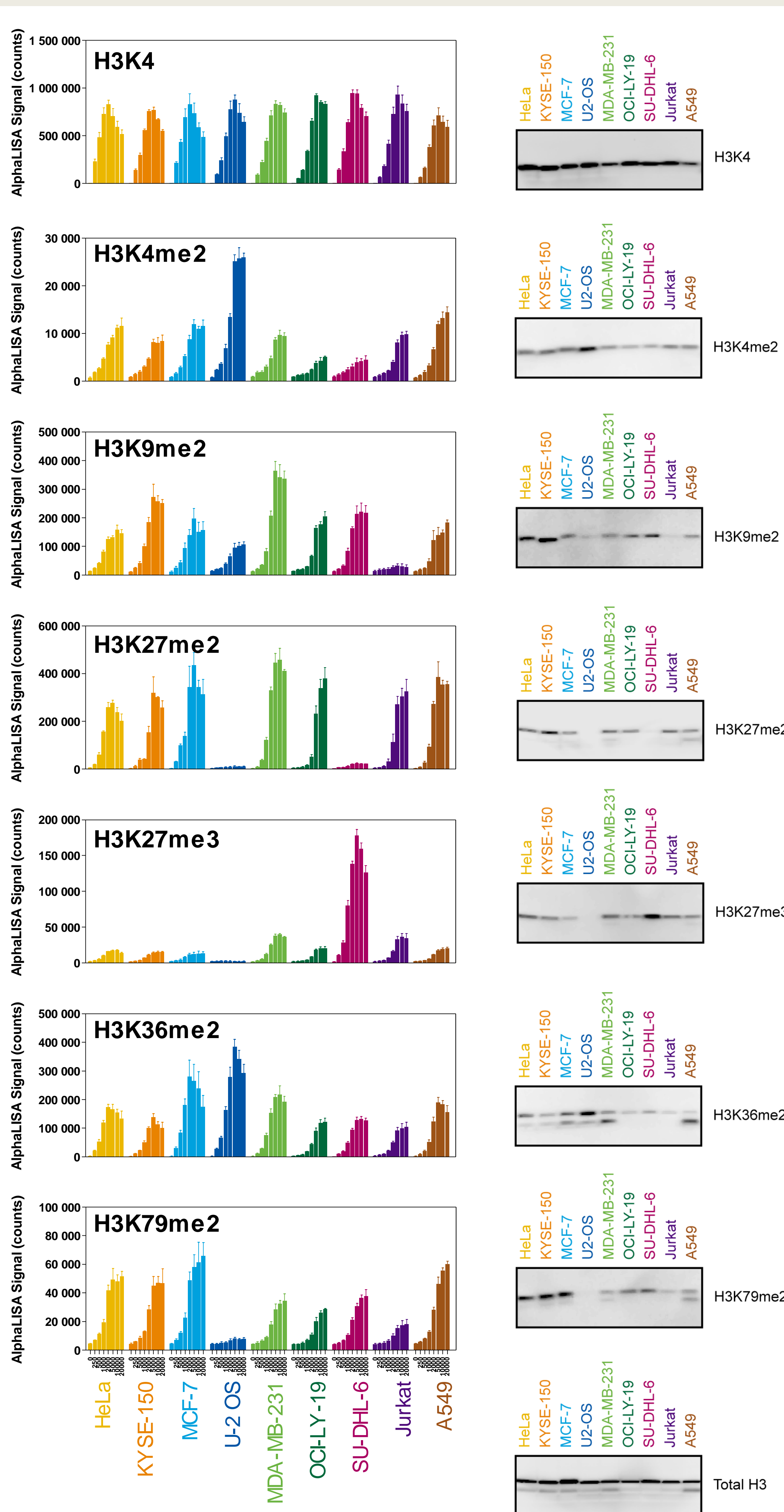
Cells were seeded in white opaque CulturPlate-384 (PerkinElmer # 6007680) and cultured in the following media (HyClone) supplemented with 10 % FBS: MEM/EBSS (SH30024; HeLa, MCF7 and Jurkat); HAM's F12 (SH30026; KYSE-150 and A549); DMEM (SH30022; MDA-MB-231); McCoy's 5a (SH30200; U-2 OS); or Alpha MEM (SH30265) supplemented with 15% FBS (OCI-LY-19 and SU-DHL-6). Compounds were from Sigma: Deferoxamine mesylate salt (DFX; D9533), UNC0638 hydrate (U4885), 5-Carboxy-8-hydroxyquinoline (IOX1; SML0067) except 3-Deazaneplanocin A (DZNep; Cayman Chemical 13828). Western blot experiments were performed with PerkinElmer reagents: PolyScreen® PVDF Hybridization Transfer Membrane for mini-gels (NEF1003001PK); Western Lightning™ ECL Pro (NEL120001EA); Goat HRP-Labeled secondary antibodies Anti-Mouse (NEF822001EA) and Anti-Rabbit IgG (NEF812001EA).

