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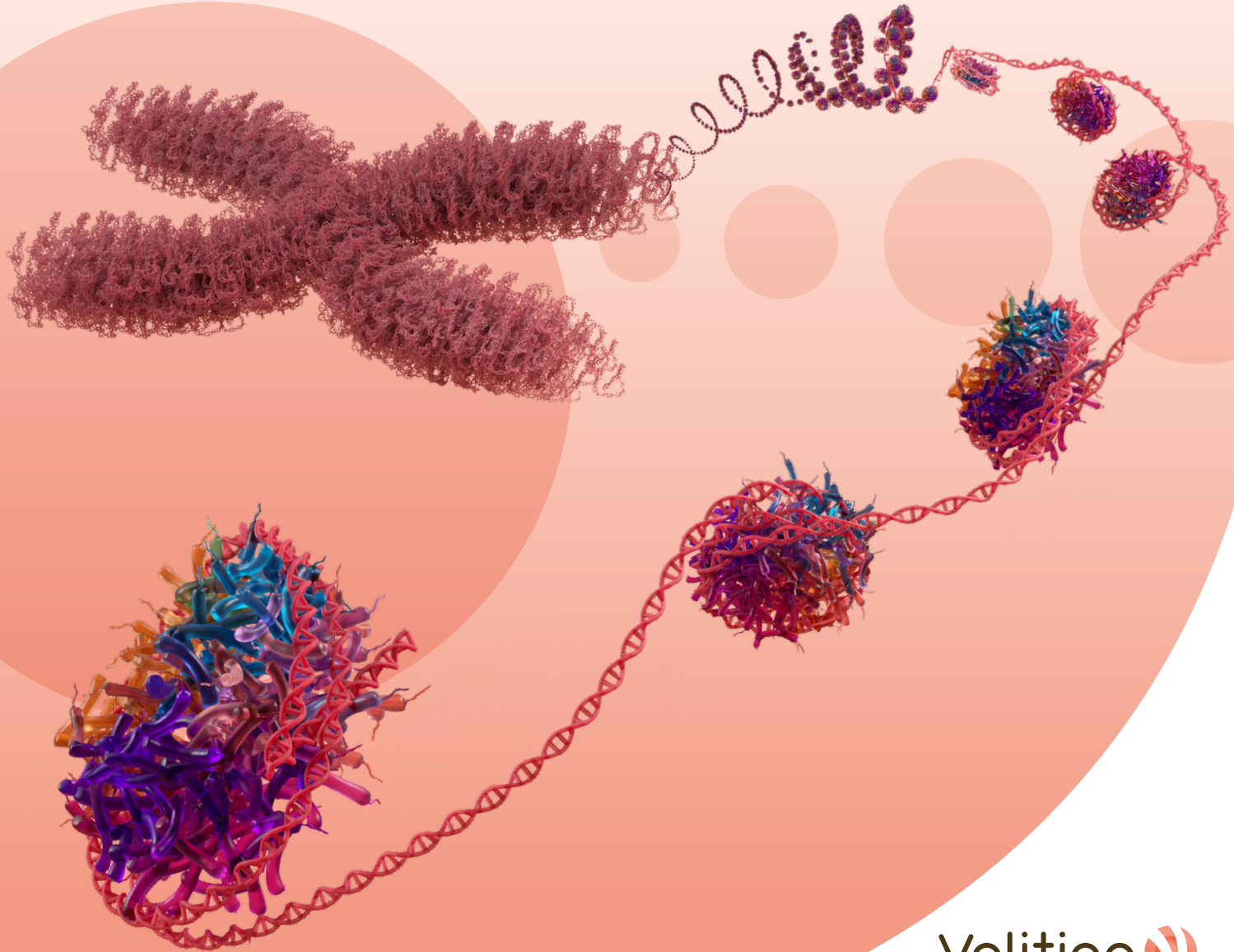
Using Nu.Q® Discover to profile histone post-translational modifications following treatment with epigenetic inhibitors.

