

Reaction Biology Corporation (RBC) has created **HMT HotSpot™**, a new assay service to fill an unmet need in drug discovery. For the first time, researchers can do extensive low-cost profiling and HTS of HMT's with higher quality data than available from other assay formats

This paper gives the background of HMT profiling and various assay methods, and summarizes the advantages of **HMT HotSpot™** for researchers.

For a price quote on assay services or collaboration on special assay development, call 1-877-347-2368 or email sales@reactionbiology.com.

HMT background:

Epigenetic factors and drugs development

Epigenetics involves the study of changes in the regulation of gene activity and expression independent of gene sequence. It has become an emerging frontier for drug discovery. As an example, Vidaza (5-Azacytidine, DNA methyltransferase inhibitor) (Kaminskas et al 2005) was recently approved for the treatment of all subtypes of myelodysplastic syndrome (MDS). Also, zolinza (suberoylanilide hydroxamic acid, a histone deacetylase inhibitor) was approved for cutaneous T-cell lymphoma (CTCL) in Oct. 2006. According to the NIH Roadmap (Fig. 1, http://nihroadmap.nih.gov/epigenomics/epigenetic_mechanisms.asp), epigenetic mechanisms are influenced by several factors and processes including development in utero and in childhood, environmental chemicals, drugs and pharmaceuticals, life style choices, aging, and diet. DNA methylation (Figure 1) occurs when methyl groups, an epigenetic factor, are attached to DNA, leading to the alteration of gene expression. DNA methylation usually occurs on CpG islands and contributes to the process of aging and age-dependent processes such as cancer development.

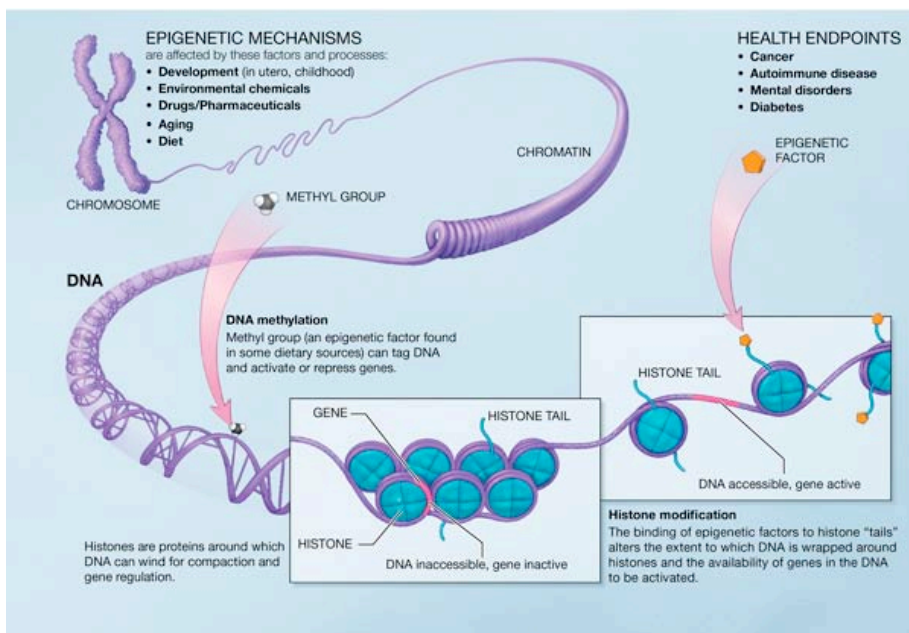


Fig. 1: A Scientific Illustration of How Epigenetic Mechanisms Can Affect Health (copied from NIH Roadmap program: http://nihroadmap.nih.gov/epigenomics/epigenetic_mechanisms.asp)

The DNA methylation and histone modifications are the key regulators for gene activities.

