Characterization of Intractable Epigenetics Targets Using Homogenous Immunoassays

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1 Abstract

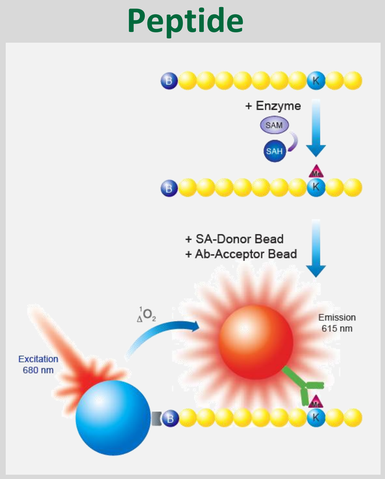
The human genome sequence project revealed the existence of a plurality of genes encoding proteins responsible of DNA remodeling. Because of their implication in key cellular processes and in the etiology of the most important diseases including cancer, neurodegenerative and developmental disorders; epigenetics enzymes are now among the most important pharmaceutical targets. Significant efforts are underway in numerous academic and industrial laboratories to identify and validate new reader, writer or eraser enzymes as well as their histone substrates. Shedding light on the functions of new epigenetics enzymes may lead to the generation of enabling drugs to treat the aforementioned diseases.

While various molecular biology techniques are used to identify and validate epigenetics enzymes and their substrates, biochemical assays are gold standard approaches to perform pharmacological characterization of histone tails modifications by reader, writer or eraser enzymes. ^{3}H-SAM and ^{3}H-acetyl CoA incorporation assays are widely used to characterize epigenetics enzymes; however these techniques can't measure methylation levels and identify the specific amino acids modified. Immunoassays relying on sequence specific antibodies allow one to reach that level of information.

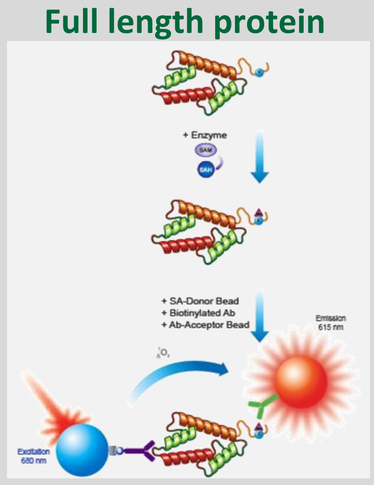
AlphaLISA® / AlphaScreen ® (abbr. as "Alpha" below) homogenous immunoassays can detect a wide range of binding partners, either small (eg. nucleotides) or very large protein complexes (> 6M Da), interacting with high (pM) or low (mM) affinities. Using Alpha technologies, various assays were developed to measure the activity of different reader, writer or eraser enzymes in both biochemical and often intractable cellular setups. In this contribution, multiple examples are presented showing how scientists can generate higher quality and biologically relevant data from epigenetics studies using Alpha technologies.