4 Methods

All AlphaLISA cellular assays were performed in accordance with kit literature. Briefly, cells were seeded at the indicated densities in the presence or absence of compound. On the next day or following compound treatment, Cell-Histone™ Lysis and Extraction buffers were sequentially added to the cells in the culture medium, AlphaLISA Acceptor beads along with the biotinylated antibody in Cell-Histone Detection buffer were added. Following a 60 min incubation at room temperature, Streptavidin-conjugated Donor beads were then added and a final 30 min incubation was performed prior to plate reading with EnVision® Multilabel Plate Reader (PerkinElmer).

For Western blot analysis, cells were harvested, and lysed in Cell-Histone Lysis followed by addition of Cell-Histone Extraction buffer. Cell lysates were separated by SDS-PAGE on a 10%-20% gradient gel. Following transfer, modified histone H3 proteins were detected using the same antibody present on the Acceptor beads. For total histone H3, an antibody recognizing a histone H3 C-terminal epitope was used.

5 Methyl mark levels in different cancer-derived cell lines

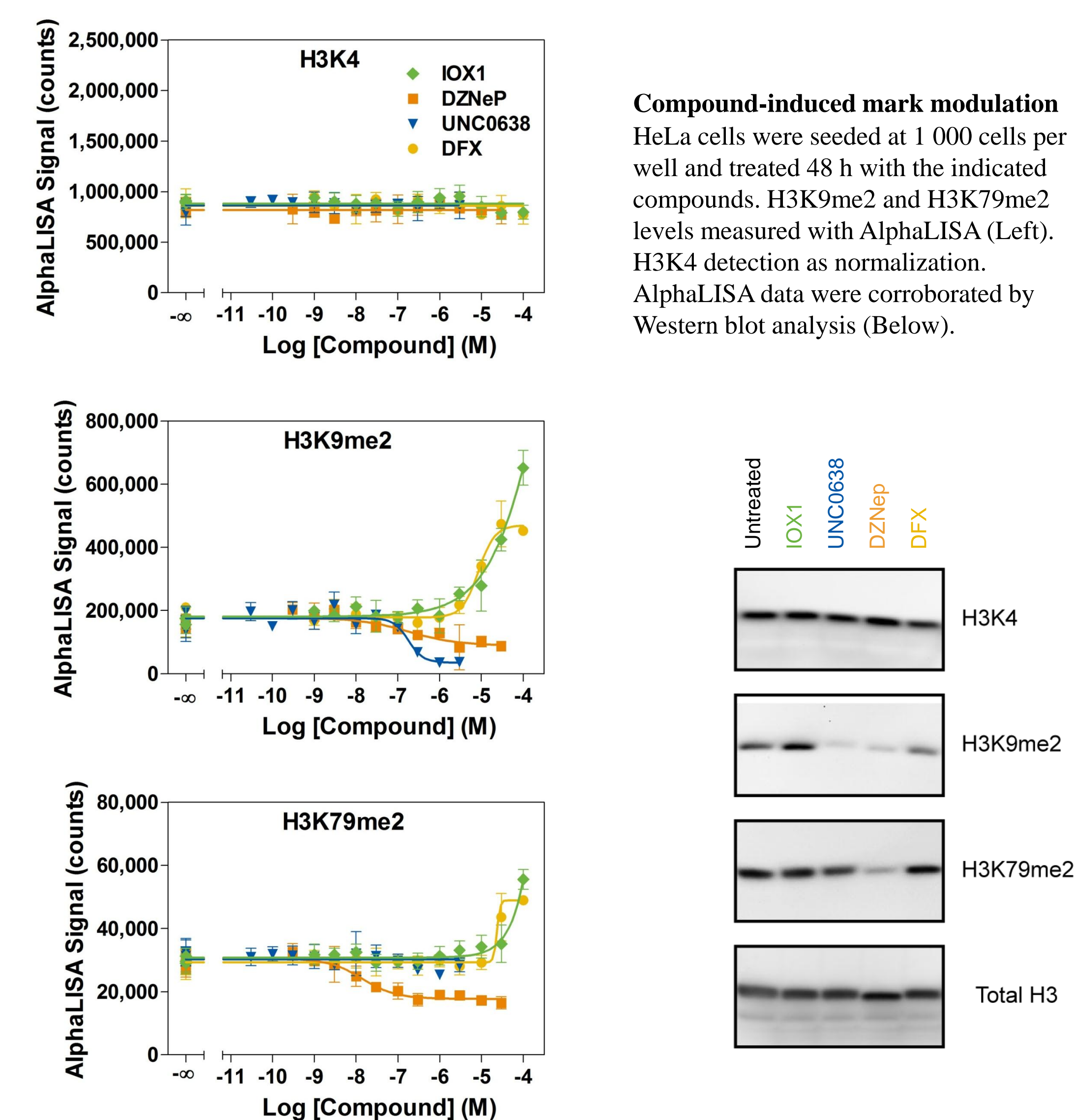


Endogenous mark levels

A panel of nine cancer-derived cell lines were each seeded in 384-well plates from 0 to 20 000 cells per well and submitted to the histone extraction method described in Assay Principles. Materials and Methods sections. The appropriate detection reagents (Acceptor, Donor beads, and biotinylated antibody) were added to the plate for measurement of target mark levels. Results were corroborated by Western blot analysis with same mark-specific antibodies (Right).