Post-translational modification of histone, including methylation, acetylation, phosphorylation and ubiquitination, are all important epigenetic factors (Fig. 1). Histones are proteins around which DNA can wind for compaction and gene regulation. Two copies of each, histones H2a, H2B, H3 AND H4, assemble to form one nucleosome core by wrapping 147 base pairs of DNA. The histone H1 binds the nucleosome at the entry and exit sites of the DNA to lock DNA into place. The tight nucleosome helps to pack the entire genome into the cell nucleus, and can restrict access of nuclear factors. Therefore, this inherently

restrictive environment must be tightly regulated to allow permissive cellular processes such as gene transcription, replication, recombination, and repair.

Histone methylation and cancer:

The histone lysine methyltransferases (HKMTs) are a large group of enzymes that specifically methylate histone using S-adenosyl-L-methionine (AdoMet or "SAM", Fig. 2) as a methyl donor. Most HKMTs belong to SET-domain (Su(var) 3-9 (suppressor of position effect variegation), E(z) (enhancer of zeste), and Trx (triThorax) protein methyltransferase family. They methylate a range of lysine residues in various histones, such as K4, K9, K27, K36 and K79 in histone H3, K20 in histone H4, K59 in the globular domain of histone H4 and K26 of histone H1B. Currently, there are 7 known main families of SET domain proteins, SUV39, SET1, SET2, EZ, RIZ, SMYD, SUV4-20 and a few orphan members such as SET7/9 and SET8. Proteins within each family have similar sequence motifs surrounding the SET domain and they also share a higher level of similarity in the SET domain.

Histone	Lysine residues	HKMT's
H3	K4	SET7/9, MLL1/HRX/ALL1, SMYD3, SUV39H1, MLL2/HRX2, MLL3/HRX2, ASH1, Meisetz/PRDM7, SET1,
	K9	G9a, SUV39H2, EuHMTase, SETDB1/ESET, RIZ1/PRDM2
	K27	EZH2, G9a, EZH1
	K36	SET2/HYPB, NSD1
	K79	DOT1
H4	K20	PR-SET7/SETD8 (Mono), SUV4-20H1, SUV4-20H2, NSD1
H1B	K26	EZH2

Many SET domain-containing proteins have been associated with human cancers suggesting that they play an important regulatory roll in the cell. The mixed-lineage leukemia gene MLL1, (a SET 1 family enzyme) is a histone H3 Lys 4-specific methyltransferase and involved in 11q23 translocations in acute leukemia. EZH2 is ubiquitously expressed during early embryo genesis, and becomes restricted to the central and peripheral nervous systems and sites of fetal hematopoiesis during later development. EZH2 is responsible for methylation of Lys 9 and, more prominently, Lys 27 of histone H3 and Lys 26 on histone H1. EZH2 is overexpressed in many type of cancers, such as metastatic prostate cancer and breast cancer; RIZ1 is a histone H3 Lys 9-specific methyltransferase and a tumor suppressor, which has been found to be inactivated by mutations or DNA methylation in a wide variety of human tumors including colorectal cancer, gastric carcinoma, breast cancer, liver cancer, lung cancer, lymphomas, and melanomas. SMYD3 interacts with the RNA polymerase II complex and activate target genes via methylation of histone H3 Lys 4. It is overexpressed in colorectal carcinomas and hepatocellular carcinomas. Introduction of SMYD3 into NIH3T3 cells enhanced cell growth, whereas genetic knockdown with small-interfering RNAs (siRNAs) in cancer cells derived from hepatomas and colorectal cancers resulted in significant growth suppression.

The HKMTs that have no SET-domain are the newly discovered DOT1 family, which was originally found in *S. cerevisiae* as a disruptor of telomeric silencing, with a S-adenosyl-L-methionine binding motif. A year later, Dot1 was identified by many labs as a HKMT that specifically methylates K79 in the globular region of histone H3 instead the lysines in the tails region as the SEK family does. Recently, hDOT1 has been associated with Leukemogenesis.