Nu.Q® Discover Cell Culture White Paper

Brandi Atteberry, Dorian Parmart, Justin Cayford, Terry Kelly



Volition

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Introduction

Epigenetic modifications are critical in regulating cellular processes and influencing gene regulation. Histone methylation and acetylation are two of the most well characterized histone post-translational modifications (PTMs), with acetylation most often associated with gene activation and methylation associated with either gene expression or inactivation, depending on which histone residue is modified. The accurate detection and analysis of these epigenetic changes hold significant implications for understanding disease mechanisms, developing targeted therapies, and advancing precision medicine.

The primary objective of this study was to assess the reliability and versatility of the Nu.Q® Discover assays in targeting these epigenetic marks within a controlled experimental system. Specifically, the research focused on Nu.Q® Discover assay's ability to detect and measure epigenetic changes in the widely studied K562 (Leukemia) cells treated with a variety of drugs. The Nu.Q® Discover assays, designed to investigate multiple epigenetic marks present in plasma, hold the potential to be a valuable set of tools for gaining deeper insights into epigenetic patterns in a variety of matrices, including nuclear lysates.

Here we demonstrate how the Nu.Q® Discover assays can be used as part of drug development programs related to global PTM alterations by showing changes in histone PTM levels in a time and dose dependent manner following treatment with drugs targeting histone methyltransferases, histone demethylases as well as histone deacetylases ***(Table 1)***. This investigation confirms the suitability of the Nu.Q® Discover assays as reliable tools for elucidating epigenetic modifications and their potential implications in cellular responses to drug treatments.

1.

Target	Drug
KMT inhibitor	BIX-01294
KDM inhibitor	2-PCPA
HDAC inhibitor	Valproic Acid
HAT inhibitor	Curcumin
Protein Kinase C inhibitor	Chelerythrine Chloride

Table 1: List of Drug targets and compound selected for evaluation. KMTi: Lysine Methyltransferase; KDM: Lysine Demethylase; HDAC: Histone Deacetylase; HAT: Histone Acetyltransferase

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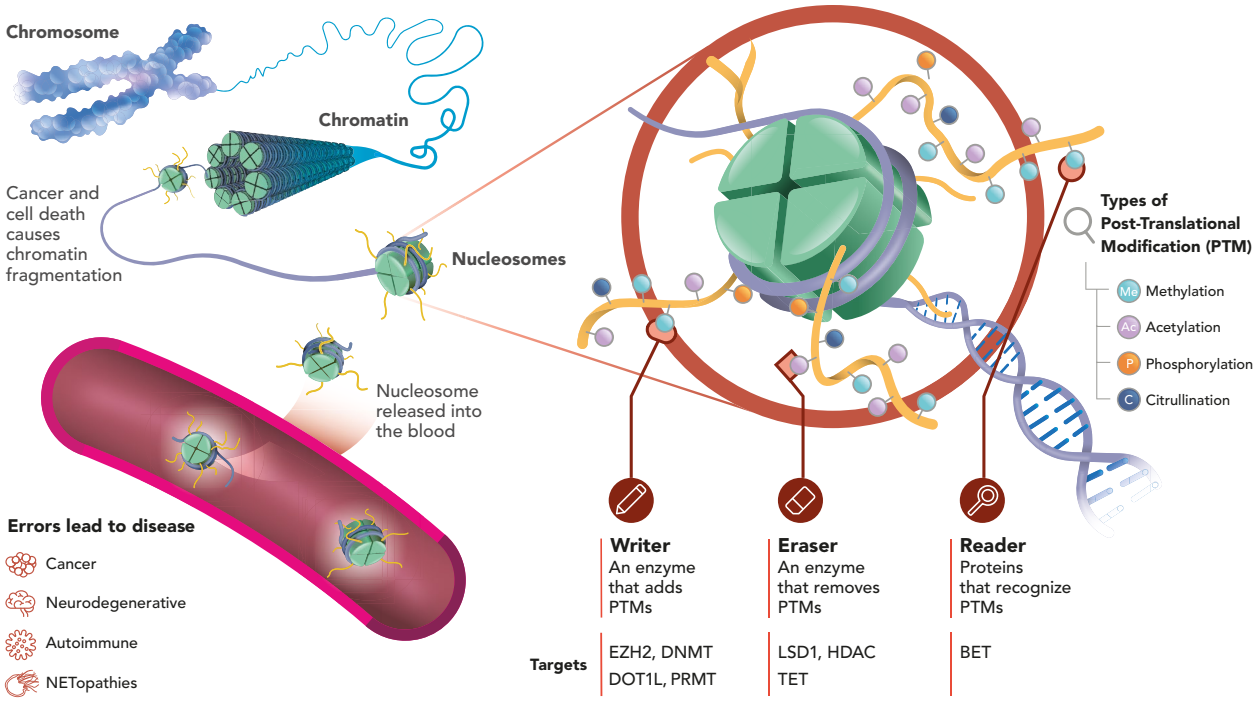
Measuring and monitoring nucleosome levels and modifications in circulating blood has the potential to aid diagnosis, prognosis and monitoring of many human diseases.



