Published in final edited form as:

Methods Enzymol. 2012 ; 512: 161–185. doi:10.1016/B978-0-12-391940-3.00008-1.

High throughput strategy to identify inhibitors of histone-binding domains

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Abstract

Many epigenetic proteins recognize the posttranslational modification state of chromatin through their histone binding domains, and thereby recruit nuclear complexes to specific loci within the genome. A number of these domains have been implicated in cancer and other diseases through aberrant binding of chromatin; therefore, identifying small molecules that disrupt histone binding could be a powerful mechanism for disease therapy. We have developed a high throughput assay for the detection of histone peptide:domain interactions utilizing AlphaScreen technology. Here, we describe how the assay can be first optimized and then performed for high throughput screening of small molecule binding inhibitors. We also describe strategies for biochemical validation of small molecules identified.

Keywords

Epigenetics; AlphaScreen; Histone Code; High throughput screen; PHD finger; Tandem Tudor domain; small molecule

1. Introduction

Within the nucleus of eukaryotic cells, DNA wraps around histone proteins to form nucleosomes, the most basic level of organization and compaction in chromatin (Felsenfeld and Groudine, 2003). The flexible N-terminal “tails” of histones are enriched for sites of posttranslational modification, which include acetylation and methylation of lysine residues, methylation of arginine residues, and phosphorylation of serine and threonine residues (Jenuwein, 2001). Posttranslational modification of histones both influences intrinsic chromatin structure and serves to recruit gene regulatory complexes to specific loci within the genome (Kouzarides, 2007). The latter function of histone modifications is achieved through specialized protein domains that have evolved to “read”, or recognize and bind a

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specific histone modification state (Taverna et al., 2007). It is partly through these “reader” domains that gene expression can be controlled in a highly dynamic and specific manner.

There are several classes of reader domains that bind to covalently modified histone residues, such as acetylated lysine, methylated lysine, methylated arginine and phosphorylated serine/threonine. These include plant homeodomain (PHD) fingers, tudor and tandem tudor (TTD) domains, chromodomains (Sanchez and Zhou, 2011, Yap and Zhou, 2011, Bonasio et al., 2010, Maurer-Stroh et al., 2003), bromodomains (Zeng et al., 2010, Mujtaba et al., 2007) and WD40 repeats (Couture et al., 2006). Modification-specific binding by these domains is achieved through discrete structural features, such as aromatic cages (methyl-lysine binding) and side chain-specific polar and ionic contacts (acetylation and phosphorylation) (Taverna et al., 2007). PHD fingers, which generally recognize the methylation status of lysines, are the most numerous; according to the Conserved Domain Database (NCBI), there are approximately 150 in humans alone. Altogether, the hundreds of histone-binding domains predicted across the human genome generate a remarkable amount of chromatin “reading” power.

The misregulation of many reader domains is strongly linked to cancer (Baker et al., 2008). Whether by mutation or overexpression, histone reader domains are associated with or directly cause cancer through aberrant binding of chromatin, which results in inappropriate activation or repression of genes. Several notable examples include the genetic fusion of the third PHD finger of JARID1A with nucleoporin 98 in acute myeloid leukemia, as well as the overexpression of TRIM24 in breast cancer, UHRF1 in lung cancer, and DNA methyltransferases in gastric cancer (Wang et al., 2009, Tsai et al., 2010, Unoki et al., 2010, Ding et al., 2008). Even though the mechanism of oncogenesis differs at the transcriptional level, each of these proteins is thought to contribute to malignancy through improper binding of chromatin by a histone-binding domain.

Because of their role in cancer, histone reader domains are attractive targets for drug development. Cancer therapeutics targeting DNA methyltransferases and histone deacetylases have met with success, suggesting that targeting the epigenome holds great potential for drug development (Zheng et al., 2008). While classical drug targets are generally enzymes or receptors, histone-binding domains utilize similar structural features, which include a small, discrete interaction interface and specialized cavities that coordinate the modified histone side chain. Therefore, we propose that histone “reader” domains are “druggable” cancer targets, and represent a new paradigm in drug development. In fact, a recent study targeting bromodomains identified a small molecule that disrupts histone binding and displays anti-tumor activity, lending credence to such a strategy (Filippakopoulos et al., 2010).

High throughput screening (HTS) provides a powerful strategy to identify small molecule inhibitors of histone-binding domains. While HTS can address a vast chemical space, there are challenges specifically associated with histone-binding domains during the development of a HTS histone-binding assay. Importantly, histone “reader” domains typically engage histone tail peptides with binding affinities ranging from 0.5 to 30 μ M. These relatively weak affinities can be prohibitive to some direct binding assays, such as fluorescence

polarization. Other methods commonly used to detect binding of histone peptides by these domains, such as isothermal titration calorimetry or bead-based GST pull-downs, are not high throughput. Consequently, development of suitable HTS assays for histone-binding domains is of great importance.

