Chapter 13

The dCypher Approach to Interrogate Chromatin Reader Activity Against Posttranslational Modification-Defined Histone Peptides and Nucleosomes

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Abstract

Bulk chromatin encompasses complex sets of histone posttranslational modifications (PTMs) that recruit (or repel) the diverse reader domains of Chromatin-Associated Proteins (CAPs) to regulate genome processes (e.g., gene expression, DNA repair, mitotic transmission). The binding preference of reader domains for their PTMs mediates localization and functional output, and are often dysregulated in disease. As such, understanding chromatin interactions may lead to novel therapeutic strategies, However the immense chemical diversity of histone PTMs, combined with low-throughput, variable, and nonquantitative methods, has defied accurate CAP characterization. This chapter provides a detailed protocol for *dCypher*, a novel approach for the rapid, quantitative interrogation of CAPs (as mono- or multivalent Queries) against large panels (10s to 100s) of PTM-defined histone peptide and semisynthetic nucleosomes (the potential Targets). We describe key optimization steps and controls to generate robust binding data. Further, we compare the utility of histone peptide and nucleosome substrates in CAP studies, outlining important considerations in experimental design and data interpretation.

Key words Chromatin binding assay, Histone code, Histone posttranslational modifications, Histone PTMs, Histone PTM binding specificity, Histone peptides, Reader domain, Semisynthetic nucleosomes

1 Introduction

Chromatin is an essential regulatory component of multiple cellular processes, including transcriptional state [1–4] and disease development [5–7]. Its structures are highly dynamic, comprising a complex network of modifications to the DNA (e.g., cytosine methylation) and histone proteins (e.g., lysine methylation/acylation/ubiquitylation, arginine methylation/citrullination, serine phosphorylation; collectively termed posttranslational modifications [PTMs]). These covalent changes are mediated and

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interpreted by specific chromatin-associated "writers," "readers," and "erasers" to control local genome access and downstream function [8, 9]. This systems-level regulatory information is termed the "histone code," and its elucidation is key to understanding chromatin function [8, 10, 11].

A range of reader domain families and their preference for various PTM classes has been described, including (but not limited to): bromo- and YEATS domains for lysine acylations [12–17]; chromo, TUDOR and PWWP domains for lysine methylations [18–22]; and ubiquitin-dependent recruitment regions (UDRs) for lysine ubiquitylations [23, 24]. Deciphering the binding preference of particular chromatin readers for their histone PTMs (residue and class: e.g., H3 lysine 4 trimethyl [H3K4me3]) is a growing area of research, and will permit the targeting of specific pathways with therapeutic intent [7, 25–27]. However, these efforts have been challenged by the sheer diversity of PTMs [28], which may work alone, in combination, or in opposition, to engage multi-domain chromatin-associated proteins (CAPs) [29, 30]. Thus, methods to interrogate chromatin interactions must be highly efficient and easily modifiable to accommodate different reader domain classes and their modes of engagement, as well as to enable screening against diverse targets.

Historically, histone PTM-binding specificities have been studied using peptide microarrays (e.g., *EpiCypher* EpiTriton™), where libraries of modified histone peptides are spotted onto glass slides [31–33]. The format allows researchers to screen protein QUERIES against hundreds of single or combinatorial PTMs (the potential TARGETS). Histone peptide arrays have been used to characterize many classes of chromatin readers (e.g., chromo-, bromo-, Tudor domains; for a full review see [34]) and modifying enzymes (e.g., lysine methyltransferases G9a [35] and NSD1 [36]). However, the resulting data are largely qualitative, with low sensitivity, narrow signal-to-background windows, and suffer from the high levels of variation inherent to microarrays [37]. This format also requires a large amount of purified Query (μM range), making it difficult to titrate concentrations and explore buffer formulations/cofactor additions. Such optimizations are essential to reduce background, improve assay reliability, and thus begin to generate the quantitative analyses required for cross-Query comparisons.

There is an additional major concern: the historical focus on histone peptides disregards the significance of nucleosome structure in modulating chromatin binding events. In typical portrayals of nucleosome structure, the histone N-terminal tails extend from the nucleosome core and are thus easily accessible. Yet, multiple approaches show the tails often make extensive contacts with nucleosomal DNA [38]. Certain PTMs, such as acetylation and phosphorylation, may act to weaken these contacts, allowing

readers of other PTMs on the same tail to engage their target [38–41]. Further, many chromatin readers and enzymes make multivalent contacts with histone PTMs, nucleosomal DNA (e.g., via AT-hooks or PWWPs [42–45]), and/or the nucleosome core (the H2A/H2B acidic patch being a particular hub [46, 47]). Such multivalent interactions are often involved in histone PTM cross talk and can promote or inhibit chromatin binding. Thus, it is no great surprise that many chromatin-modifying enzymes require a nucleosome substrate for activity (e.g., NSD2 [42], LSD1 [48, 49], and DOT1L [50]), or show dramatically different kinetics to nucleosomes vs. peptides (e.g., SetD8 [51]). As a result of these complexities, most putative chromatin readers domains, and the means by which they act in concert in a given CAP, remain uncharacterized [52, 53], and nucleosome-based data will almost certainly be required for maximal insight.

To address these issues, we developed *dCypher*® as a novel and highly adaptable system for high-throughput CAP profiling. The approach uses chemiluminescent bead-based, no-wash Alpha technology (*see* **Note 1**), and delivers massive gains in sensitivity, flexibility, and throughput relative to histone peptide arrays. Of particular note, *dCypher* is fully compatible with PTM-defined histone peptides and semisynthetic nucleosomes (Fig. 1). In brief, biotinylated peptide or nucleosome substrates (the potential TARGETS) are coupled to streptavidin-coated "Donor" beads, while epitope-tagged proteins (QUERIES; from single domains to

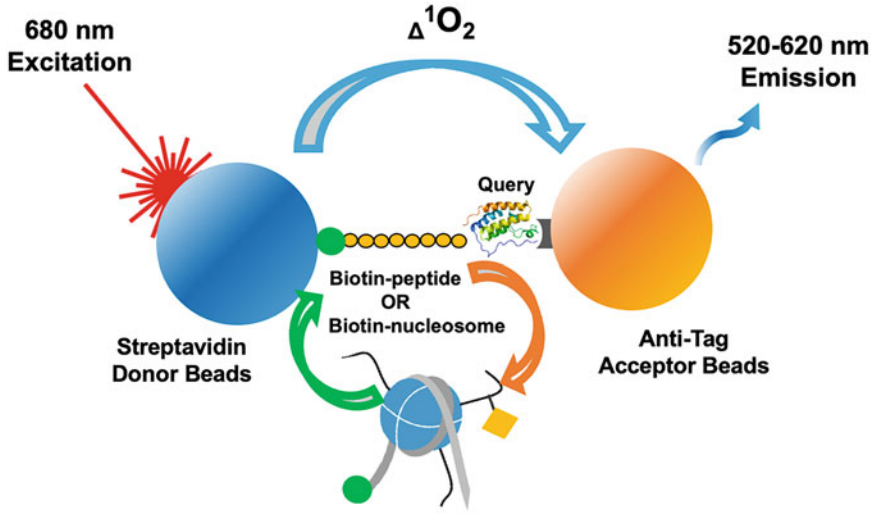


Fig. 1 Depiction of amplified luminescence proximity homogeneous assay (Alpha) technology (*see* **Note 1**). Alpha Donor and Acceptor beads are brought into proximity via [Target: Query] binding. Laser excitation (680 nm) of the Donor releases singlet oxygen that causes emission (520–570 nm) in proximal (within 200 nm) Donor beads; this luminescent signal is directly proportional to the amount of [Donor-Acceptor] complex bridged by the [Target: Query] interaction [23, 54–56]

complexes) are bound to anti-tag "Acceptor" beads (*see* **Note 2**). After mixing potential reactants, the Donor beads are excited at 680 nm, releasing a singlet oxygen that causes emission (520–620 nm) in proximal (within 200 nm) Acceptor beads; this luminescent signal is directly correlated to interaction/binding affinity [23, 54–56]. *dCypher* assays are performed in 384-well plates, enabling high-throughput analysis of potential [Query: Target] interactions.