2 Choice of Substrate Matters

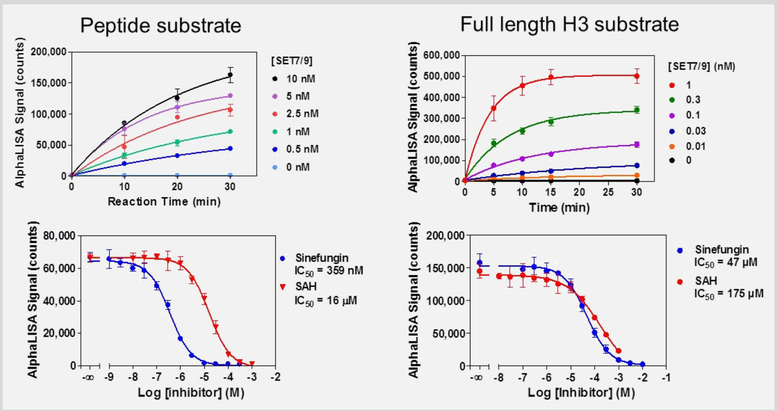
Peptide



Full length protein

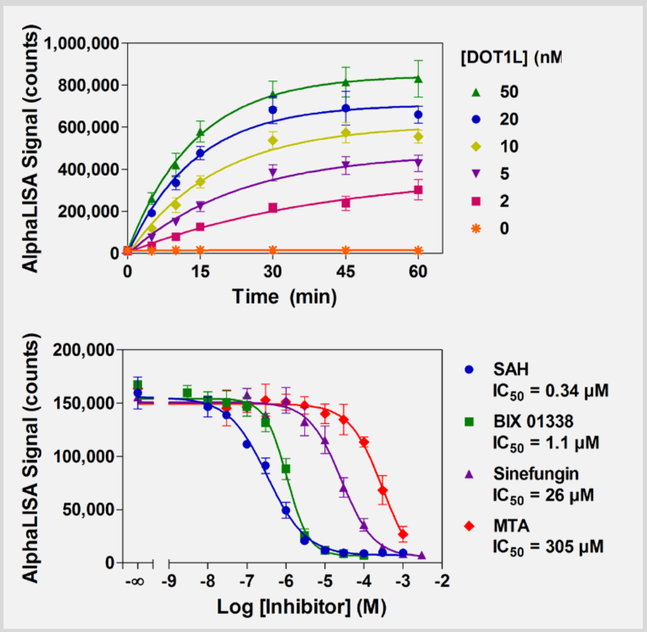


Biotinylated peptide substrates are captured onto Streptavidin coated Donor beads whereas full-length substrates are detected using biotinylated anti-H3 antibodies captured onto Streptavidin coated Donor beads. In both cases, specific epigenetic marks are detected using anti-mark antibodies conjugated to Acceptor beads. Both Donor and Acceptor beads are brought into proximity by the presence of specific substrate modifications allowing Acceptor beads to emit a signal detectable at 615 nm following excitation of the Donor beads at 680 nm and consequent release of singlet oxygen needed to trigger Acceptor bead chemiluminescence.



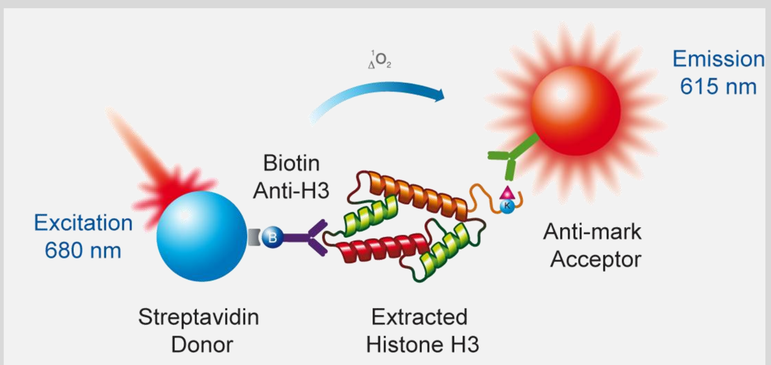
SET7/9 methyl transferase activity was measured using either H3-derived peptide or full length H3 substrates and H3K4me1-2 specific antibodies. Time-course / enzyme titration experiments show that full length H3 was the preferred SET7/9 substrate since higher counts, representative of product capture, were observed at lower enzyme concentrations. Both SAH and Sinefugin inhibitors showed marked differences in potency and rank order of potency when tested with peptide or full length H3 substrates .

3 Testing Reconstituted Nucleosomes

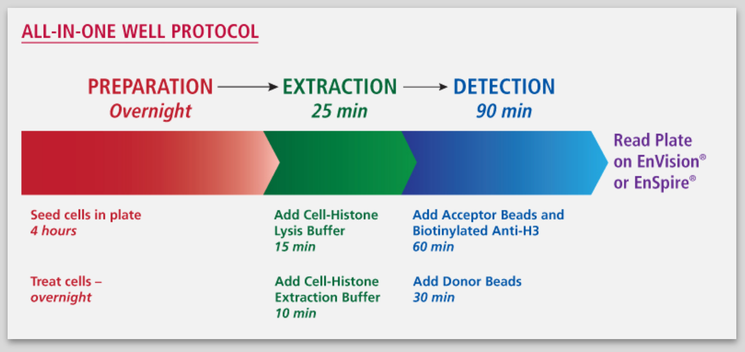


To validate the Alpha platform in a more biologically relevant context, nucleosomes purified from HeLa cells were tested as substrate for DOT1L. When used from 2 to 50 nM, DOT1L was shown to generate robust H3K79 methylation over a time course of 1 hour. DOT1L activity was inhibited by various methyltransferase inhibitors with a rank of order of potency comparable to that described in previous literature reports.

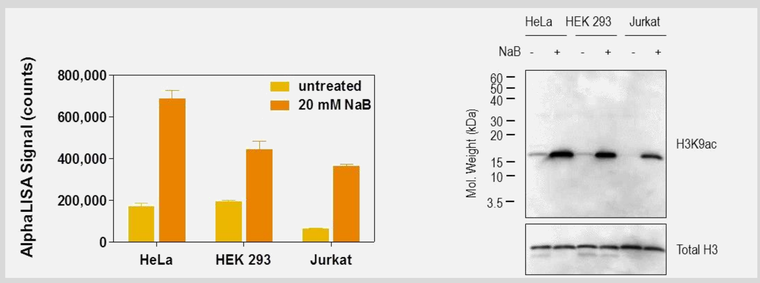
4 All-in-one Well Cell-based Assays



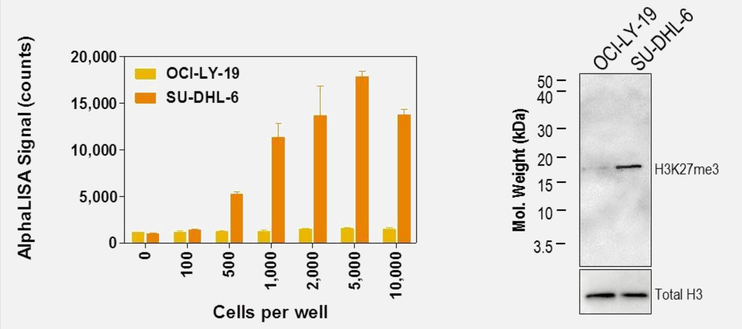
Endogenous (extracted) Histone H3 molecules are detected using a biotinylated anti-H3 antibody captured onto Streptavidin coated Donor beads and specific anti-mark antibodies conjugated to Acceptor beads. Both Donor and Acceptor beads are brought into proximity by the presence of specific mark-bearing Histones allowing Acceptor beads to emit a signal detectable at 615 nm following excitation of the Donor beads at 680 nm and consequent release of singlet oxygen needed to trigger Acceptor bead chemiluminescence.