Our laboratory and others have successfully used AlphaScreen® technology (Perkin Elmer) for the high throughput screening of histone-binding domains. The technology functions as a bead-based proximity assay, where the interaction between a protein and its ligand tethers two affinity beads together. During detection, chemical amplification of the binding interaction is translated to fluorescence that can be measured on a plate reader. A number of affinity capture matrices can be linked to AlphaScreen beads, including antibodies and streptavidin. AlphaScreen technology is well suited to low-affinity interactions due to the avidity of the assay beads, which makes it useful for detecting histone-binding interactions. Our laboratory uses a hexahistidine-tagged “reader” domain and biotinylated histone peptides, which are captured by nickel chelate and streptavidin-coated AlphaScreen beads, respectively. To date, other research groups have successfully used this strategy for the high throughput screening of a malignant brain tumor (MBT) repeat domain and a BET bromodomain (Wigle et al., 2009, Filippakopoulos et al., 2010). We have successfully utilized this general assay platform for a number of other histone-binding domains, including PHD fingers and tandem tudor domains. Here, we describe how the AlphaScreen-based, histone peptide binding assay can be first optimized and then utilized for high throughput screening of histone-binding domains for the identification of small molecule inhibitors. We also describe strategies for biochemical validation of small molecules identified from high throughput screening.

2. AlphaScreen Assay Principles

Detecting the binding of epigenetic proteins and their binding targets can be accomplished through various assays, such as fluorescence polarization, *in vitro* pull down assays and isothermal calorimetry. While these assays are generally useful methods for characterizing binding interactions, each approach has significant disadvantages for high throughput analysis, such as the requirement of large quantities of protein, laborious wash steps, and inner filter interference from fluorescence compounds. AlphaScreen technology permits fast, homogenous experiments, and requires minimal sample material to measure binding.

The AlphaScreen assay utilizes proximity-based fluorescence detection through the tethering of donor and acceptor beads by a protein-ligand interaction (Figure 1) Initially developed underneath the name LOCI® (luminescent oxygen channeling assay) (Ullman et al., 1996, Ullman et al., 1994), the reagents and bead technologies for drug discovery are currently exclusively commercially available under the name AlphaScreen by Perkin Elmer. In this assay, the photosensitizer phthalocyanine is dissolved on a polystyrene donor bead. Excitation with 680 nm light induces phthalocyanine to convert ambient oxygen to singlet oxygen molecules with a 4 μ s half-life. These molecules can diffuse ~200 nm freely through solution. If a polystyrene acceptor bead is within the lifetime of the singlet oxygen species, the singlet oxygen will react with thioxene derivatives on the bead, resulting in a dioxetane product followed by a diester fluorescent product. This energy is transferred to a number of

energy acceptors, with rubrene as the final emitter at 520–620 nM (Ullman et al., 1996, Ullman et al., 1994).

The detection of the chemiluminescent readout depends on binding of the protein and its cognate ligand. Typically, the donor bead captures a ligand while an acceptor bead captures the binding partner. The interaction of protein and ligand results in chemical energy transfer of acceptor and donor beads, culminating in a luminescent signal. Lack of binding fails to bring acceptor and donor beads into sufficiently close proximity and the singlet oxygen decays without the production of light. Because the beads are coated with hydrogel, non-specific interactions are minimized, providing a large signal-to-background assay window. The relatively small size of the beads (250 nm) allows them to remain in suspension and be dispensed by automated liquid handlers.

We, and others (Wigle et al., 2010, Quinn et al., 2010), have used this approach to investigate interaction of biotin-labeled histone peptides and histidine-tagged epigenetic protein partners with the AlphaScreen Histidine detection kit. This kit contains streptavidin-coated donor beads for immobilizing biotin and nickel-chelated acceptor beads to affinity capture histidine residues, making it a versatile assay to detect binding of many different epigenetic proteins and their preferred modified histone peptide. In addition to the Histidine detection kit, several AlphaScreen assays accommodate binding interactions with proteins that have other common tags such as a GST-tag in the AlphaScreen GST-detection kit. Together, these properties make AlphaScreen a “ready-to-go” assay for screening epigenetic interactions.

3. Materials and instrumentation

The AlphaScreen Histidine Detection Kit (cat. no. 6760619C), the Alpha Screen TruHits kit (cat. no. 6760627D), 384-well white Optiplates (cat. no. 6007299), and Enspire Alpha Plate reader (cat. No.2300-001A) are from PerkinElmer. The StabilCoat Immunoassay Stabilizer buffer (SC01-1000) is available through SurModics. All amino acid derivatives can be purchased from Novabiochem, unless otherwise indicated. Peptides were either synthesized in-house on an Intavis robotic synthesizer or on a Prelude instrument at the University of Wisconsin Biotechnology Center peptide synthesis facility. The Synergy H4 Hybrid Multi-Mode Microplate reader, 570/100 nM filter (Part no. 7082264) 680/30 nM filter (cat. no. 7082229), filter wheel plug (Part no. 708673), and Half-Size, Ex. 640–780, Em. 400–630 dichroic mirror (Part no. 7139635) are from Biotek. Compound screening and use of the Beckman Coulter Biomek® 2000 liquid handler was carried out at the University of Wisconsin-Madison Small Molecule Screening facility. All other reagents are available through Sigma-Aldrich or Fisher Scientific, unless otherwise specified.