We have now used *dCypher* to characterize multiple classes of potential chromatin binding domains in mono- or multivalent format against peptide and nucleosome substrates [23, 54–56]. Beyond exploring binding preference, *dCypher* was recently used to characterize a selective/potent inhibitor of nucleosomal H3K36me2/3 binding by the NSD2 N-terminal PWWP domain (representing a potential pathway to a high value therapeutic) [57]. Our extensive studies emphasize the need for rigorous assay optimization when exploring CAP capability, and the central importance of nucleosome context (see below). *dCypher* is uniquely suited for such work. Due to its high sensitivity, we are often able to use 100–1000-fold less Query protein compared to peptide arrays. Indeed, the ability to screen in multiwell plates allows the user to independently titrate the concentration of Query proteins, salt, and potential cofactors/competitors (e.g., free DNA), against potential Targets (nucleosome, peptide, or DNA) in parallel reactions. The resulting data show CAPs can be profoundly impacted by context. As an example, while *dCypher* confirms that the HP1β chromodomain binds all three H3K9 methyl states (me1, me2 and me3) on histone peptides (and makes no discrimination between me3 and me2) [58], it reveals an absolute preference for H3K9me3 nucleosomes (compare Fig. 2a, b). We propose the revised specificity on nucleosome substrates to be the more likely physiological state, and potentially driven by multivalent interactions (enhancing and inhibitory) between histone tails and other nucleosome surfaces. It also has profound implications: methyltransferases or demethylases that convert the H3K9me3 state are now of central importance to mechanistic studies of HP1β function.

Notably, some proteins require a more extensive *dCypher* workflow to reveal their true binding specificity on nucleosomes. In initial assays profiling the DNMT3A PWWP domain, we observed only weak binding to H3K36 methylated nucleosomes (their reported Target [22, 59]). We thus performed an extensive 2D [Query vs. Salt] titration, analyzing the impact of salt (NaCl) concentration on DNMT3A PWWP binding against H3K36me2 (Target) and unmodified (Control) nucleosomes. This showed the domain was highly salt-sensitive, exhibiting PTM selectivity within a narrow range (Fig. 3a). Running the assay at 100 mM NaCl provided the window to probe a large nucleosome panel and identify the exquisite selectivity of DNMT3A PWWP for H3K36me2/3

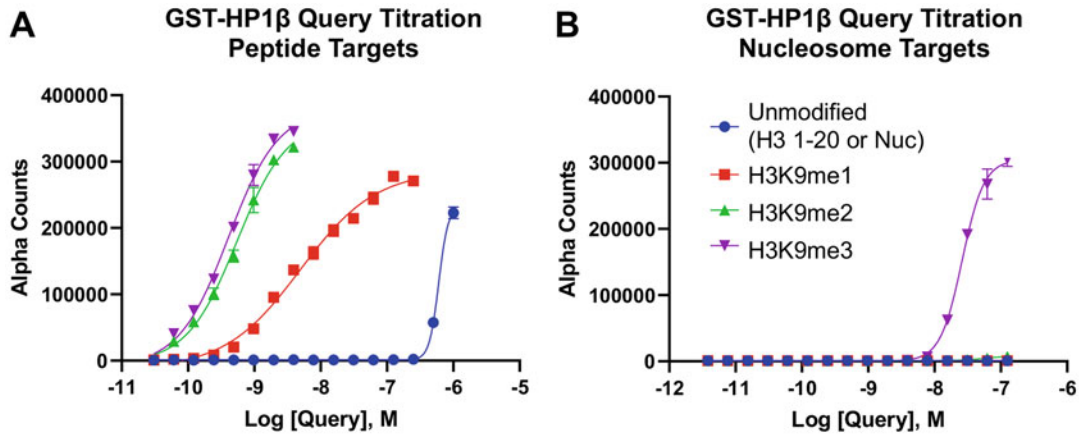


Fig. 2 (**a**) Titration of GST-HP1β chromodomain (UniProt P83916; aa1-185) against peptide Targets (key in **b**) reveals equivalent binding to H3K9me3/me2 and reduced binding to H3K9me1. (**b**) GST-HP1β chromodomain shows a dramatically refined specificity on nucleosomes, binding only H3K9me3 (*see* **Note 8**)

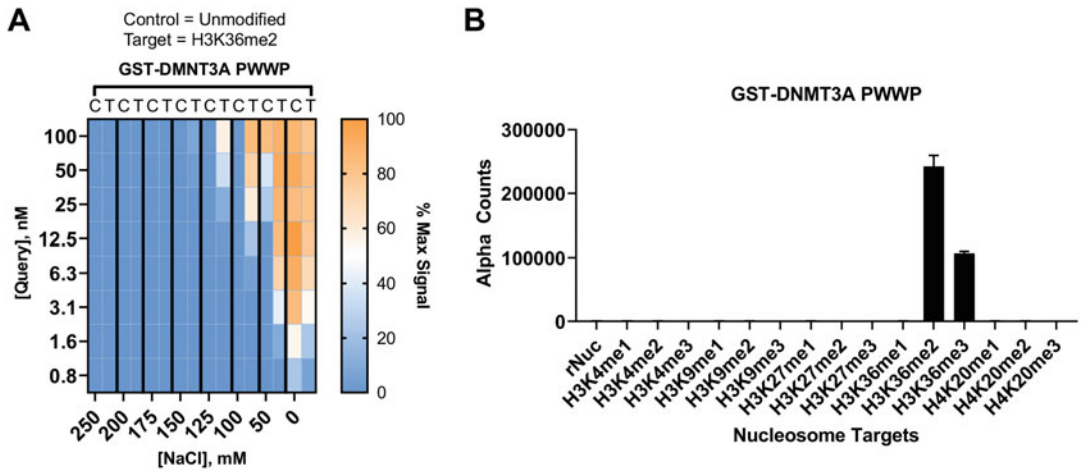


Fig. 3 (**a**) 2D [Query vs. Salt] titration of GST-DNMT3A PWWP (UniProt Q6Y6K1; aa278-432) on nucleosomes identifies NaCl sensitivity (rapid signal drop off >100 mM). Buffer supplemented with 100 mM NaCl was determined as the optimal signal window (note selectivity for H3K36me2 over unmodified nucleosomes). Data is normalized to the maximal Alpha signal on the plate. (**b**) Discovery Screen testing of DNMT3A PWWP in nucleosome assay buffer with 100 mM NaCl identifies a preference for H3K36me2 > me3 and no discernable interaction with all other lysine-methylated nucleosome in the panel (*X*-axis; rNuc, unmodified Nucleosome)

(Fig. 3b). This provided the mechanistic link from specific histone PTMs (H3K36me2/3) to DNMT3A recruitment, and thus how de novo DNA methylation is recruited to intergenic regions in vivo [54]. Furthermore, it explains the similar developmental pathologies associated with loss of function in H3K36 (*NSD1*: Sotos Syndrome) and DNA (*DNMT3A*: Tatton-Brown–Rahman syndrome) methyltransferases.