Adapted from: Li X and Li XD. Integrative Chemical Biology Approaches to Deciphering the Histone Code: A Problem-Driven Journey. Acc Chem Res 2021 54(19), 3734-3747; Regnier FE, Kim J. Proteins and Proteoforms: New Separation Challenges. Anal Chem 2018 Jan 2;90(1):361-373

Methods

Cell Culture:

K562 cell line (ATCC #CCL-243) was grown in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FBS (ATCC # 30-2020) at 37°C with 5% CO_2 per ATCC recommended growth conditions. Viability was maintained at 98%. K562 cells were seeded at $1x10^6$ cells per well in 6 well plates (Corning #150239). The normal doubling time of K562 cells line is 24 hours and cells were treated for 12h, 24h and 48h. Cells were treated with the following drugs at the concentrations indicated:

- BIX-01924 (Active Motif #14072): 0µM, 0.5µM, 1µM, 2µM, 4µM, 8µM
- 2-PCPA (Sigma #3905058): 0µM, 1µM, 2µM, 4µM, 8µM, 16µM
- Curcumin (Sigma #C1386): 0mM, 2.5mM, 5mM, 10mM, 20mM
- Valproic Acid (Sigma #P4543): 0mM, 0.5mM, 1mM, 2mM, 4mM, 8mM
- Chelerythrine Chloride (Sigma #220285): 0mM, 2.5mM, 5mM, 10mM, 20mM, 40mM

The concentrations listed above were based on publicly available IC_{50} estimates of each drug. All drugs were reconstituted in dH_20 based on manufacturer directions, except Curcumin which was reconstituted in DMSO. In the curcumin treatment, a DMSO control was included and DMSO concentration was kept constant in all wells. K562 cells were harvested from each well at the required time point and centrifuged at 12,000 x g for 5 minutes. Cells were then resuspended in cold 1xPBS (Gibco #10010023) and placed on ice for chromatin extraction processing.

Chromatin Extraction:

Briefly, nucleosome isolation was done by incubating cells in lysis buffer for 20 minutes in duplicate, followed by centrifugation at 3,000 x *g* for 10 minutes. Nuclei were then digested with 0.0125 U/µl MNase (Sigma #N5386-200UN) at 37°C for 15 minutes. The reaction was stopped using 0.5M EDTA and incubated on ice for 5 minutes. Nucleosomes were isolated by centrifugation at 5,000 x *g* for 5 minutes at 4°C then stored on ice. 100 µl of each sample was removed for quality control (QC) testing, and the remaining sample was stored at -80°C. The following QC tests were completed for each sample: 30 µl was incubated with Proteinase K at 56°C for 2 hours, followed by DNA purification using Zymo DNA Clean and Concentrator-5 Kits (Zymo #D4013). DNA concentration and size was verified by Qubit and TapeStation, and nucleosome isolation was confirmed by running Volition's H3.1 Nu.Q® Kit. The final QC was to complete western blot for specific antibodies.

Discover Assay:

Nu.Q® Discover assays targeting H3.1 and the following histone modifications: H3K27me3, H3K36me3, H3K4me1, H3K4me2, H3K9me1, H3K9me3, H3K27Ac, H3K9Ac, and H3K14Ac were run in triplicate. Assays with missing values or where replicates exceeded 30% were excluded from further analysis. Histone PTM levels were assessed by calculating the ratio of Nu.Q® Discover assay to H3.1 and plotted using GraphPad Prism.