4. Design and preparation of histone peptides and histidine tag fusion proteins

The AlphaScreen-based histone-binding assay relies on the streptavidin-coated donor bead and nickel-chelate acceptor beads to be in close proximity to measure binding interactions. Therefore, the target epigenetic protein and target histone peptide must contain a histidine

fusion tag and a biotin group, respectively, in order to immobilize these constructs onto the bead surfaces. Ideally, four different peptides are required to measure several assay parameters. Most histone-binding domains bind histone peptides with a preference for a particular modification state; we refer to this peptide as the “high affinity” peptide. For example, the tandem tudor domain (TTD) of UHRF1 binds histone H3 trimethylated at lysine 9. However, as is the case with many histone-binding domains, UHRF1 TTD can bind other histone H3 peptides with reduced affinity (“low affinity peptides”). In our studies, we prefer to use high and low affinity histone peptides as controls within a high throughput histone-binding experiment because they delimit the expected binding range of the chromatin reader. We also synthesize non-biotinylated versions of these peptides and perform peptide competition controls during HTS to confirm specific inhibition of the histone-binding domain. It may also be valuable to utilize a biotinylated peptide that is not bound whatsoever by the chromatin reader (i.e. a scrambled peptide) as a means of defining the absolute background of the assay.

In addition to the TTD of UHRF1, we have successfully adapted this histone-binding assay to a number of other reader domains, including a number of PHD fingers (ING2, RAG2, BHC80, AIRE, JARID1A), and bromodomains (Brdt, TRIM24).

4.1. Design and construction of histone peptides

In general, longer peptides are more difficult to synthesize versus shorter peptides. As such, we synthesize peptides 10–20 amino acids in length. Additionally, we have found that peptides containing trimethylated lysines have reduced coupling efficiency during synthesis compared to their unmodified counterparts. In order to minimize synthetic difficulties, previous structural and biochemical studies of epigenetic proteins with target peptides should be taken into consideration to determine which amino acids to include within the peptide sequence. Shown below and in figure 2 are example peptides we have used to perform AlphaScreen with the TTD of UHRF1. This domain has been shown to bind an H3 K9me3 peptide with a K_d value of $\sim 20 \mu\text{M}$, while K4me3 is disruptive to binding (a K_d value of $\sim 90 \mu\text{M}$ has been measured for an H3 K4me3/K9me3 peptide) (Nady et al., 2011). Each peptide ends with a C-terminal tryptophan for quantification purposes. Biotinylated peptides are synthesized with a glutamate conjugated to a PEG-biotin group, located immediately N-terminal to tryptophan, while unbiotinylated peptides contain unmodified glutamate.

H3K9me3 biotinylated peptide (high affinity peptide):

ARTKQTARK(me3)STGE(PEG-biotin)W

H3K4me3 biotinylated peptide (low affinity peptide): ARTK(me3)QTARKSTGE(PEG-biotin)W

H3K9me3 competition peptide (high affinity competitor): ARTKQTARK(me3)STGEW

H3K4me3 competition peptide (low affinity competitor): ARTK(me3)QTARKSTGEW

Peptides can be synthesized on an automated solid phase peptide synthesizer, using standard Fmoc-based solid phase peptide synthesis (Bodanszky, 1993). Alternatively, there are a number of commercial suppliers (i.e. Anaspec, Bachem) that offer custom peptide services,

including the synthesis of biotinylated peptides. In our lab, we have utilized the Res Pep SL (InTavis) robotic synthesizer to generate peptides on a 5 μmol scale and a Prelude instrument (Protein Technologies) to generate peptides on a larger scale (12.5 μmol). Because the histone-binding assay requires pico-to nanomol amounts of peptide, 5 and 12.5 μmol synthesis should be sufficient. Carry out synthesis on Fmoc-rink amide resin (Applied Biosystems, 0.66 mmol/g load capacity), using 5 molar equivalents of amino-acid derivative, 5 molar equivalents of O-Benzotriazole-N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU)/1-Hydroxybenzotriazole(HOBT) activators and 6 molar equivalents of Diisopropylethylamine (DIEA) base at each coupling step. Use standard side chain-protected Fmoc-amino acids (Novabiochem) to incorporate amino acids and carry out Fmoc deprotection of amino acids with 20% piperidine. To incorporate trimethylated lysine, use *N*- α -Fmoc-*N*- ϵ -(methyl)₃-L-Lysine chloride (Novabiochem). For peg-biotin glutamate, use Fmoc-Glu(biotinyl-PEG)OH (Novabiochem). We have found an extended coupling step at trimethyllysine (4 hours) and double coupling (30 min each coupling step) at residues following (C to N directionality) improves purity and yield of the peptide. After synthesis, cleave the peptides from the resin with the appropriate TFA/scavenger mixture (for the above peptides, 92.5% TFA; 5% thioanisole, 2.5% ethanedithiol) using standard cleavage protocols. Perform MALDI to verify the identity of the peptide. Peptides should be purified over semi-preparative reverse phase HPLC. For peptide purification, we use a Vydac C18 HPLC column (Cat. no. 218TP510), and perform a 0–30% acetonitrile linear gradient in 0.05% TFA over a 60 minute time course. Typically, biotinylated peptides elute later than unbiotinylated counterparts due to the hydrophobic nature of the biotin group. Peptides can be resuspended and stored in water at -20°C for short-term storage (<1 month) and should be desiccated for long-term storage at -20°C . Measure the absorbance of the peptide at 280 nM and use the extinction coefficient of $5502\text{ M}^{-1}\text{cm}^{-1}$ to obtain the peptide concentration. Typical yields after purification is 20–50%, depending on the initial purity of the peptide synthesis.