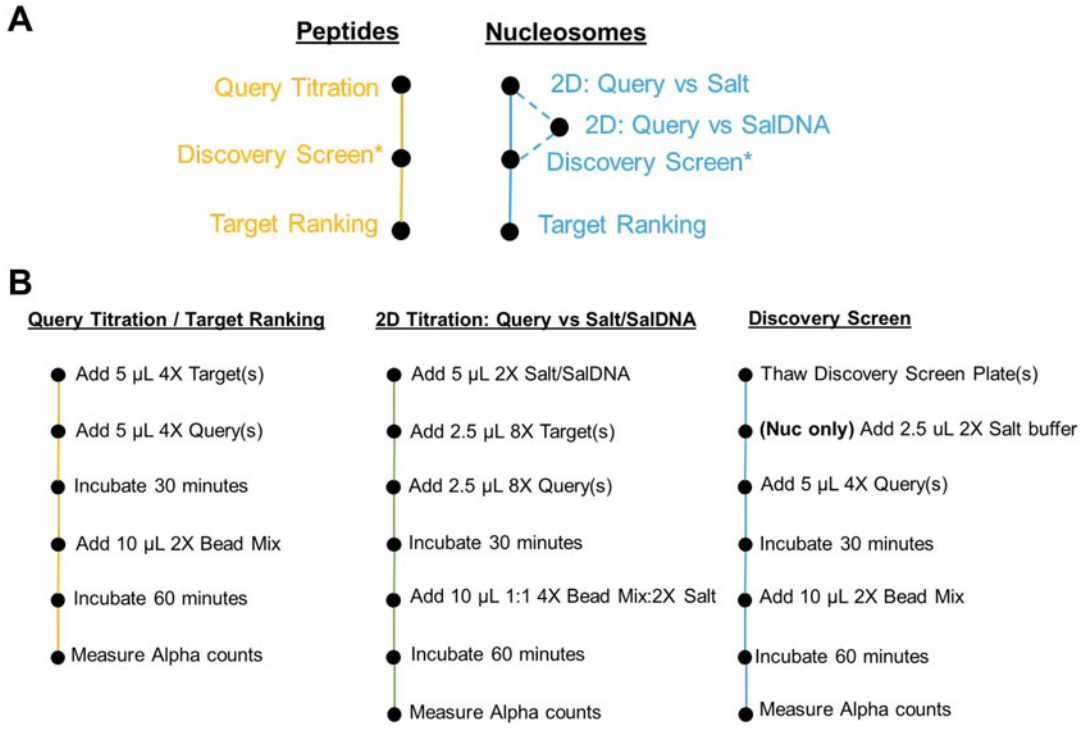


Fig. 4 Stepwise workflow for *dCypher* assay procedures (see Subheadings 3.1–3.5). (**a**) Comprehensive workflow overview to interrogate a chromatin reader. A typical study starts with titrating Query to a predicted histone peptide Target(s) to confirm activity and identify optimal probing concentration (i.e., good signal-over-background, on linear part of binding curve), and then progresses down the peptide branch or moves directly to nucleosomes. For Queries with no known Target a **Discovery Screen** (Subheadings 3.4 and 3.5) to the histone peptide panel at high and low concentrations (chosen from other reader domains of the same family; or *see* **Note 26**) can be performed, and the workflow then restarted with any hits to dial in optimal conditions. (**b**) Experimental guide for various study modules (i.e., order of addition, relative volumes, and incubation times)

This chapter contains a full outline of our *dCypher* pipeline (and its various modules) for testing Queries to PTM-defined peptide and nucleosome Targets (Fig. 4a, b). Of note, peptide-based *dCypher* assays generally do not require an exploration of salt concentration (and thus use a standard buffer). In contrast, salt titrations are always performed when developing nucleosome-based assays, as this often has a profound impact on Query binding (as above for DNMT3A PWWP). We also frequently use exogenous salmon sperm DNA (SalDNA) to interrogate DNA binding by Queries [57], particularly when moving to multidomain (and potentially multivalent) proteins with poorly characterized regions. These experimental modules highlight the complexity inherent to nucleosome studies.

dCypher has proven a powerful approach to interrogate CAP binding, and its application has revealed novel insight to the mechanisms underlying chromatin structure and function

[23, 54–56]. Many results (e.g., Fig. 2) raise important questions about current standards in chromatin methodology, particularly the continued reliance on a reductionist approach of isolated reader domains as Queries and PTM-defined histone peptides as Targets. With its ability to incorporate PTM-defined nucleosomes, *dCypher* will provide the means for physiologically relevant epigenetic discovery.

The *dCypher* workflow has been developed to guide the interrogation of CAP Queries against PTM-defined histone peptides and nucleosome Targets. The approach can be broken into various experimental modules (Fig. 4a), which are optimized to run sequentially in rapid throughput while controlling material consumption (Fig. 4b). Prior to performing the assay, it is necessary to select your protein Query and potential Targets. Epitope-tagged recombinant protein domains (or full-length proteins) can be expressed in-house or obtained from commercial sources. We have found the GST-tag (~220 aa/~26 kDa) to be reliable and produce robust results, but other epitope-fusions (6His and FLAG [DYKDDDDK]) are also compatible (*see* **Note 2**). For biotinylated Targets, PTM-defined histone peptides and semisynthetic nucleosomes are available from *EpiCypher* and were used to develop the *dCypher* platform.

The **first** step of the *dCypher* workflow involves optimization of Query binding to known or predicted PTM-defined targets: this includes exploring protein concentration, buffer conditions (e.g., salt), and potential supplements (e.g., exogenous SalDNA). **Second**, the optimized assay is used to evaluate Query binding against a large panel of potential Targets to determine preference, secondary interactions (e.g., with DNA or the acidic patch [by coupling biotinylated DNA or acid patch mutant nucleosomes to the Acceptor beads]), and the impact of neighboring PTMs. In the **third** and final step, identified Targets are ranked by their relative EC_{50} (EC_{50}^{rel}) values (*see* **Note 3**). This process is usually performed sequentially: first using histone peptides for initial testing and Target confirmation (since this can formally confirm activity of the Query protein based on prior literature); then using nucleosomes as physiological substrates. However, the peptide screen is not required, and may be omitted in favor of focusing on nucleosomes.

2 Materials

A complete list of consumables and equipment needed to perform *dCypher* assays. All peptides and nucleosomes are biotinylated. All solutions are prepared using ultrapure water (deionized to 18 MΩ-cm at 25 °C).