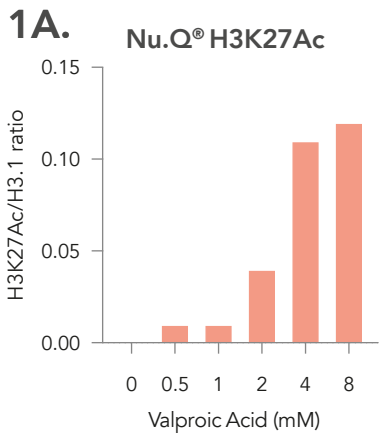
Results

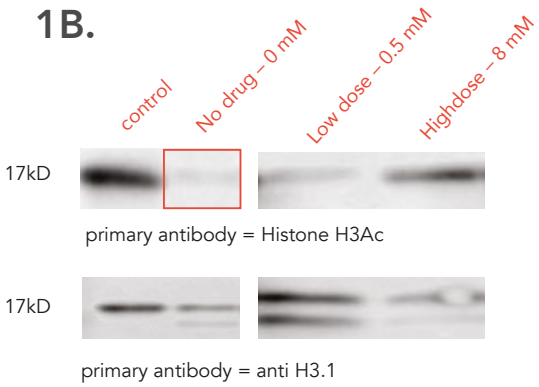
The Nu.Q® Discover assays demonstrated their ability to capture epigenetic changes in K562 cells following a variety of drug treatments and time courses used in the study.

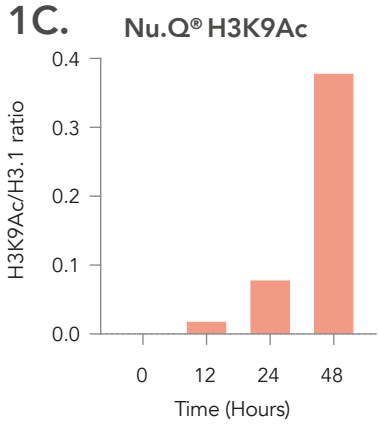
This highlights Nu.Q® Discover's potential as a valuable tool for studying epigenetic modifications as both commonly used methylation and acetylation targets were studied. A protein kinase C inhibitor (PKCi) was included as a control to show that epigenetic targets can be assessed even when the small molecules being studied are not known to target epigenetic modifications.

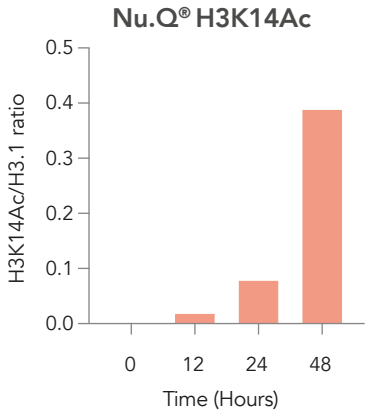
Acetylation:

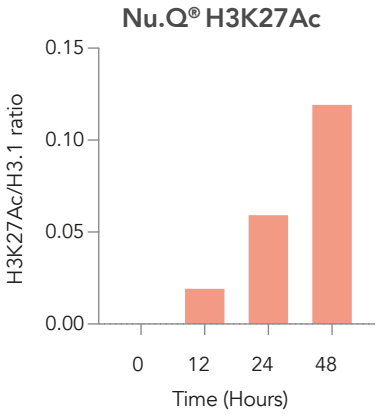
Two drugs were tested to determine if global acetylation changes could be detected using Nu.Q® Discover assays. Valproic Acid was used to test the effect of a histone deacetylase inhibitor (HDACi) and Curcumin was used to test histone acetyltransferase inhibition (HATi) ***(Table 1)***. These two drugs are believed to influence global acetylation in K562 cells. A Nu.Q® Discover assay targeting H3K27ac, showed the expected dose dependent increase in acetylation ***(Figure 1A)***. The increase in acetylation on H3 upon HDACi treatment was confirmed by western blot ***(Figure 1B)***. Nu.Q® Discover assays targeting H3K27ac, H3K9ac, and H3K14ac showed an accumulation of acetylation levels with time ***(Figure 1C)***. Interestingly the increase in H3K27ac was accompanied by a concomitant decrease in methylation as measured by the H3K27me3 Nu.Q® Discover assay ***(Figure 1D),*** which supports potential compensatory changes in chromatin structure following drug treatment.