4.2. Protein expression and purification

Produce a purified protein construct containing the histone-binding domain of interest fused to a histidine affinity tag. The following describes a general procedure using a T7 *E. Coli* overexpression system to generate the protein fusion construct, although individual recombinant proteins will likely require optimization of the purification and expression conditions.

Clone the histone-binding domain into a T7 vector (i.e. pQE80 or a pET plasmid), which contains a hexahistidine affinity tag. Transform the plasmid into a BL21(DE3) competent *E. coli* cells. Inoculate 1–3 liters of media containing the appropriate antibiotic with transformed cells. Grow cells at 37°C and induce at $A_{600}=0.6$ with isopropyl-D-thiogalactopyranoside (IPTG). Following cell harvest, lyse cells, centrifuge away cellular debris and add nickel-chelate resin to clarified supernatant. After binding His-tagged protein to resin, elute protein with high imidazole containing buffer. This eluted sample should be concentrated, dialyzed into storage buffer and aliquoted for storage. Samples should be run on an SDS-PAGE gel to examine protein expression and purity, and protein yield quantified (i.e. extinction coefficient or Bradford Assay). Qiagen and GE Healthcare provide useful

handbooks on the expression and purification of histidine-tagged fusion proteins should additional information be required.

5. Initial optimization of an AlphaScreen-based histone-binding assay

Initial optimization of the AlphaScreen-based histone binding assay is critical to ensure robust signal to background measurements while simultaneously minimizing reagents utilized. Additionally, as histone-binding domains demonstrate some affinity for most histone peptide sequences, it is important to optimize protein and peptide concentrations in order to discern binding of preferred modifications over those that are less preferred. Thus, the following parameters of the assay should be investigated: buffer conditions, protein concentration, histone peptide concentration, bead quantities utilized in each assay, and order of reagent addition. To increase signal to background measurements and reduce nonspecific binding, we recommend including a low concentration (0.01–0.1 % (v/v)) of detergent (i.e. Triton X-100, NP40, Tween-20) and 1–10 mg/mL (w/v) BSA in the buffer. Alternatively, we have also found the commercially available StabilCoat buffer (Surmodics) reduces nonspecific binding and works well with most proteins we have assayed thus far. Any portion of the assay that involves assay bead handling should be performed in low light conditions, as natural and fluorescent lighting can interfere with the bead light output signal. The following procedure is described as a starting point to begin using the assay for further optimization.

5.1. Preliminary histone-binding experiment

Prepare 100 nM stocks of His-tagged protein, 100 nM high affinity biotinylated peptide and 100 nM low affinity biotinylated peptide in Stabilcoat buffer. These concentrations are 2× stocks of protein and biotinylated peptide. To a 384-well Optiplate (PerkinElmer), add 9 µL of His-tagged protein followed by 9 µL of high or low affinity biotinylated peptides, such that the final concentration of each component is 50 nM. Wells containing only His-tagged protein or biotinylated peptides serve as absolute baseline signal controls. Perform each reaction in triplicate for error determination. Cover plates with aluminum foil plate sealant and incubate the plate at ambient temperature with shaking (200–300 RPM) for 30 minutes to allow binding equilibration. During this time, make a solution containing 150 µg/mL each of streptavidin donor beads (stock 5 mg/ml) and nickel chelate acceptor beads (stock 5 mg/mL) in Stabilcoat buffer and protect from light. At the end of the incubation, add 2 µL of the mixed bead cocktail to each well (final bead concentration of 15 µg/mL), re-cover with foil sealant and incubate at ambient temperature for an additional 30 minutes with shaking. Remove foil sealant and measure AlphaScreen luminescence near 570 nm on a Perkin Elmer Enspire or Biotek Synergy H4 plate reader. The Enspire Alpha Plate Reader is “plug and play”, such that one only needs to load the plate into the instrument and run the preset “Alpha 384-well” protocol. The Biotek Synergy H4 instrument requires more initial setup, with detailed procedures of setup described below.

5.2. Setting up an AlphaScreen detection protocol for the Biotek Synergy H4

The Biotek Synergy H4 plate reader can be used for the detection of AlphaScreen-based experiments; however, the Gen5 software-based protocol requires very specific parameters