In addition to their roles in cancer pathogenesis, the HKMTs are also involved with other disease forms. For example, alterations in GABAergic mRNA expression play a key role for prefrontal dysfunction in schizophrenia and other neurodevelopment disease, and the GABAergic mRNA expression is influenced by the histone H3-lysine 4 methylation, which is the substrate of MLL1.



Despite the importance of HKMT's, few recombinant proteins and assays kits are commercially available.

Current assays for HKMTs and their limitations

HKMTs all use S-adenosyl-L-methionine (AdoMet or SAM) as the methyl donor, and the final products are the methylated histone and the S-adenosyl-homocysteine (AdoHcy) (Fig. 2). Detection strategies have focused on the production of the methylated histone and/or AdoHcy. To study the methylation activities of HKMTs, a few labs have published formats that are efficient for lab research purposes involving electrophoresis, but a standard, affordable 384-well plate HTS assay that is suitable for large scale of drug discovery and compound profiling has not been available up till now. Below are 5 common approaches used for research labs.

1: Fluorescent polarization (FP) assay: The fluorescent tracer conjugated AdoHcy can be recognized by AdoHcy to produce a high FP complex. The native AdoHcy produced in the methylation process has higher affinity toward the same antibody, and will displace the fluorescent tracer conjugated AdoHcy, to produce a low FP signal.

The advantages: 1) HTS-compatible and 2) universal for methyltransferases.

The disadvantages: 1) low sensitivity (only moderate detection sensitivity when ≥ 0.5 μ M AdoHcy has been generated); 2) Potential interference from fluorescent compounds and most importantly, 3) High [AdoMet] interferes with assay since anti-AdoHcy antibody can cross-react with AdoHcy, therefore [AdoMet] has to be kept low ($\leq 3\mu$ M). For example, the kinetic experiments to determine competition with AdoMet cannot be run.

2: Fluorescent coupling assay: This assay utilizes S-adenosylhomocysteine hydrolase (SAHH) to hydrolyze the methyltransfer product AdoHcy to homocysteine (Hcy) and adenosine (Ado). The Hcy concentration is then determined through conjugation of its free sulfhydryl moiety to a thiol-sensitive fluorophore.

The Advantages: 1) High throughput compatible, 2) Universal assay and 3) Ability to vary AdoMet.

The Disadvantages: 1) can detect inhibitors of S-Adenosylhomocysteine Hydrolase and Adenosine Deaminase enzymes to produce too many false positives; 2) Assay is sensitive to reducing agents (e.g. DTT); 3) Potential interference from fluorescent compounds and Sh-containing compounds; 4) the cost is high, and 5) high reaction volume with multiple reaction steps.

3: Mass Spectrometry Assay:

The Advantages: 1) Universal detection.

The Disadvantages: 1) low throughput; 2) high cost; and 3) special instrument and knowledge.

4: Streptavidin coated FlashPlates assay by using radioisotope materials and biotin peptide substrate:

The Advantages: 1) Medium to high throughput, and 2) Universal assay.



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The Disadvantages: 1) large volume radioisotope based reactions; 2) hard to use protein as substrate; 3) Potential interference from fluorescent compounds; and 4) high cost of FlashPlates.

5: Gel electrophoresis assay by using radioisotope materials: This is the classical assay for detecting methyltransferase ratio, but is not well suited for drug screening.

RBC's HMT HotSpot™ and its advantages

RBC's new radioisotope based HMT assay service provides a new "Gold Standard" level of HMT activity detection. Using proprietary low-volume methods to enhance the traditional tritium-based assay, RBC is able to offer the following advantages:

1. The only assay technology that can use nucleosome, histone protein and peptide as substrates;
2. High quality, high sensitivity with flexible SAM concentration;
3. No need for substrate labeling;
4. No need for antibodies;
5. Minimum false positives and negatives;
6. No intermediate dilution;
7. No interference from fluorescent compounds;
8. Only nanoliters of compound needed.
9. Low cost for HTS applications and profiling.