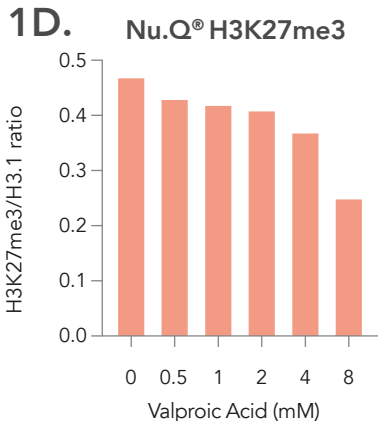


Figure 1: Histone PTM Changes in response to treatment with the HDACi Valproic Acid. (A): Nu.Q® Discover assay showing increase in acetylation with increasing HDACi. (B): Western blot confirming increase in acetylation. (C): Time course of increasing acetylation at a variety of histone residues upon treatment with Valproic Acid. (D): Together with (A) As increasing Valproic acid treatment increases acetylation at K3K27, there is a corresponding decrease in methylation at the same histone residue.

With the HATi, Curcumin, we were not able to observe a consistent decrease in acetylation, which is likely due to a general low abundance of all the acetylation marks, making it difficult to see a further reduction upon treatment ***(Figure 2)***. Interestingly, at 20uM we see an increase in acetylation levels, but this could be an artifact of poor cell viability at that concentration. Based on these results, we would not recommend using these assays for experiments which involve HATi's, unless one is working in a system with elevated acetylation levels beyond what is seen in K562 cells.

2.

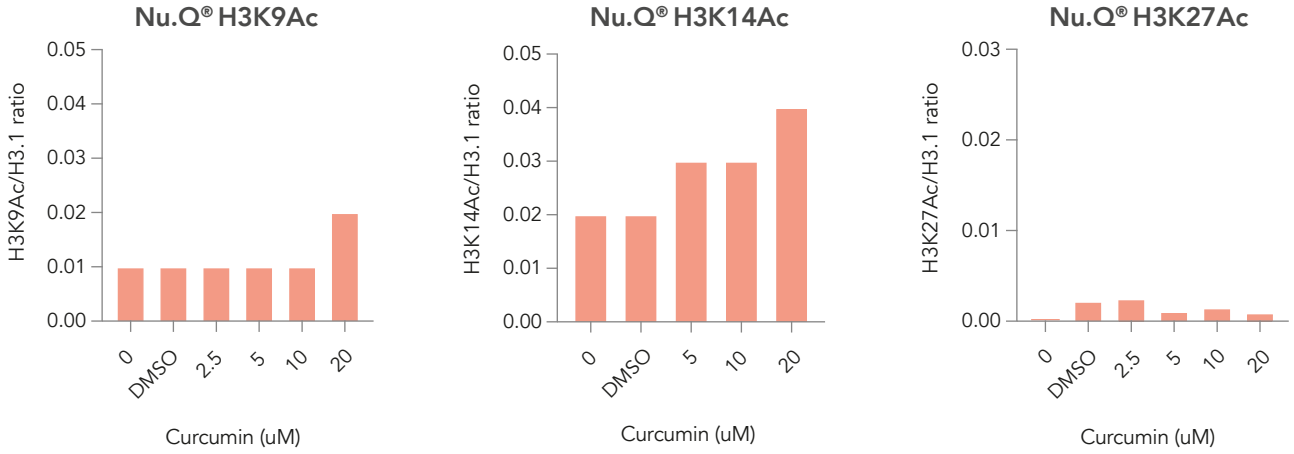


Figure 2: K562 Acetylation Responses to Curcumin (HATi)

Methylation:

The drugs BIX-01294 and 2-PCPA were used to measure the effects of inhibition of histone lysine methyltransferase (KMTi) and histone lysine demethylases (KDMi), respectively. The Nu.Q® Discover assay targeting H3K27me3 showed an expected decrease in methylation in a dose responsive manner with increasing concentrations of BIX-01294 having a larger effect ***(Figure 3A)***. Inverse to the pattern seen with Valproic acid, the decrease in H3K27me3 corresponded to an increase in acetylation in H3K27Ac ***(Figure 3B)*** demonstrating a change in the epigenetic signatures in response to epigenetic therapies could have broader impact on genomic function and transcription beyond the modification directly targeted.