that are not currently in the user manual. This procedure assumes that a Biotek Synergy H4 instrument is already installed and will be adapted for performing AlphaScreen. Install a 680/30 nM filter in position 1 and a filter wheel plug in position 2 into an excitation filter wheel. Install a filter wheel plug in position 1 and a 570/100 nM filter in position 2 into an emission filter wheel. The plugs are essential for establishing the necessary time delays during detection. Screw these wheels into the instrument wheel chamber and update these filters as described in the Synergy 4 user manual. Install a Half-Size, Ex. 640–780, Em. 400–630 dichroic mirror into the instrument, as instructed by the user manual. The AlphaScreen detection protocol should be built according to the following procedure. First, create a new protocol under the main Synergy 4 menu. In the procedure menu, choose 384 well plate type. Select “Plate” under “synchronized modes,” which will create a “plate mode with time control” dialogue. Inside this window, create a two-step procedure (Figure 3) with a 5-minute Delay step and a Read step. The Read step parameters should be adjusted as shown in figure 3, such that “Step label”= default, “Detection method”=fluorescence, “Read Type”= Endpoint, “Read Step”=Normal, “Light Source”=Tungsten, “Top Probe Vertical Offset”=7.00 mM. Filter 1 set should be set to “Excitation”=680/30, “Emission”=Plug, “Optic Position”=Top 635 nM, “Sensitivity”=190. Filter 2 set should be set to “Excitation”=Plug, “Emission”=570/100, “Optic Position”=Top 635 nM, “Sensitivity”=190. Run this experiment using this built protocol. It should be noted that the tungsten lamp requires 3 minutes to warm-up; this will occur at the start of the experiment.

5.3. Data analysis

A good description of statistical analysis of data in HTS is available in *High Throughput Screening: Methods and Protocols*, edited by William Janzen and Paul Bernasconi (Janzen and Bernasconi, 2009). Along with mean (M) and standard deviations (SD), two statistical parameters of particular interest are signal to background (S/B) and Z' factor (Z') (Zhang et al., 1999, Janzen and Bernasconi, 2009).

$$S/B = M_{\text{signal}} / M_{\text{background}}$$

For S/B, M_{signal} is the mean signal with the high affinity biotinylated peptide and $M_{\text{background}}$ is the mean signal with the low affinity biotinylated peptide. We usually observe S/B=5–50 in the optimized AlphaScreen assay. By defining a low affinity biotinylated histone peptide as “background” relative to the high affinity biotinylated histone peptide, one establishes the greatest amount of stringency within the assay.

$$Z' = 1 - 3 * (SD_{\text{signal}} + SD_{\text{background}}) / (M_{\text{signal}} - M_{\text{background}})$$

The Z' factor takes into account both the robustness of signal over background and the variability of the assay. Z' factor values of 0.5–1 are generally accepted in HTS (Zhang et al., 1999). For our histone-binding assay, we usually observe Z' factor values of 0.6–0.7. Higher Z' factors can be achieved by increasing assay volumes and utilizing 96-well plates,

although these adjustments are made at the expense of increased reagent usage and decreased high throughput format.

5.4. Peptide-Protein concentration matrices

In order to characterize the dynamic range of peptide binding within the assay and select appropriate concentrations of protein and peptide for future experiments, perform biotinylated peptide: protein matrices in which peptide and protein concentrations are simultaneously varied. One should maximize assay measurements with the high affinity biotinylated peptide while keeping the low affinity peptide signal relatively low. Additionally, one should aim to utilize the lowest concentrations possible to conserve reagents. Shown is a 3×3 concentration matrix with the TTD of UHRF1 and high affinity H3K9me3 or low affinity H3K4me3 biotinylated peptides (Figure 4). Here, a near maximal assay signal is observed with 6.25 nM biotinylated H3K9me3 and 6.25 nM of UHRF1 TTD (Figure 4a). At 25 nM biotinylated H3K9me3 and 25 nM of UHRF1 TTD, a “hooking effect,” in which there is a decrease of signal at higher reagent concentrations, can be observed. This phenomena is due to the complete saturation of beads with protein and peptide, with excess protein or peptide becoming inhibitory to bead interaction.

In addition to observing the magnitude of the raw assay signal, the S/B should also be taken into consideration. The H3K9me3 biotinylated peptide S/B is ~16-fold greater than the H3K4me3 biotinylated peptide signal at 6.25 nM protein and 6.25 nM peptide concentrations. However, at higher protein and peptide concentrations, the S/B decreases due to greater binding of UHRF1 TTD to the lower affinity H3K4me3 peptide (Figure 4b). Based on these results, 6.25 nM peptide and 6.25 nM protein is the optimal concentration to achieve near-maximal signal, while retaining sufficient dynamic range between the high and low affinity histone peptides.

5.5. Bead concentration optimization

The Perkin-Elmer AlphaScreen product guidelines suggest 20 $\mu\text{g/mL}$ of each bead type in the assay. To make the assay more cost-effective, try several lowered bead concentrations (5, 10, and 15 $\mu\text{g/mL}$) to identify a bead condition that does not significantly reduce the signal. We have found that 10 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$ maintains 5 to 40-fold S/B readings and reduces bead cost by 25–50%. In addition, lowering bead amounts has little effect on the Z' factor.

5.6. Peptide competition

Peptide competition demonstrates that the His-tagged histone binder and cognate biotinylated histone peptide interaction can be effectively disrupted in a modification specific manner, thereby resulting in a loss of binding signal. We have successfully measured competition when competitor peptide is added following addition of protein and biotinylated peptides and prior to bead addition. This order of addition is important for two reasons. First, adding competitor peptide after protein and biotinylated peptide addition mimics the compound addition step during library HTS. Second, bead avidity phenomena may actually prevent competition if beads are added prior to competitor peptide. Because the final concentrations of protein and biotinylated peptide will usually be far lower than the

K_d , the IC_{50} value (concentration of compound where there is a 50% reduction in the fraction bound value) measured in the competition assay should be reflective of the dissociation constant of the competitor (Cer et al., 2009). Therefore competitor peptide concentrations should range from $0.2 \times K_d$ to $20 \times K_d$ in the competition assay. Shown in figure 5 is an example competition assay in which H3K9me3 and H3K4me3 peptides are titrated in the histone-binding assay containing UHRF1 TTD and biotinylated H3K9me3 peptide. The measured IC_{50} value is 6 μ M for the H3K9me3 competitor peptide and 95 μ M for the H3K4me3 competitor peptide. This measured IC_{50} value concentration from the competition assay is the recommended concentration to utilize in the competitor peptide control of the small molecule primary screen.