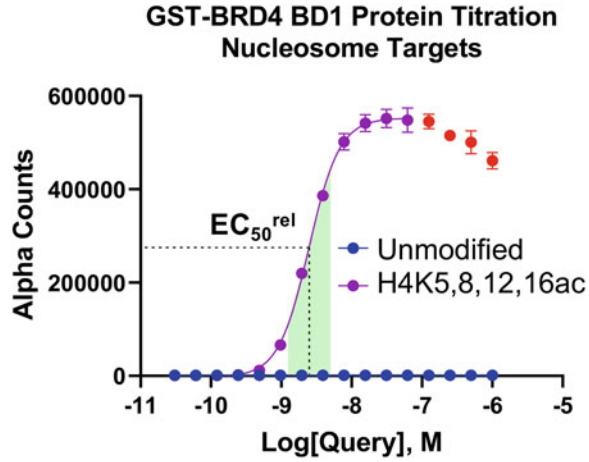


Fig. 6 Titration of GST-BRD4 BD1 (UniProt O60885; aa41-180) against unmodified and H4K5,8,12,16 ac Target nucleosomes. Dashed lines represent the relative EC_{50} (EC_{50}^{rel}, 2.5 nM) for BRD4 BD1 binding to H4K5,8,12,16 ac. Red circles represent assay points removed from analysis due to the hook point being reached (*see* **Note 15**): this indicates bead saturation/declining signal as excess nonbead bound Query is now competing with that on the Acceptor beads for Target binding. Green shaded area represents the optimal probing concentration range (shown is relative EC_{20}–EC_{80}) (*see* **Note 15**)

2.1 General Reagents

1. GST-, 6His-, or FLAG-tagged Query (*see* **Note 2**).
2. GST-HP1β (as in Fig. 2: UniProt P83916; aa1-185).
3. GST-DNMT3A (as in Fig. 3: UniProt Q9Y6K1; aa278-432).
4. GST-BRD4 BD1 (as in Fig. 6: UniProt O60885; aa41-180).
5. Biotinylated Peptides (*see* **Note 4**).
6. Biotinylated Nucleosomes (e.g., *EpiCypher* #16-9001).
7. Poly-L-lysine.
8. Salmon Sperm DNA (SalDNA).
9. Streptavidin Donor Beads (*see* **Note 5**).
10. Glutathione Acceptor Beads.
11. Nickel-Chelate Acceptor Beads.
12. Protein-A Acceptor Beads.
13. Anti-FLAG Antibody.

2.2 Buffers

Assay buffers (compositions as noted) are prepared fresh for each experiment and kept at room temperature (unless otherwise specified).

1. Peptide reconstitution solution: 0.01% BSA in ddH_2O.
2. Peptide assay buffer: 50 mM Tris pH 7.5, 50 mM NaCl, 0.01% Tween 20, 0.01% BSA, 1 mM TCEP, 0.0004% poly-L-lysine (*see* **Note 6**).
3. Nucleosome assay buffer: 20 mM Tris pH 7.5, 0–250 mM NaCl, 0.01% NP-40 alternative, 0.01% BSA, 1 mM DTT.

2.3 Equipment

1. AlphaPlate-384 (Assay Plate; *PerkinElmer* 6005350) or similar product.
2. 384 Deep Well Plate (Dilution plate; *Greiner Bio-One* 781270) or similar product.
3. 1.5 mL Microtubes (Lo-bind).
4. 50 mL conical tubes.
5. Divided Reservoirs.
6. TopSeal A-PLUS (*PerkinElmer* 6050185) or similar product.
7. TopSeal A Black (*PerkinElmer* 6050173) or similar product.
8. MicroAmp Adhesive Film (Storage seals: *Applied Biosystems* 4311971) or similar product.
9. Set of single channel pipettes (0.1–1000 μL).
10. 16-channel pipette (1–10 μL).
11. 16-channel electronic pipette (5–50 μL).
12. Microplate centrifuge.
13. Personal Incubator.
14. EnVision Plate Reader (*PerkinElmer* 2105-0010) (*see* **Note 1**).
15. AlphaScreen Mirror D640as (*PerkinElmer* barcode #444) (*see* **Note 1**).
16. AlphaScreen/AlphaLISA Emission Filter (*PerkinElmer* barcode #244) (*see* **Note 1**).
17. AlphaLISA Emission Filter M615 (*PerkinElmer* barcode #203) (*see* **Note 1**).

3 Methods

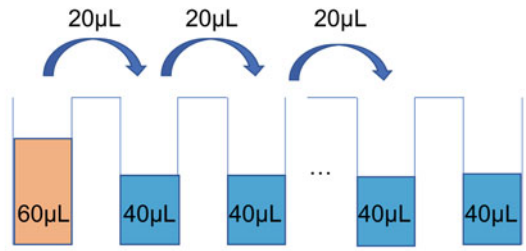
These protocols provide conditions, volumes, and concentrations compatible with GST-tagged protein Queries, although 6HIS or FLAG-tagged proteins may be substituted (*see* **Note 2**). All volume calculations are precise (so desired dead volumes must be added). All procedures are performed at room temperature and incubation steps at 23 °C unless otherwise noted. After each reagent addition, Assay plates should be firmly tapped on the lab bench, a plate seal applied (*see* **Note 7**), and centrifuged briefly (600 × *g* for 10–15 s). It is *highly recommended* that each experiment includes positive control reactions with a similarly tagged Query (*see* **Note 8**).

3.1 Query Titration, Target Ranking

The following protocol describes titration and target ranking experiments using a *GST-tagged* Query and a Target and Control substrate (*see* **Note 9**). For peptide-based assays with a known or suspected Target, we recommend starting with Query titrations (Subheading 3.1: e.g., Fig. 2a). However, when using nucleosomes, a 2D [Query vs. Salt] titration is the suggested first step, as precise salt conditions are often critical for appreciable binding to nucleosome substrates (Subheading 3.2: e.g., Fig. 3a). Query titrations assess binding to an anticipated Target, related PTMs, and a negative control. Results from these assays provide essential information for additional testing: (1) confirm Query functionality and target preference; (2) determine optimal probing concentration (and if salt, competitor DNA, or other additives are needed; *see* Subheadings 3.2 and 3.3); (3) identify any hook point (i.e., maximal Query concentration before bead saturation); and (4) calculate EC_{50} relative (EC_{50}^{rel}) values. Query binding to PTM-defined peptide/nucleosome targets are ranked using their respective EC_{50}^{rel} values, enabling quantitative comparisons across Queries and Targets. This information informs the design of subsequent discovery screens with a larger set of potential Targets (Subheadings 3.4 and 3.5).

1. Plan the desired plate layout and calculate the needed quantities of buffer, Query (or Queries), and Target (or Targets) (*see* **Notes 8–10**).
2. Thaw GST-tagged Queries and peptides/nucleosomes on ice (*see* **Note 11**).
3. Prepare 10 mL of peptide or nucleosome assay buffer immediately before use in a 50 mL conical tube (*see* **Note 6**).
4. Peptide Targets: Prepare 4× (400 nM) peptide dilutions by adding 2.4 μL of each peptide (20 μM) to 117.6 μL peptide assay buffer in 1.5 mL tubes.

   Nucleosome Targets (*see* **Note 12**): Prepare 4× (40 nM) by adding 6.4 μL of each nucleosome (1.5 μM) to 113.6 μL nucleosome assay buffer in 1.5 mL tubes. Prepare these dilutions immediately before use and keep at room temperature.
5. Prepare the highest concentration of Query dilution (4×) by adding 12 μL of Query (20 μM) to 228 μL assay buffer in a 1.5 mL tube.
6. In a Dilution plate (Subheading 2.3), serially dilute the 4× Query (threefold; 20 μL Query into 40 μL assay buffer) to prepare 11 testing concentrations plus a buffer-only control (12th point) (*see* **Note 13**/Fig. 5).
7. Add 5 μL of 4× diluted Target(s)/Control to their assigned well positions in the Assay plate.
8. Add 5 μL of serially diluted Query to the Assay plate.