The pan demethylase inhibiting activity of 2-PCPA is demonstrated by dose dependent increases in H3K4me1 and H3K4me2 Nu.Q® assays ***(Figures 3C and 3D)***.

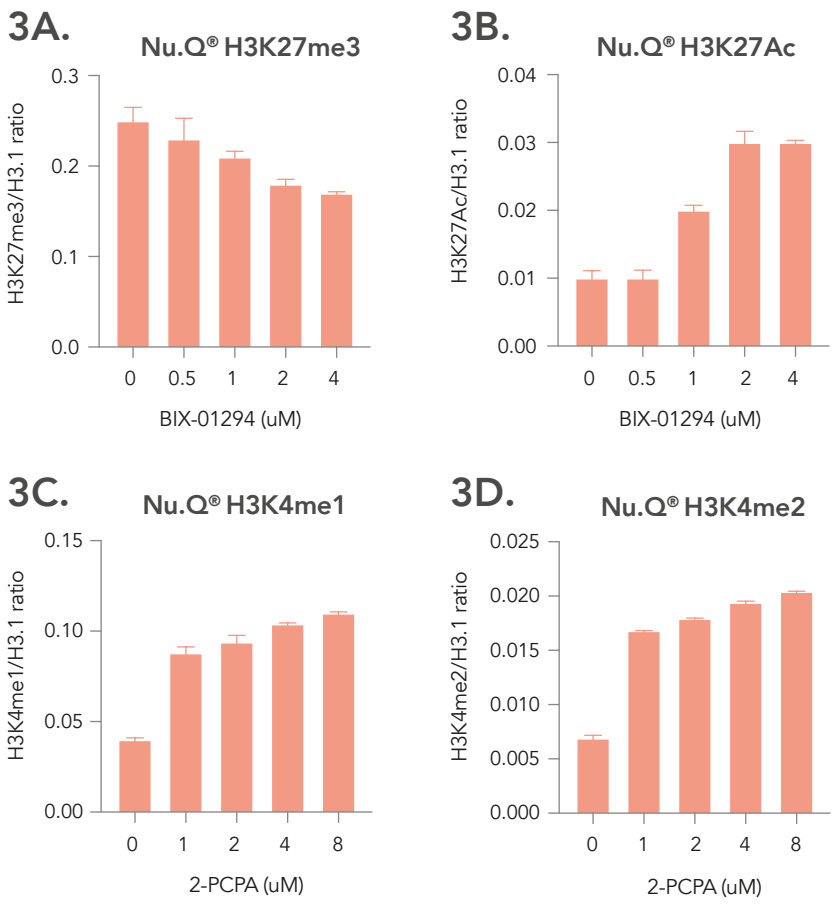


Figure 3: K562 Methylation Responses to BIX-01294 (KMTi) and 2-PCPA (KDMi): (A): Nu.Q® Discover assays showing that increasing KMTi results in decreasing methylation. (B): Nu.Q® Discover assay showing that as increasing KMTi decreases methylation at histone lysine 27, a corresponding increase in acetylation at H3K27 can be seen. (C): Nu.Q® Discover assays showing increase in H3K4 me1 with increasing KDMi. (D): Nu.Q® Discover assays showing increase in H3K4 me2 with increasing KDMi.

Non-Epigenetic Control, PKCi:

Chelerythrine Chloride, a known protein kinase C inhibitor, was included as a control targeting a non-epigenetic regulatory mechanism and showed little variability between all the marks was expected ***(Figure 4)***. There was quite a uniform response with the methylation targets, as little variability within each assay was seen ***(Figure 4)***.