6. Primary screening

Following optimization, the AlphaScreen-based histone-binding assay can be used to screen small molecule libraries. The following DMSO-treated controls should be performed with each screening plate: binding of the high affinity biotinylated histone peptide, binding of the low affinity biotinylated histone peptide, and peptide competition with the high affinity peptide. These controls establish the dynamic range in binding of the two biotinylated peptides, and confirm specific inhibition by peptide competition. If a known small molecule inhibitor is available, this can also be a useful control. Individual controls should be distributed across the plate to account for plate-wide effects; our screening facility designates the first and last two columns of a 384-well plate for controls. Many HTS facilities offer a number of compound libraries, typically including large “diversity” libraries and smaller clinical drug libraries. Each type of library offers unique features, which a combined screen of a diversity and clinical library can encompass. We typically screen compounds at a concentration between 25 and 100 μ M. If desired, compound concentration during screening can be adjusted following a pilot screening experiment to increase or decrease screening stringency. We have performed multiple screens of smaller compound libraries (<1,500 compounds) using the following strategy:

1. Dispense 18 μ L cocktail of reader domain and biotinylated peptide at the optimized concentrations to 384-well plates using a robotic liquid handler (Biomek® 2000, Beckman Coulter).
2. Centrifuge plates for 30 s, $800 \times g$.
3. Dispense compounds from a 5–10 mM screening stock plate through a 30 second robotic pin transfer (Biomek® FX, Beckman Coulter).
4. Seal plate and agitate for optimized time period on orbital shaker (200–300 rpm).
5. Dispense 2 μ L AlphaScreen bead cocktail by multichannel pipette under reduced light.
6. Seal plate and agitate for optimized time period on orbital shaker (200–300 rpm).
7. Measure emission at 570 nm on Synergy™ H4 (Biotek) or EnSpire® (Perkin Elmer) plate reader.

Converting to robotic liquid handling from manual/multichannel liquid handling is easily achieved for each reagent addition, but there are considerations to address during this transition. Importantly, the so-called “dead volume” for liquid handlers can be prohibitive in regards to the cost of AlphaScreen beads; therefore, it may be preferable to dispense beads using a multichannel pipette in order to minimize waste, especially if a relatively small library (<1000 compounds) is being screened. Another consideration during primary screening is the timing for handling each plate. The Biotek Synergy™ H4 reader requires approximately 15 minutes to read an entire 384-well plate, as opposed to 5 minutes or less for the Perkin Elmer EnSpire® reader. If the Biotek reader is used, it may be advisable to either perform screening over multiple experiments, or within a single experiment, stagger individual plates to ensure non-overlapping detection periods.

Following primary screening, calculate the raw signal for each compound as a fraction of the DMSO-treated binding control (relative binding). When the relative binding for each compound is ranked from smallest to largest, independent of position or plate, the data will assume a sigmoidal curve, with the majority of compounds not affecting relative binding (relative binding = 1), some subset reducing relative binding (<1), and the rest increasing relative binding (>1) (Figure 6). We have sometimes observed a downward shift of the median relative binding value (0.8–0.9 rather than 1). This suggests either an “edge effect” (i.e. temperature difference at edge wells) is occurring in our screening controls, or that most compounds have a slight negative effect on the assay. This can be corrected by averaging the signal for all wells, excluding the negative binding or peptide competition controls, and using this value to recalculate relative binding for each compound.

7. Hit selection and secondary screening

Following primary screening, secondary screens, in which hit compounds selected from the primary screen are tested in a second, independent experiment, can reveal assay-based or handling-based artifacts during primary screening and narrow the pool of compounds to be studied for validation. To select compounds for secondary screening, we recommend using a minimum cut-off value calculated from the standard deviation of the DMSO-treated binding control from the primary screen. This strategy allows the identification of compounds that reduce binding signal reliably outside of the intrinsic error of that plate. We typically use a three-standards of deviation selection value. For example, if the standard deviation associated with the DMSO-treated high affinity binding control was 8% of the mean value for that control, compounds are selected for secondary screening if the relative binding is reduced by greater than 24% compared to the DMSO-control. The cut-off value is calculated for each plate, rather than averaged across multiple plates. Since the binding control typically yields <10–15% standard deviation relative to the mean, it can be helpful to establish a second criterion to select compounds for validation, such as a minimum 30% reduction in relative binding in addition to the three standards of deviation cut-off. This strategy is especially useful if a) the standard of deviation for the DMSO-treated control is small, and b) a large number of compounds appear to have a negative effect on binding.