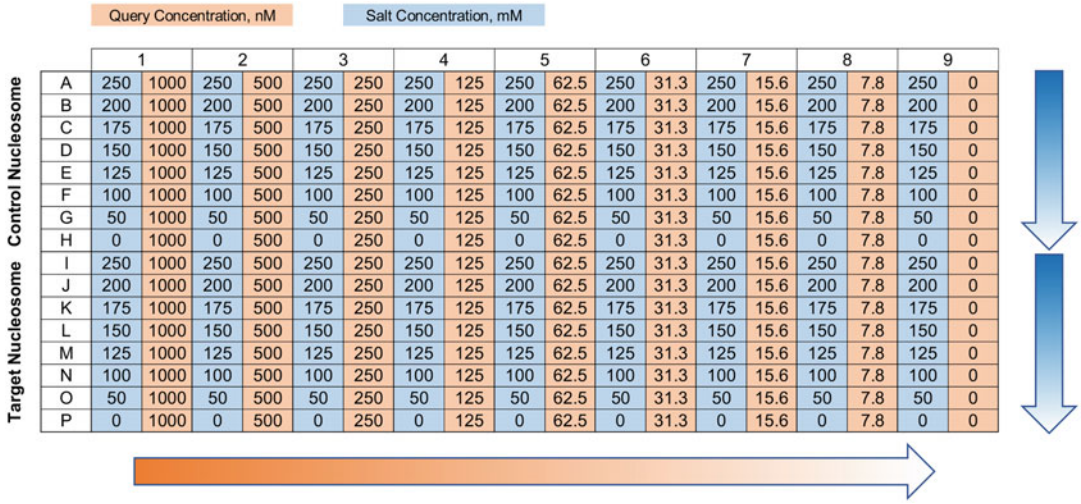
Serial #	1	2	3	11	12
[Query] in 5μL	4 μM	1.3 μM	444 nM	67.7 pM	0
[Query] in 20μL	1 μM	333 nM	111 nM	16.9 pM	0

Fig. 5 Threefold serial dilution of Query in a 384-well deep Dilution plate. Query concentration in 5 μL is when added to the plate; concentration in 20 μL represents that after all reagents have been added

9. Place Assay plate in 23 °C incubator for 30 min.
10. During the incubation, move to a subdued lighting area and prepare a 2× mixture by adding 0.48 μL of 5 mg/mL AlphaLISA Glutathione Acceptor beads (5 μg/mL) and 0.96 μL of 5 mg/mL Alpha Streptavidin Donor beads (10 μg/mL) to 478.6 μL appropriate assay buffer in a 1.5 mL tube (*see* **Notes 2**, **5**, and **14**).
11. Prepare a 4× bead mixture by adding 1.44 μL of 5 mg/mL AlphaLISA Glutathione Acceptor (10 μg/mL) and 2.88 μL of 5 mg/mL Alpha Streptavidin Donor beads (20 μg/mL) to 715.7 μL of appropriate assay buffer in a 1.5 mL tube.
12. Add 10 μL of the bead mixture to each well of Assay plate under subdued lighting.
13. Place Assay plate in 23 °C incubator for 60 min. Biotinylated peptide/nucleosome Targets will bind Donor beads and the GST-tagged Query will couple with Acceptor beads.
14. Remove the plate seal and measure Alpha counts using an *EnVision* plate reader (or similar) using Alpha settings (*see* **Note 1**).
15. Analyze data using *GraphPad Prism* (or similar) to: (1) identify hook point (if present); (2) determine optimal protein probing concentration; and (3) compute EC_{50}^{rel} for each Target (*see* **Note 15**/Fig. 6).

3.2 2D [Query vs. Salt] Titrations

This experimental module is designed to optimize both Query and Salt concentrations for *dCypher* assays. Given the salt sensitivity inherent to Query-nucleosome interactions (e.g., Fig. 3a) this module is a strongly recommended first step for testing Queries



	1		2		3		4		5		6		7		8		9	
A	250	1000	250	500	250	250	250	125	250	62.5	250	31.3	250	15.6	250	7.8	250	0
B	200	1000	200	500	200	250	200	125	200	62.5	200	31.3	200	15.6	200	7.8	200	0
C	175	1000	175	500	175	250	175	125	175	62.5	175	31.3	175	15.6	175	7.8	175	0
D	150	1000	150	500	150	250	150	125	150	62.5	150	31.3	150	15.6	150	7.8	150	0
E	125	1000	125	500	125	250	125	125	125	62.5	125	31.3	125	15.6	125	7.8	125	0
F	100	1000	100	500	100	250	100	125	100	62.5	100	31.3	100	15.6	100	7.8	100	0
G	50	1000	50	500	50	250	50	125	50	62.5	50	31.3	50	15.6	50	7.8	50	0
H	0	1000	0	500	0	250	0	125	0	62.5	0	31.3	0	15.6	0	7.8	0	0
I	250	1000	250	500	250	250	250	125	250	62.5	250	31.3	250	15.6	250	7.8	250	0
J	200	1000	200	500	200	250	200	125	200	62.5	200	31.3	200	15.6	200	7.8	200	0
K	175	1000	175	500	175	250	175	125	175	62.5	175	31.3	175	15.6	175	7.8	175	0
L	150	1000	150	500	150	250	150	125	150	62.5	150	31.3	150	15.6	150	7.8	150	0
M	125	1000	125	500	125	250	125	125	125	62.5	125	31.3	125	15.6	125	7.8	125	0
N	100	1000	100	500	100	250	100	125	100	62.5	100	31.3	100	15.6	100	7.8	100	0
O	50	1000	50	500	50	250	50	125	50	62.5	50	31.3	50	15.6	50	7.8	50	0
P	0	1000	0	500	0	250	0	125	0	62.5	0	31.3	0	15.6	0	7.8	0	0

Fig. 7 Example of a 2D [Query: Salt] titration 384-well plate map (columns 10–24 not shown) for a known Target and Control nucleosome. The Control is expected to represent a nonbinder for Query and is usually an unmodified nucleosome, though this depends on the mode of engagement (e.g., we occasionally use a nucleosome deleted of all histone tails [tailless] or with acid-patch mutations: **Note 9**). Salt concentration is titrated from top to bottom while protein is titrated from left to right (*see* **Note 17**)

to potential nucleosome Target(s). Results from this assay provide essential information to: (1) confirm protein functionality and target preference; (2) identify any hook point; (3) determine optimal probing concentration; (4) determine optimal salt condition; and (5) determine EC_{50}^{rel} values. In our experience this module is not generally required for peptide-based *dCypher* studies (which use a standard buffer).

1. Plan the desired plate layout and calculate the needed quantities of buffer, Query (or Queries), and Target (or Targets) (see **Notes 16** and **17**/Fig. 7).
2. Thaw biotinylated nucleosomes and GST-tagged Queries on ice.
3. Prepare 1 mL of 8 different 2× salt nucleosome assay buffers *with* 500, 400, 350, 300, 250, 200, 100, and 0 mM NaCl in 1.5 mL tubes. Transfer to a Dilution plate for easier pipetting (*see* **Note 17**).
4. Prepare 1× nucleosome assay buffer *without* salt.
5. Prepare 8× (80 nM) nucleosome dilutions by adding 9.6 μL of 1.5 μM nucleosome to 170.4 μL 1× nucleosome assay buffer *without* salt in 1.5 mL tubes.
6. Prepare the highest 8× (8 μM) Query dilution and controls by adding 36 μL of 20 μM Query to 54 μL 1× nucleosome assay buffer *without* salt in a 1.5 mL tube (*see* **Notes 17** and **18**).

