Histone Methyltransferase Profiling & Screening

Biochemical Assays for Drug Discovery

INTRODUCTION

Histone methyltransferases (HMTs), which methylate the lysine or arginine residues of histones and other proteins, constitute one of the major classes of epigenetic modifiers. Aberrant HMT activity has been implicated in cancer,[1] inflammatory diseases,[2] and neurodegenerative disorders,[3] and preclinical studies indicate that HMTs are a promising drug target class.[4, 5]

The human genome encodes over 70 HMTs, all of which use *S*-adenosyl-L-methionine (SAM) as the methyl donor, which—following transfer of its methyl group to the histone substrate—is converted to *S*-adenosyl-L-homocysteine (SAH). Histone lysine methyltransferases add up to 3 methyl groups to a lysine residue, while protein arginine methyltransferases (PRMTs) can be separated into two classes: type I PRMTs modify arginine residues via mono-methylation or asymmetrical dimethylation; type II PRMTs, via mono-methylation or symmetrical dimethylation.

Due to the diversity of methylation sites and states, choosing an assay platform for screening and profiling against HMTs requires special considerations. This report will describe the advantages and limitations of common biochemical assay platforms, particularly with regard to their utility in high throughput screening (HTS).

BIOCHEMICAL ASSAY PLATFORMS FOR HMT SCREENING

Radiometric Assays

Radiometric assays represent a well-established platform for drug discovery, offering high sensitivity and reliable detection of enzyme activity. The test compound is incubated with HMT, substrate, and radiolabeled SAM (either ^{3}H-SAM or ^{14}C-SAM). Detection of the radiolabeled, methylated product is performed via filter binding methods or scintillation counting.

Radioisotope Filter Binding

The radioisotope filter binding platform is preferred for its high sensitivity, low incidence of compound interference, and universal application: any HMT and any substrate—including nucleosomes, histone octamers, other proteins, and peptides—can be tested. Reaction mixtures are incubated and spotted onto filter papers, which are then washed to remove unreacted SAM, leaving the bound radiolabeled product. Due to the wash step requirement and the costs and safety concerns associated with the use of radioisotope materials, the radioisotope filter binding assay has traditionally been limited in its application to HTS. To address these limitations, Reaction Biology has developed HotSpot℠, a biologically relevant, nanoliter-scale radioisotope filter binding platform, which offers high throughput capacity and low material requirements.

Scintillation Proximity

In scintillation proximity platforms, the substrate is immobilized on scintillation beads or scintillant-coated wells, most commonly via biotin-streptavidin binding. When the radiolabeled methyl function is transferred from SAM to the scintillant-bound substrate, scintillation is triggered. Unreacted SAM in solution will not trigger scintillation; therefore, no wash step is required, making the assay easy to adapt to HTS. Though various types of substrate can be used in scintillation proximity assays, substrate modification is required for capture on the scintillant beads or wells. Due to the relatively large amount of radioisotope material used and the detection instrumentation required, the platform can be costly.

Mass Spectrometry

Mass spectrometry (MS) is a highly reliable method by which to identify and distinguish between various epigenetic modifications. MS systems with automated cycles as fast as 6 seconds per sample have been developed, making the platform a good option for HTS.[6, 7] Liquid chromatography/MS-based SAH detection has also been utilized for HMT screening and selectivity profiling.[8] Though high-throughput MS systems are expensive and have a steep learning curve, the platform is favored for its utility in time-course studies and the ability to distinguish between multiple methylation sites and states.

Anti-Methylation Antibody-Based Detection

A number of anti-methylation antibody-based assays, utilizing a variety of technologies, have been developed. Generally, substrate and the anti-methylation antibody are paired with donor and acceptor chemiluminescent or fluorescent molecules. When the substrate is methylated to produce an epitope recognized by the anti-methylation antibody, the donor and acceptor molecules are brought into close proximity, producing signal when triggered by wavelength-specific excitation of the donor molecule.