Once candidate compounds are selected from the primary screen, several strategies may be employed for the secondary screening experiment. In order to eliminate artifacts from liquid

or plate handling, the histone-binding assay can be repeated with each compound in triplicate. Alternatively, assay-specific false positives/negatives can be identified at an early stage using a secondary high throughput method, such as fluorescence polarization. Similar statistical cut-off criteria should be employed following the secondary screen in order to select compounds for additional biochemical validation.

8. Concentration-dependence validation studies

Concentration dependence studies following secondary screening using the AlphaScreen-based histone-binding assay can expose concentration-independent assay artifacts and define relative compound potency. At this stage of validation, it is important to purchase new compounds from a commercial supplier to correct for chemical defects that arise from freeze-thawing screening stock plates and general time-dependent instability (i.e. oxidation, hydrolysis). To determine the IC_{50} value of the compound, perform a complete inhibition curve, similar to the peptide competition procedure described in section 5.6. Figure 5 shows an inhibition curve of UHRF1-TTD with an undisclosed compound identified from HTS. This compound does not interfere with the AlphaScreen assay (described in section 9). The inhibition analysis yielded an IC_{50} value of 20 μ M, demonstrating effective competition of biotinylated H3K9me3 peptide. If there are still a large number of compounds following secondary screening, it may be more practical to initially test fewer concentrations.

9. Assay-specific compound interference

Assay-specific compound interference may occur by a number of mechanisms within the AlphaScreen format. Compound interference can arise from compounds that bind the nickel chelate beads, as well as compounds that react with singlet oxygen during detection, or green/blue compounds that interfere with fluorescence excitation or emission. Other mechanisms of interference may also occur, including compounds that resemble biotin, thiophene, or phthalocyanine.

To address compound interference, Perkin Elmer has a TruHits™ kit that uses streptavidin-coated donor beads and biotinylated acceptor beads to tether the two beads together. While this kit can address fluorescence interference, singlet oxygen quenching, and biotin mimetics, it cannot identify compounds that interfere by binding nickel chelate beads. Our laboratory developed an alternative assay that can identify interference by any one of the above mechanisms, including nickel chelation.

Each AlphaScreen nickel chelate histidine detection kit provides a biotinylated hexahistidine peptide as a positive control reagent. We developed an assay-based counterscreen using this peptide, which will tether the donor and acceptor beads together for a positive binding signal (Figure 7). Compounds can be dispensed to a binding reaction containing this peptide, followed by standard AlphaScreen bead addition and detection. The peptide-based counterscreen assay is conducted as follows:

1. Dilute the biotinylated hexahistidine peptide to 10 nM in StabilCoat buffer. Prepare enough solution to test all compounds of interest in triplicate, plus a DMSO-treated control. Dispense 18 μ L to each well of a 384-well white OptiPlate.

2. Dispense compounds to wells in triplicate at several concentrations. Include a DMSO-treated control.
3. Proceed as with the histone-binding assay, including incubation times, bead addition, and detection.

Figure 8 demonstrates three examples of compound interference as identified by the above assay.

Compounds that interfere with fluorescence excitation or emission can be characterized through absorbance spectrum scans or by eye (blue/green color). The Perkin Elmer AlphaScreen manual suggests that it is possible to identify nickel-binding compounds by preincubating the compound with the nickel chelate acceptor bead prior to adding the streptavidin donor beads and biotinylated hexahistidine peptide/fusion protein. Similarly, biotin mimetics may be identified by preincubating the compound with the streptavidin donor beads prior to adding the rest of the assay components. Singlet oxygen quenching compounds are more difficult to definitively characterize other than by structural features (ascorbate-like compounds).

Although a compound may interfere with AlphaScreen, it does not definitively eliminate that compound as a genuine inhibitor of the histone-binding domain protein. We have identified several compounds that interfere with AlphaScreen but inhibit histone peptide binding by a reader domain in independent biochemical assays. One limitation of the above counterscreen experiment is that it cannot account for the presence of the histidine-tagged reader domain. Thus, it is essential to have other strategies in place to test compound activities before eliminating them solely on the basis of AlphaScreen interference.

10. Additional assays for validation

Low to medium throughput methods can be employed once a pool of small molecules has been identified and validated by the AlphaScreen binding assay. These include but are not limited to isothermal titration calorimetry (ITC), traditional *in vitro* pull downs, fluorescence polarization (FP), and structural studies. ITC can be used to determine dissociation constants of compounds binding to the histone reader domain. The exquisite sensitivity and highly quantitative nature of ITC makes it a powerful technique, but its utility can be limited by compound solubility. Briefly, a solution of compound (titrant) is serially injected into a solution of the reader domain protein contained in a sample cell, where heats of binding are measured (Pierce et al., 1999). Histone peptide pull downs are less quantitative but more accessible since no elaborate instrumentation is required. Biotinylated histone peptides are immobilized to streptavidin-coated beads; a fusion-tagged reader domain is allowed to bind the peptides with increasing amounts of small molecule inhibitor present. The reader domain is eluted, resolved by gel electrophoresis and detected immunologically by western blotting.