7. Transfer Query solution to a Dilution plate, serially dilute 8 times (twofold; 45 μL Query into 45 μL buffer) in 1× nucleosome assay buffer *without* salt. Add a ninth point as a buffer-only control (*see* **Notes 13** and **19**).
8. In an Assay plate, add 5 μL of each 2× salt condition (from Dilution plate) where the concentration is fixed left to right and decreasing top to bottom (*see* **Note 17**/Fig. 7).
9. Add 2.5 μL of 8× nucleosomes to assigned wells (72 wells total/nucleosome).
10. Add 2.5 μL of serially diluted Query (from Dilution plate) where the concentration is decreasing left to right and fixed top to bottom (*see* **Note 17**/Fig. 7). The final Query concentrations will be 1 μM (1×) to 7.8 nM.
11. Place Assay plate in 23 °C incubator for 30 min.
12. During the 30-min incubation, move to a subdued lighting area and prepare a 4× bead mixture by adding 1.44 μL of 5 mg/mL AlphaLISA Glutathione Donor beads (10 μg/mL) and 2.88 μL of 5 mg/mL Alpha Streptavidin Donor beads (20 μg/mL) to 715.7 μL of nucleosome assay buffer *without* salt in a 1.5 mL tube (*see* **Note 14**).
13. Prepare a 1:1 mixture of 4× bead mix plus each 2× salt condition by mixing 45 μL of each 2× salt condition with 45 μL of the 4× bead mix for a total of 8 bead–salt mixes. Move mixes to a Dilution plate for easier transfers.
14. Add 10 μL of the bead–salt mixes in the same method as **step 8**.
15. Place Assay plate in 23 °C incubator for 60 min. Biotinylated nucleosome will bind Donor beads and the GST-tagged Query will couple with Acceptor beads.
16. Remove the plate seal and measure Alpha counts using an *EnVision* plate reader (or similar) using Alpha settings (*see* **Note 1**).
17. Analyze data using *GraphPad Prism* (or similar) to identify: (1) hook point (if present); (2) optimal probing concentration; (3) optimal salt condition; and (4) compute EC_{50}^{rel} for each Target (*see* **Notes 16** and **20**).

3.3 2D [Query vs. Salmon Sperm DNA (SalDNA)] Titration

The 2D [Protein vs. SalDNA] protocol is used in scenarios where Queries are thought to be contacting nucleosomal and/or linker DNA and masking histone PTM interactions [57]. DNA engagement typically manifests as equal binding to all nucleosomes regardless of PTM (assuming the same DNA length is used). If DNA binding is suspected, titrate Query against biotinylated nucleosome (s) and DNA Targets. If DNA binding is observed, the following procedure can be used to challenge the Query-DNA interaction and reveal underlying histone PTM interactions (if they exist).

Results from this assay provide essential information to: (1) confirm protein functionality and PTM target preference; (2) identify hook point (if present); (3) determine optimal probing concentration; (4) determine optimal SalDNA condition; and (5) determine EC_{50}^{rel} values.

1. Plan the desired plate layout and calculate the needed quantities of buffer, Query (or Queries), and Target (or Targets) (similar to **Note 18**).
2. Thaw biotinylated nucleosome(s) and Query on ice.
3. Prepare 1× nucleosome assay buffer using the optimal salt condition (Subheading 3.2: If unclear from initial studies supplementing with 150 mM NaCl is a good starting point).
4. Prepare 2× SalDNA dilution by adding 0.3 μL of 10 mg/mL SalDNA stock to 149.7 μL nucleosome assay buffer in a 1.5 mL tube.
5. Serially dilute 2× SalDNA by adding 50 μL SalDNA into 100 μL buffer (threefold). Prepare a total of 7 serial dilutions, including the 2× stock (i.e., 20, 6.67, 2.22, 0.74, 0.25, 0.08, 0.027, and 0 μg/mL) plus a buffer-only control (similar to **Note 13**).
6. Prepare 8× (80 nM) nucleosome dilutions by adding 9.6 μL of 1.5 μM nucleosome to 170.4 μL nucleosome assay buffer in 1.5 mL tubes.
7. Prepare the highest 8× (8 μM) Query dilution and controls, by adding 36 μL of 20 μM Query to 54 μL nucleosome assay buffer in a 1.5 mL tube (*see* **Note 19**).
8. Transfer Query solution to a dilution plate, serially dilute 8 times (twofold; 45 μL Query into 45 μL buffer) in nucleosome assay buffer and add a ninth point as a buffer-only control (similar to **Note 13**, *see* **Note 19**).
9. Add 5 μL of 2× SalDNA serial dilution where the concentration is fixed left to right and decreasing top to bottom over 9 columns (similar to **Note 17**).
10. Add 2.5 μL of 8× nucleosomes to assigned wells.
11. Add 2.5 μL of 8× serially diluted Query where the concentration is decreasing left to right and fixed top to bottom (similar to **Note 17**).
12. Place Assay plate in 23 °C incubator for 30 min.
13. During the 30-min incubation, move to a subdued lighting area and prepare a 4× bead mixture by adding 1.44 μL of 5 mg/mL AlphaLISA Glutathione Donor beads (10 μg/mL) and 2.88 μL of 5 mg/mL Alpha Streptavidin Donor beads (20 μg/mL) to 715.7 μL of nucleosome assay buffer in a 1.5 mL tube (*see* **Note 14**).

14. Prepare 1:1 mixture by combining 45 μL of each 2× SalDNA concentration with 45 μL of the 4× bead mix for a total of 8 bead–SalDNA mixes. Transfer mixes to a Dilution plate for easier transfers.
15. Add 10 μL of the bead–SalDNA mixes in the same method as **step 9**.
16. Place Assay plate in 23 °C incubator for 60 min. Biotinylated nucleosomes will bind Donor beads and the GST-tagged Query will couple with Acceptor beads.
17. Remove the plate seal and measure Alpha counts using an *EnVision* plate reader (or similar) using Alpha settings (*see* **Note 1**).
18. Analyze data using *GraphPad Prism* (or similar) to identify: (1) hook point (if present); (2) optimal probing concentration; (3) optimal SalDNA condition; and (4) EC_{50}^{rel} for each Target (*see* **Notes 15** and **21**).

3.4 Preparation of Discovery Screen Plate(s)

This protocol details the production of Discovery Screen plates (typically prepared in batch for greatest efficiency). The resulting peptide/nucleosome plates are intended to be stored at −80 °C, thawed once, and used to screen Queries against a broad set of targets (*see* **Notes 22–25**). Peptides and nucleosomes are *not* recommended for simultaneous testing given their different buffer requirements.

1. Generate a Discovery Screen plate map of the intended peptide/nucleosome targets and determine the number of plates to be prepared. Using each target concentration, calculate the volumes required (*see* **Note 22**).
2. Thaw all biotinylated peptides and/or nucleosome stocks on ice.

   Peptides: Prepare a modified peptide assay buffer *without* poly-L-lysine.