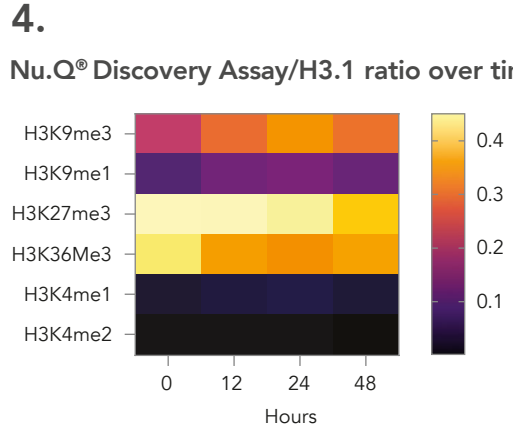


Figure 4: Heatmap of various Nu.Q® Discover Assays in response to a protein kinase C inhibitor (chelerythrine chloride): Nu.Q® Discover assay methylation response over time with increasing concentration of PKCi.

Discussion

This study evaluated numerous Nu.Q® Discover Assays on the K562 leukemia cell line treated with a variety of drugs targeting epigenetic regulatory enzymes. Leveraging this widely employed cell line, the project demonstrated the ability of Nu.Q® Discover to discern fluctuations in epigenetic marks following drug treatment.

Nu.Q® Discover assays demonstrated changes in Histone PTM levels in a dose-dependent manner across various assays tested. Each experimental condition evaluated five to nine Nu.Q® Discover Assays. Using the H3K27ac Nu.Q® Discover assay, we demonstrated dose-response profiles by comparing treated and untreated groups, encompassing concentrations above and below the drug's reported IC_{50}. While literature acknowledges IC_{50} variability among different drugs, our initial attempts yielded promising outcomes. Importantly, concentrations ranging from 0 – 8 mM of the histone deacetylase inhibitor, Valproic Acid, established the feasibility of generating a dose-dependent response. These insights were also shown through Western blot analyses, confirming the direct correlation between elevated HDACi concentrations and increased acetylation levels. Simultaneously, this examination highlighted a temporal aspect, as heightened acetylation manifested over specific time intervals (0-hour, 12-hour, 24-hour, and 48-hour) under maximum inhibition conditions (8mM). Interestingly, this was accompanied by a concurrent reduction in methylation levels which could warrant exploration in the future.

H3K27 showed an inverse relationship where an increase in acetylation was matched with a diminished methylation response, in response to valproic acid. Exploring the effects of the histone acetyltransferase inhibitor curcumin revealed complexities. The modest baseline levels of acetylation in untreated cells rendered the Nu.Q® Discover assays less responsive, making it challenging to detect subtle acetylation changes – highlighting the assays' detection limitations.

Histone lysine demethylase inhibition validated Nu.Q® Discover assay's reliability in detecting heightened methylation alongside increasing inhibitor concentrations. Conversely, the application of a histone lysine methyltransferase inhibitor, BIX-01294, demonstrated marked reductions in methylation patterns, a consistent trend across diverse Nu.Q® Discover assays corroborated by Western blot validations. This finding accentuated the assay's sensitivity in tracking methylation shifts and underscored its ability to monitor concomitant acetylation alterations.

Assessment of non-epigenetic controls, exemplified by the protein kinase C inhibitor chelerythrine chloride, revealed no significant effect on histone PTMs even with escalating concentrations over defined timeframes.

Considering this is the initial endeavor utilizing Nu.Q® Discover assays to decipher inhibitor-mediated epigenetic changes within cell lines, the findings underscore the assays' robust sensitivity, aligning well with anticipated outcomes stemming from effective inhibition. Subsequent investigations can refine the approach to produce more focused datasets. This exploratory study establishes a foundational understanding of optimization requirements.

Conclusion

In summary, this study has yielded several significant insights into the potential use of Nu.Q® Discover assays in deciphering epigenetic modifications. Foremost among these findings is the superiority of methylation targets in comparison to acetylation targets. This contrast in performance could be attributed to the inherent abundance of methylation marks, rendering them more detectable and responsive within the assay framework. Equally noteworthy is the partial success observed with acetylation marks. While the current scope of the study limits the extent of this observation, it underscores the potential for detecting acetylation changes under different conditions or with alternate compounds, inviting further exploration.