Anti-methylation antibody-based assays allow for detection of specific methylation states and sites. Identifying highly specific anti-methylation antibodies, however, is often a bottleneck in assay development; for instance, antibody cross-reactivity between mono-, di-, and tri-methylated lysine residues is common.[9] Anti-methylation antibody-based assays are generally not suitable for kinetic studies[10] or for profiling against a wide range of HMTs with diverse methylation sites.[11] Additionally, signal interference must be considered when using fluorescent and chemiluminescent detection methods.[11, 12] Time-resolved fluorescence resonance energy transfer (TR-FRET) methods reduce interference by using ratiometric measurements and by delaying signal measurement to 50–150 µs after excitation, thereby mostly avoiding interference from compounds with short fluorescence lifetimes.

Enzyme-Coupled SAH Detection

Detection of SAH avoids the challenges of detecting diverse methylation products. However, due to the difficulty of developing antibodies that recognize SAH but not SAM (which differs from SAH by only a single methyl group), most platforms employ one or more coupling enzymes to convert SAH to a more easily detectable molecule—most commonly homocysteine, AMP, ATP, or hydrogen peroxide. Like radiometric and MS methods, SAH detection assays are universal to all HMTs and can be used with various types of substrate. However, test compounds should be counter-screened for activity against the coupling enzyme(s) in order to identify false positives and false negatives.[10]

Selecting an HMT Assay: 5 Factors to Consider

Universal vs. Specific Detection

Universal assays detect total HMT activity, while anti-methylation antibody-based platforms allow for site-specific, state-specific methylation detection. MS both detects total HMT activity and distinguishes between the various types of methyl modifications. Universal assays are preferred for profiling against panels of HMT targets.

Substrate Compatibility

HMTs may methylate nucleosomes, histone octamers, and other proteins, but some platforms are compatible only with peptide substrates. Radiometric, MS, and SAH detection assays generally accommodate a range of substrates.

Signal Interference

Interference from fluorescent and fluorescence-quenching compounds must be considered when using fluorescent platforms. Similarly, in using enzyme-coupled assays, test compounds must be screened for interfering activity against the coupling enzyme(s).

Activity vs. Binding

Though binding and inhibition are often correlated, binding assays typically detect only inhibitors that bind the active site. Activity assays, on the other hand, also identify uncompetitive and non-competitive inhibitors.

Throughput Capacity

Several simple, low-volume assays have been developed for HTS applications. Single step “mix and read” platforms, such as scintillation proximity are convenient and amenable to HTS. Reaction Biology’s HotSpot℠ radioisotope filter binding platform for HMT assay services has also been optimized for HTS and rapid turnaround.

Protease-Coupled Detection

Protease-coupled assays are based on the activity of either endo-lysC or endo-argC. These proteases cleave proteins at lysine or arginine residues, respectively, but do not cleave at methyllysine or methylarginine residues. Protease coupling has been employed in mobility shift[13] and fluorescence lifetime[14, 15] assays for HMTs. The major drawback of protease-coupled assays is that they require the use of synthetic peptide substrates that are specially designed to be uncleavable upon methylation. Using peptide substrate can substantially alter HMT activity, even when the peptide is based on native target sequences.[16] Additionally, test compounds must be screened for interfering activity against the protease.[10, 11]

Competition Binding

Unlike the activity assays mentioned thus far, competition binding assays do not measure catalytic product (or a derivative thereof), but rather the binding of ligands to the SAM-binding active site. One example of a competition binding assay is the probe displacement assay, which measures the displacement of a fluorescent standard inhibitor probe from the active site.[17] A drawback of competition binding assays is their inability to detect uncompetitive, non-competitive, and substrate-competitive HMT inhibitors (i.e. those that do not bind the SAM-binding site).

CONCLUSION

With the wide variety of platforms available, each with advantages and limitations, selecting an assay for HMT drug discovery must be carefully considered. Mass spectrometry and anti-methylation antibody-based assays are useful for identifying and distinguishing between the various types of HMT methylation products. On the other hand, radiometric assays provide highly sensitive and robust options for universal detection of HMT activity and are the preferred option for profiling against panels of methyltransferases. Optimizing the radioisotope filter binding platform for HTS, Reaction Biology has developed HotSpot^{SM}, a miniaturized assay format that requires no substrate modification, coupling enzymes, or detection antibodies.

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