   Nucleosomes: Prepare a modified nucleosome assay buffer *without* NaCl. Keep buffers on ice.
3. Prepare 400 nM peptide or 80 nM nucleosome dilutions.

   Peptides: Add 2 μL of 20 μM peptide to 98 μL of modified peptide assay buffer.

   Nucleosomes: Add 2.7 μL of 1.5 μM nucleosome to 47.3 μL modified nucleosome assay buffer. Keep each diluted target on ice (*see* **Note 23**).
4. Transfer Targets to Assay plate(s).

   Peptides: Transfer 5 μL of each in duplicate to 10 Assay plate(s).

   Nucleosomes: Transfer 2.5 μL of each in duplicate to 10 Assay plate(s).

5. Once all material is added, tap microplates firmly on bench, carefully apply a storage seal, and centrifuge each Assay plate to settle any droplets (*see* **Note 24**).
6. Prepared plates can be stored for up to 3 months at −80 °C (*see* **Note 25**).

3.5 Discovery Screen

At this stage, optimal buffer conditions and probing concentrations have been identified for the Queries of interest (Subheadings 3.1–3.3). Profiling Queries using the Discovery Screen plates (Subheading 3.4) will provide a breadth of binding data to many PTM targets (100 in this example) at a single Query concentration. It is recommended to quantitatively rank targets (using EC_{50}^{rel}) from discovery screens by titration testing (Subheading 3.1). In cases with no binding target (i.e., Subheading 3.5 is being entered blind), we suggest testing with both high and low Query concentrations (*see* **Note 26**) and then restarting the workflow with identified Targets (to optimize the system).

1. Plan the desired plate layout and calculate the needed quantities of buffer and Query (or Queries).
2. Thaw Discovery Screen plate(s) on ice and centrifuge (600 × *g* for 1 min) to settle any droplets. Adjust to room temperature for about 10 min.
3. Thaw Queries on ice.
4. Prepare peptide assay buffer or nucleosome assay buffer with optimal salt and DNA (if latter is required).
5. Nucleosomes only: Prepare 500 μL of 2× salt nucleosome assay buffer.
6. Nucleosomes only: Add 2.5 μL of 2× salt nucleosome assay buffer to each well with substrates or buffer control.
7. Prepare 1 mL of Query at 4× the optimal probing concentration in assay buffer (*see* **Note 15**).
8. Add 5 μL of 4× Query dilution to all wells containing substrates or buffer.
9. Place plate in 23 °C incubator for 30 min.
10. During the incubation, move to a subdued lighting area and prepare a 2× bead mixture by adding 4 μL of 5 mg/mL AlphaLISA Glutathione Donor beads (5 μg/mL) and 8 μL of Alpha Streptavidin Donor beads (10 μg/mL) to 1988 μL assay buffer in a tube (*see* **Note 14**).
11. Add 10 μL of the bead mixture to each well under subdued lighting.
12. Place Assay plate in 23 °C incubator for 60 min. Biotinylated nucleosomes will bind Donor beads and the GST-tagged Query will couple with Acceptor beads.

13. Remove the plate seal and measure Alpha counts using an *EnVision* plate reader (or similar) using Alpha settings (*see* **Note 1**).
14. Analyze data using *GraphPad Prism* (or similar) to identify Potential Targets (by signal-over-background: e.g., Fig. 3b).
15. Identified Targets are titration tested (Subheading 3.1) under optimized conditions (Subheadings 3.2 and 3.3) to rank order (*see* **Note 27**).

4 Notes

1. Amplified luminescent proximity homogenous assay (Alpha, *PerkinElmer*) technology is a bead-based, no-wash chemiluminescent approach. The no-wash and signal amplification elements provide dramatically enhanced sensitivity relative to fluorescence-based histone peptide arrays. Prior to performing Alpha-based experiments, it is critical to ensure instrumentation/optics are compatible with the intended Acceptor beads (AlphaScreen or AlphaLISA). We use an EnVision 2104 instrument (Subheading 3.3) equipped with the Alpha 680 nm laser, AlphaScreen mirror (*PerkinElmer* barcode #444), and the AlphaScreen/AlphaLISA Emission filters (barcodes #244 and #203 respectively). The specific emission filter requirements are due to different luminescent chemistries on each Acceptor bead: AlphaScreen uses rubrene (broad ~520–620 nm emission), while AlphaLISA utilizes europium (narrow 615 nm emission). Because of this, AlphaScreen requires the #244 emission filter (570 nm/100 nm bandwidth) for accurate measurement, while AlphaLISA can be measured with the #244 or #203 (615 nm/8.5 nm bandwidth) emission filters.

   Although we perform *dCypher* assays using each Acceptor bead type, AlphaLISA tend to emit brighter, and can provide several-fold improvement in assay sensitivity. However, this comes at a price, with AlphaLISA Acceptor beads costing significantly more than AlphaScreen. Alpha streptavidin Donor beads are compatible with each Acceptor assay format (AlphaScreen and AlphaLISA). Of note, Alpha beads are much smaller in diameter (~250 nm) relative to typical bead-based assays (usually >5 μm). Due to this small size, Alpha beads will remain in suspension for the duration of the assay and do not require resuspension prior to signal measurement.
2. *dCypher* is optimized for use with GST-, 6His-, and FLAG-tagged Queries. Other epitope tags (or primary antibodies) can be used but must first be optimized. It is important to consider

that tags have the potential to modify Query behavior (e.g., GST can induce dimerization). Each tag requires a unique combination of detection reagents.

(a) GST-tags: Use 2.5 μg/mL glutathione Acceptor beads and 5 μg/mL Alpha Streptavidin Donor beads. Glutathione Acceptor beads are only available in AlphaLISA format.

(b) 6His-tags: Use 5 μg/mL Nickel-chelate Acceptor beads and 10 μg/mL Alpha Streptavidin Donor beads. Nickel-chelate Acceptor beads are available as either AlphaScreen or AlphaLISA formats.

(c) FLAG-tags: Use 1:400 anti-FLAG antibody, 5 μg/mL Protein A Acceptor beads and 10 μg/mL Alpha Streptavidin Donor beads. Protein A Acceptor beads are available as either AlphaScreen or AlphaLISA formats.

3. To compare and rank targets we use a four-parameter logistical (4PL) model and compute the relative EC_{50} (EC_{50}^{rel}) values for each target. Although K_d values are typically used for reporting binding affinity, specific conditions must be met to determine a K_d when using Alpha technology: a Query concentration at least 5× below bead binding saturation and 10× excess of fixed target. A competition assay can be performed to determine binding K_d of each interaction but will require case-by-case optimization to ensure sufficient signal-to-background of the Query and Target. It is important not to over-interpret EC_{50}^{rel} values, as they are defined as the concentration of Query required to elicit a response halfway between the maximal and baseline along the concentration–dose response curve. Further, we report EC_{50} values as relative EC_{50} because a stable maximal response (100% ± 5%) control is not included during data generation: as such we cannot ensure saturation.

4. Lyophilized peptides are dissolved in peptide reconstitution buffer (Subheading 2.2). Typically, all peptides are resuspended to the same concentration (we generally use 20 μM) to aid experimental planning.

5. Streptavidin Donor beads are light-sensitive and should only be handled under subdued lighting. After beads have been added to Assay plates, these should be covered with a black or other nontransparent seal to protect from light exposure.