Within the confines of K562 cells as the experimental model, the assays demonstrated sensitivity, particularly in the context of methylation assays. This attests to the suitability of K562 cells for investigating dynamic epigenetic changes.

The deliberate inclusion of a negative control drug validated the assays' ability to discern expected responses, further bolstering the reliability. Overall, this project effectively highlights the sensitivity and capability of Nu.Q® Discover assays in capturing the intricacies of epigenetic alterations.

In conclusion, this study underscores the potential of Nu.Q® Discover assays as tools for probing epigenetic modifications. This demonstration of sensitivity and efficacy paves the way for further refinement and collaborative efforts.

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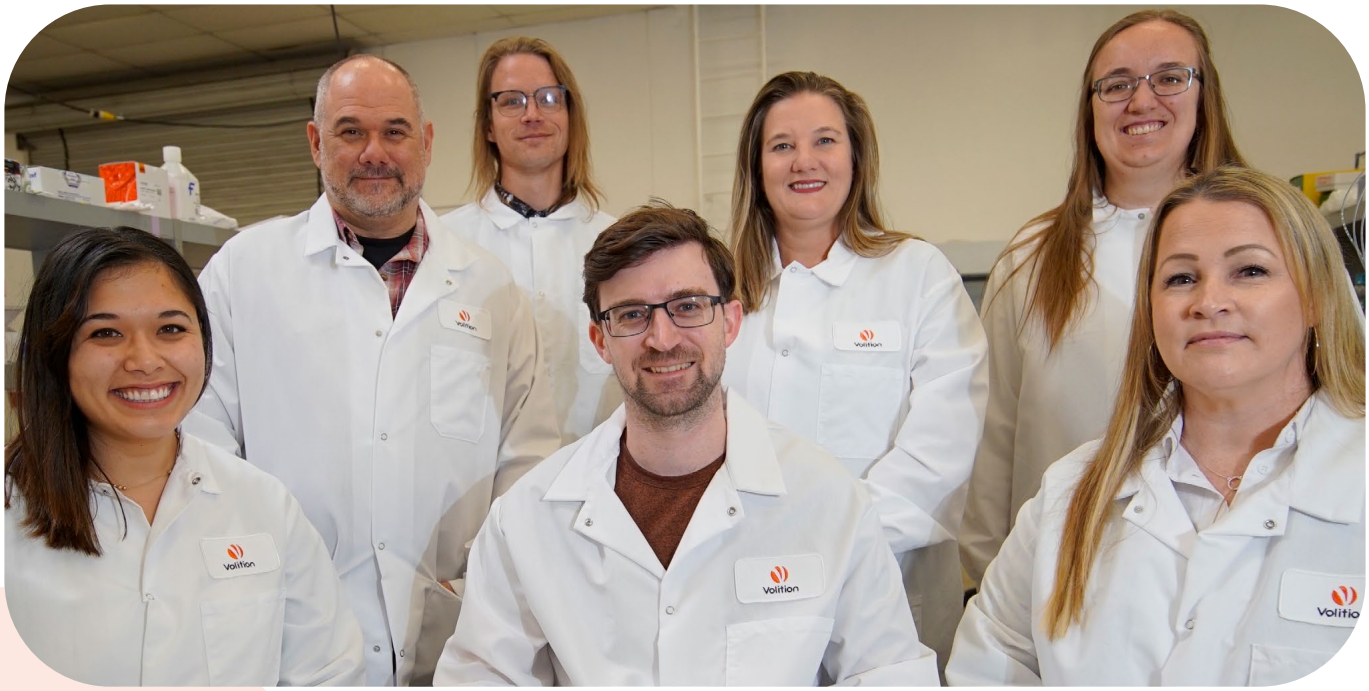

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About Volition

Volition is a multinational epigenetics company, powered by Nu.Q®, our proprietary nucleosome quantification platform. Our Nu.Q® Discover program enables drug developers and scientists access to a range of state-of-the-art assays for rapid epigenetic profiling in disease model development, preclinical testing, and clinical studies.

For details go to:
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