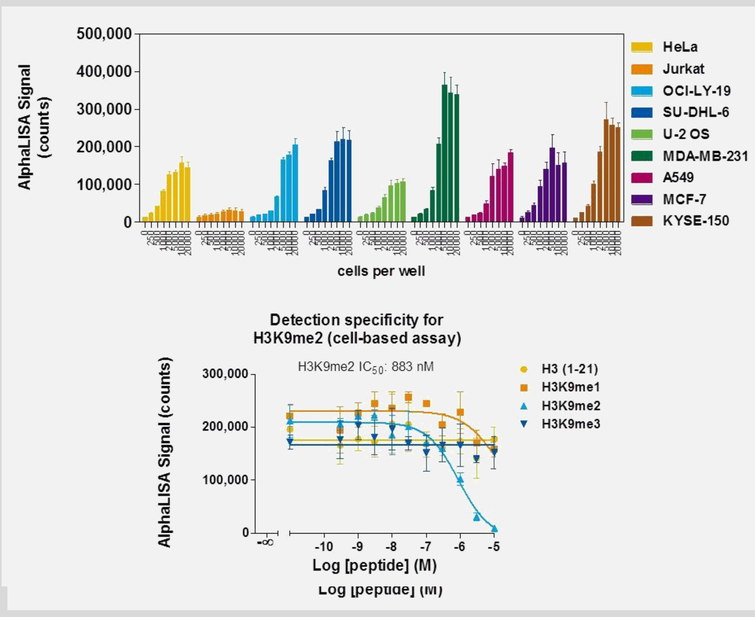
Enabling buffer formulations were developed to achieve all-in-one well detection of modified histones. Both extraction and detection buffers were formulated to allow 1) cell and nuclear membranes lysis and 2) DNA unwrapping from histones to occur in a non-chaotropic environment favorable for epigenetic mark detection with antibodies.



Sodium Butyrate was used to induce histone hyper-acetylation in 3 representative cell lines: HeLa, HEK293 and Jurkat. Histone H3 acetylation on K9 residue was tested with AlphaLISA and compared to traditional Western Blotting. Same acetylation pattern was observed with both technologies. It is worth noting that Alpha was performed with 3000 cells/assay, equivalent to 50-75 ng protein.



H3K27 methylation was measured on B cell lymphoma cell lines known to display different levels of H3K27me2 and H3K27me3. The OCI-LY-19 cell line is widely used to measure the activity of wild type EZH2 and known to accumulate H3K27me2 mostly. SU-DHL-6 cells carry the heterozygous EZH2 HMT mutation (Y641N) affecting substrate selectivity and leading to H3K27me3 accumulation essentially. AlphaLISA was capable of measuring differential methylation states on H3K27 with as little as 500 B cell lymphoma cells per well. Data were corroborated by Western blotting.



AlphaLISA was further validated on a wider range of range of cell lines commonly used to perform epigenetics research. For the 9 cell lines tested, detection of H3K9me2 mark was possible using as little as 250 cells per assay (top graph).

To assess the selectivity of the anti-H3K9me2 antibody used in the assay, a series of competition isotherms were performed using non-biotinylated peptides carrying H3K9 mono, di and tri-methyl marks. As observed on the bottom graph, only the H3K9me2 peptide was able to inhibit the interaction of H3 histones with the anti-H3K9me2 antibody. Interestingly, the IC_{50} value resulting from this competition curve is near 1 uM. It is worth noting that Alpha technologies are capable of measuring affinity constants ranging from pM to mM,

5 Summary

Using AlphaLISA, a novel suite of homogenous assays were developed to characterize intractable epigenetic targets. These assays allowed us to assess:

- The importance of selecting the right type of substrate when performing biochemical assays, since both potency and rank order of compound potencies may vary.
- The suitability of the AlphaLISA to measure epigenetic enzyme activity using reconstituted nucleosome preparations with epigenetics enzymes known to require native substrates (eg. DOT1L).
- The simplicity and sensitivity of AlphaLISA compared to traditional technologies used to perform epigenetics assays. Compared to Western Blotting, AlphaLISA:
  - ✓ Could be performed in less than 2 hours
  - ✓ Requires a few thousand cells per assay (the equivalent of 50-75 ng proteins)
- The compatibility with immortalized, primary and stem cell lines.
- The selectivity of epigenetic marks detection, in both biochemical and cellular assays, through the utilization of high quality antibodies.

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