6. Peptide assay buffer may turn slightly cloudy at room temperature but this will not impact assay performance. It is *not recommended* to store peptides long term in buffer containing poly-L-lysine.

7. A new plate seal is applied after each addition to prevent accidental cross-contamination between assay wells. Clear seals are

typically used for incubations prior to bead additions to the plate. After beads have been added, only use black or nontransparent seals (*see* **Note 5**).

8. A positive control Query should be included in each experiment to verify the assay system. The ideal control will use the same tag as the Query under interrogation. GST-, 6His-, and FLAG- tagged HP1β are commercially available and work best when paired with H3 (aa1-20; *EpiCypher* #12-0001) and H3K9me3 (aa1-20; *EpiCypher* #12-0012) peptides or unmodified (Control; *EpiCypher* #16-0006) and H3K9me3 (Target; *EpiCypher* #16-0315) nucleosomes.
9. Queries are usually assayed in duplicate or triplicate against a PTM-defined suspected Target and Control. The latter represents a predicted nonbinder for the Query, and in the case of nucleosome-based assays is usually an unmodified nucleosome, though this depends on the mode of engagement (e.g., could also be deleted of all histone tails [tailless; e.g., *EpiCypher* #16-0027], with acid-patch mutations [e.g., *EpiCypher* #16-0029, #16-0030 and #16-0031], or free DNA [e.g., *EpiCypher* #18-0005]).
10. Query titrations are typically performed at different 12 concentrations in duplicate and in two- or threefold dilution increments to cover a wide range and ensure upper and lower plateaus are captured. The 12th point is always a buffer control to assess assay background signal.
11. Peptides can be thawed at room temperature and then placed on ice. However, nucleosomes should always be thawed on ice, which will occur quickly due to the glycerol in their storage buffers.
12. Never vortex or sonicate nucleosomes to mix. Instead, gently pipet up and down until homogenous and flash centrifuge to settle any droplets on cap or sides of tube.
13. 4× Query serial dilutions are usually prepared in 384 deep well plates (Dilution plates). Sixteen-channel pipettes (if available) greatly increase efficiency and allow all dilutions to be handled simultaneously [Fig. 5].
14. Prior to adding Alpha Donor and Acceptor beads, vortex on high for ~10 s to ensure they are completely mixed and flash centrifuge to settle any droplets on cap or sides of tube.
15. Query Titration data is usually analyzed by generating nonlinear regression XY plots in *GraphPad Prism*. When determining EC_{50}^{rel}, the max and min plateaus must be visible for accurate quantification. Often a hook point is reached when using Alpha technology (Fig. 6): this indicates bead saturation/declining signal as excess nonbead bound Query is now competing with

that on the Acceptor beads for Target binding. Data points beyond the hook point must be removed for proper analysis. Sometimes in order to achieve a proper curve fit after excluding the hooked data points, a maximum signal constraint will need to be used in *GraphPad Prism*, particularly with sharp hook points (signal rapidly decreases as Query concentration increases). Each Query will have a unique hook point but molecular weight (MW) is a general predictor, where higher MW proteins tend to hook at lower molar concentrations. Optimal probing concentrations balance signal-to-background and being within the linear range of the sigmoidal curve: the EC_{50}^{rel} or EC_{80}^{rel} are typically selected. In the example [GST-BRD4 BD1: Fig. 6], the EC_{50}^{rel} can be computed for H4K5,8,12,16 ac as 2.5 nM (dotted line) but is nondeterminable for unmodified nucleosome (Control) as no binding was detected. The optimal probing concentration can be selected from a range, shown in green, which represents the EC_{20}^{rel} – EC_{80}^{rel}.

16. When performing 2D [Query vs. Salt] titrations, the typical 1× (final) concentrations are 250, 200, 175, 150, 125, 100, 50, and 0 mM NaCl (*see* Figs. 3 and 7). Antibodies to FLAG-tagged queries often show high nonspecific nucleosome interaction at low salt, so test 250, 225, 200, 175, 150, 125, 100, and 50 mM NaCl.
17. When planning/preparing each of the eight individual 2× salt buffers and Query serial dilutions, it is recommended to prepare the material in a 384 deep-well Dilution plate for easier transfer by 16-channel pipette to the Assay plate. For adding 2× salt buffer to the Assay plate, pipet the column of salt dilutions from left to right. For transferring 8× Query serial dilutions, pipet the column of serially diluted Query from top to bottom (Fig. 7).
18. In general, the highest Query concentration tested is 1 μM final in 20 μL (8× = 8 μM), though this may not be possible depending on the Query hook point (*see* **Note 15**).
19. Queries are usually diluted in twofold increments for 2D Titration to nucleosomes. In our experience Queries consistently display higher EC_{50}^{rel} concentrations with nucleosomes compared to peptides.
20. Choosing the optimal salt concentration is a combination of balancing signal-to-background of Targets vs. Controls, EC_{50}^{rel} values, and the resulting reagent consumption (considering all experimental modules). The general trend is greater salt stringency will decrease Query binding, which in some cases will help separate Targets from Controls. If this cannot be achieved and DNA binding is the suspected cause, a 2D [Query vs. SalDNA] titration may be necessary.

21. Choosing the optimal SalDNA concentration is similar to choosing optimal salt concentration (*see* **Note 19**). However, for SalDNA the ideal concentration is when maximum signal-to-background between Target and Control is achieved (usually when the DNA interaction is nearly abrogated by SalDNA). In some cases, the DNA interaction is essential for Query: nucleosome engagement and cannot be separated.
22. When preparing Discovery Screen plates, an additional dead volume of at least 10% is factored in to ensure the desired number of plates are prepared. Each substrate is usually prepared in duplicate. Typically, 5 μL of 400 nM peptide and 2.5 μL of 80 nM nucleosome is added per well. Peptides and nucleosomes are not recommended for simultaneous testing given their different buffer requirements.
23. Nucleosomes are prepared in a no-salt buffer for Discovery Screen plates to provide flexibility to adjust to the optimal salt concentration for any given Query. If desired, nucleosomes can be prepared with salt up to 250 mM NaCl (any higher may impact their long-term stability).
24. For proper storage of Discovery Screen plates, carefully apply a storage plate seal, centrifuge to settle any droplets, and store at −80 °C for up to 3 months. The recommended storage plate seals (*see* Subheading 2.3) use a high-bond pressure-sensitive adhesive. To create an extra tight seal, use a pen or marker cap to apply directed pressure around the plate perimeter. If an alternative plate seal is to be used, it is critical that the adhesives are designed for −80 °C.
25. Do not perform more than one freeze-thaw of Discovery Screen plates. They are to be used immediately after thawing.
26. For Queries of ~25–50 kDa with no known or suspected targets, use a high concentration of 1 μM and low concentration of 10 nM for a peptide discovery screen/a high concentration of 1 μM and low concentration of 50 nM for a nucleosome discovery screen. The high and low concentrations may require adjustment based on the protein size to prevent hook point issues (*see* **Note 15**). It is also recommended to start with a nucleosome assay buffer supplemented with 150 mM NaCl.
27. Using *GraphPad Prism*, the data can be organized into columns to analyze and visualize the discovery screen data. Alternatively, Targets can be rank ordered by max signal in an Excel file. A third method to visualize large quantities of ranking data is a heat map (e.g., Fig. 3). It is recommended that a target ranking experiment be performed to quantitatively rank any Targets of interest by their EC_{50}^{rel}.

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