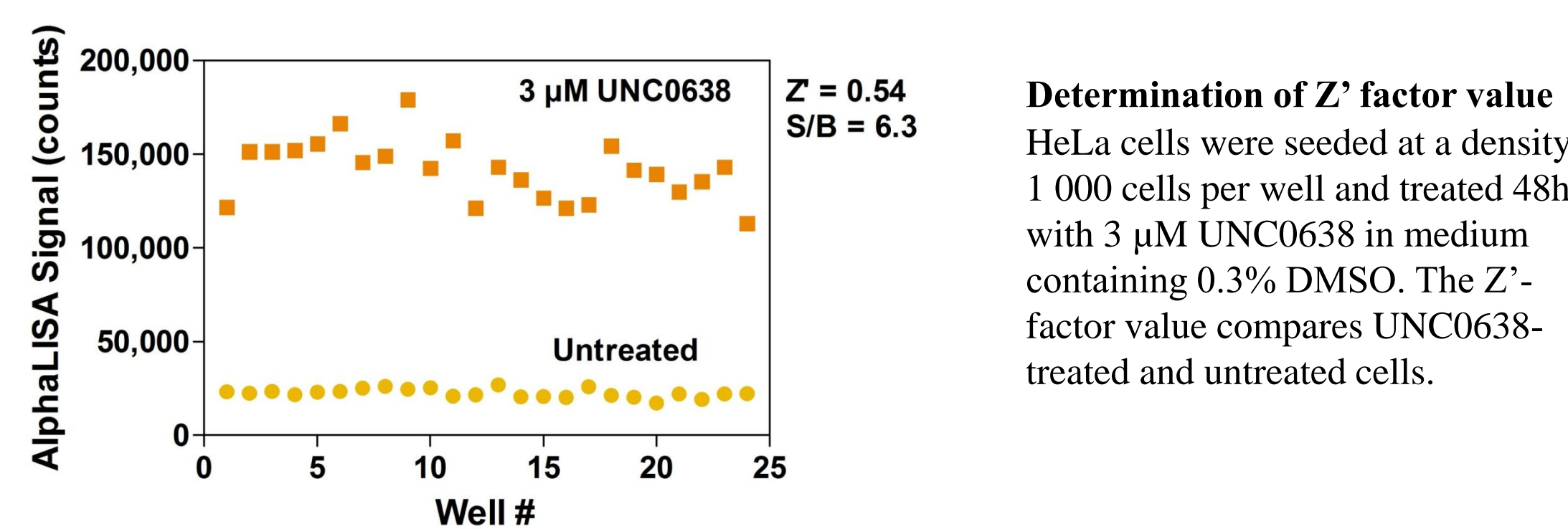
H3 methyl mark levels considerably vary between cell lines. Unmodified H3K4 detection was used to normalize the assays. Constant H3K4 levels between cell lines as well as corroboration with total H3 levels suggest that unmodified H3K4 detection can be effectively used to normalize the assays.

6 Modulation of H3K9 and H3K79 methylation



The AlphaLISA H3K9me2 and H3K79me2 detection kits were used to measure the effect of demethylase (HDM) inhibitors: deferoxamine (DFX) and 8-hydroxy-5-quinolinecarboxylic acid (IOX1); as well as methyltransferase (HMT) inhibitors: DZNep and UNC0638. Results indicate that at high concentration, HDMi were able to increase the di-methylation levels of H3 lysine 9 and 79 while the G9a-selective inhibitor, UNC0638, only modulated lysine 9 methylation levels. DZNep showed a higher potency when looking at lysine 79 modulation. Finally, none of the compounds affected total H3 levels, as measured using the unmodified K4 kit.

7 Assay performance



With a Z' factor value over 0,5, the cellular H3K9me2 assay can be considered robust. The same applies to other marks tested.

8 Summary

- Using the AlphaLISA platform we have developed rapid, and highly-specific all-in-one-well cell-based epigenetic assays to monitor histone H3 tail modifications, namely methylation on lysine residues 4, 9, 27, 36, and 79.
- The assays were used to compare methyl marks levels in 9 cancer-derived cell lines, revealing cell line-specific patterns.
- Tool compounds modulated H3K9me2 and H3K79me2 levels, but not those of unmodified H3K4, suggesting the latter could act as a normalization marker.
- These data were corroborated by Western blot analysis.
- The assays are robust and exhibit HTS-suitability.

References

- PerkinElmer *Technical Notes* for H3K9me2 (AlphaLISA 20), H3K79me2 (AlphaLISA 23) and normalization with H3K4 (AlphaLISA 24)
- Peach SE, *et al.* *Mol Cell Proteomics*. (2012) **11**:128