FP can be a particularly useful technique to validate hit compounds and quantitate the affinity of hit compounds to the histone reader. Here, we describe a general method for performing FP; a detailed description of FP and HTS is available in the following references (Janzen and Bernasconi, 2009, Lakowicz, 1983). First generate a high affinity fluorescently

labeled histone peptide. Our FP peptides have 5-carboxy-fluorescein positioned at the C-terminus, as many histone-binding proteins require the free N-terminal residue for recognition. Perform a histone binding domain saturation curve with 25 nM of FP peptide to determine the dissociation constant (K_d). At conditions of 0.5–0.8 fraction bound (equivalent to $\sim 1\text{--}2 \times K_d$ protein concentration), titrate competitor peptide or compound and measure the resulting polarization/anisotropy to determine the IC_{50} value (Cer et al., 2009). In addition to the anisotropy/polarization analysis, the total fluorescence of the FP peptide should be observed in the presence of compound to identify inner filter compound interference. Figure 9 demonstrates an FP competition assay in which unlabeled H3K9me3 peptide or compound is titrated against the UHRF1-TTD: H3K9me3-fluorescein complex. The H3K9me3 peptide IC_{50} value was 12 μM and the compound IC_{50} value was 29 μM . Therefore, these parameters obtained from FP are in good agreement with the IC_{50} values of 6 μM for the H3K9me3 peptide and 20 μM for the compound calculated from the AlphaScreen-based histone binding assay.

If small molecule binding inhibition is validated biochemically, structural studies utilizing x-ray crystallography or solution-phase NMR can provide insight into the mechanism of small molecule inhibition.

11. Summary

Many histone-binding domains are implicated in disease through improper binding of chromatin, thereby making them attractive therapeutic targets. Here, we have shown how to design and optimize an AlphaScreen-based assay to detect binding of histone peptides by reader domains, and how this platform may be used for the high throughput screening of small molecule libraries. This histone-binding assay is conducted rapidly, requires minimal sample material, and is easily translated to 384-well systems. We also discuss strategies for the biochemical validation of compounds identified from HTS using this platform and other independent assays, including fluorescence polarization, and how compound interference within the AlphaScreen-based assay may be identified.

Acknowledgments

We would like to thank Noël Peters and Song Guo of the University of Wisconsin – Madison Carbone Cancer Center Small Molecule Screening Facility for valuable advice and training. This work was supported by NIH Grant GM059785 (to JMD), the Molecular Biosciences Training Grant (EKW) and the Postdoctoral Training Grant on the Molecular and Cellular Mechanisms of Tumor Development (to BNA).

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Figure 1.
Schematic of AlphaScreen assay, probing for His-tagged histone reader binding to its cognate modified histone peptide.

Figure 2.
Structure of an H3 (1–12) K9me3 peptide synthesized with a C-terminal PEG-biotin linked glutamate for streptavidin bead binding, followed by a tryptophan for quantification. Important features are noted in the figure.

Figure 3. Creating a protocol on Synergy 4 for Alpha Screen. Shown are screen shots of the software protocol window. A. In the “Procedure Menu,” create a “Delay” step and “Read” step. The Plate Mode with Timing control can be added by choosing the “Well” button under synchronized modes (see arrow). B. Shown are the parameters to adjust in the “Read” step.

Figure 4.

Alpha screen biotinylated peptide: Protein concentration matrices. A. Raw 570/100 emission signal at 6.25 nM (medium grey), 12.5 (light grey), 25 nM (dark grey) protein concentrations. Assays were performed with H3K9me3 (solid lines) and H3K4me3 (dashed lines) biotinylated peptides. B. S/B (H3K9me3/H3K4me3 biotinylated peptide signal) from the same assay.

Figure 5. H3K9me3 (circles) and H3K4me3 (squares) Alpha Screen-based peptide competition assay with UHRF1 TTD. In addition, an undisclosed compound identified from HTS (diamonds) was titrated and competition observed. UHRF1 TTD and H3K9me3 biotinylated peptides were added to 384-well plates. Following, competitor peptides or compound were added and the AlphaScreen assay was performed. The fraction signal (fraction bound) with increasing competitor was fit to an IC_{50} binding equation.

Figure 6.

Primary screening data that measures the ability of compounds to UHRF1-TTD: H3K9me3 interaction in the AlphaScreen assay. UHRF1 TTD and H3K9me3 biotinylated peptides were added to 384-well plates. Following, compounds were added from a small molecule library and the AlphaScreen assay was performed. When sorted and graphed by relative binding, the data yields a sigmoidal curve with a median value near 1. Compounds to the left of this inflection point are candidate inhibitors of the histone-binding domains.

Figure 7.

We developed an assay-based counterscreen that takes advantage of the biotinylated hexahistidine peptide including with all nickel chelate AlphaScreen kits. This counterscreen format will reveal compound interference due to nickel chelation, excitation/emission interference, singlet oxygen quenching, and biotin mimics.

Figure 8.

An assay-based counterscreen reveals that these three compounds interfere with AlphaScreen. Manipulating the order of addition during the counterscreen and performing absorbance scans can help pinpoint the mechanism of interference by such compounds.

Figure 9.

H3K9me3 (circles) and compound (squares) as competitors in an FP-based competition assay with UHRF1 TTD. At 0.8× fraction bound, 0–200 μM of competitor peptides or compound were added and the FP assay was performed. The fraction signal (fraction bound) with increasing competitor was fit to an IC_{50} binding equation