motif|vations

ACTIVE MOTIF®

Enabling Epigenetics Research

ACTIVE MOTIF – Advancing Neuroepigenetics Research

Epigenetic modifications including histone modifications, DNA methylation, and non-coding RNAs influence chromatin structure and, ultimately, gene regulation. Epigenetic modifications are heritable but, unlike genetic modifications, are dynamic and responsive to external stimuli such as stress, toxins and learning. Neuroepigenetics is a growing area of research focused on the study of epigenetics in various neural processes, including development and plasticity, behavior, stress, aging, and brain disorders. Below are two publication summaries illustrating how Active Motif, the leading provider of innovative epigenetic research tools, is aiding scientists every day in advancing their neuroepigenetics research.

IN THIS ISSUE

THE ROLE OF EPIGENETIC MODULATION IN BRAIN DISORDERS

There is increasing evidence that several neurodevelopmental, neurodegenerative and psychiatric disorders are, in part, caused by aberrant epigenetic modifications. As a result, researchers in neurogenetics are now beginning to investigate the mechanisms underlying epigenetic regulation in neuronal gene expression and how this impacts normal brain function. Recently, Active Motif collaborated in two separate published studies where loss-of-function mutations of chromatin modifiers *Wiz* and *Kdm5C* were used as mouse models to study how disrupting the function of epigenetic modifiers contributes to brain disorders. These recent publications in *eLife* by the laboratory of Dr. Emma Whitelaw at La Trobe University in Melbourne, Australia and in *Cell Reports* by the laboratory of Dr. Yang Shi at Harvard Medical School, respectively, are summarized below.

Identification of a role for the epigenetic modifier Wiz in neural function

This following article is a summary of the research published in *eLIfe.*: Isbel *et al.*, 2016;5:e15082. DOI: 10.7554/eLife.15082.
To identify novel modifiers of epigenetic reprogramming, the laboratory of

Dr. Emma Whitelaw performed a mutagenesis mouse screen in which they identified the gene *Widely interspaced zinc finger motifs,* or *Wiz*, as a modulator of gene expression. Although little is known about Wiz, it is highly expressed in both the embryonic and adult brain and is known to mediate protein-protein and protein-DNA interactions of the G9a/GLP histone methyltransferase complex which catalyzes methylation of H3K9me1 and H3K9me2, marks that are associated with transcriptional repression. G9a is also associated with transcriptional activation independent of its histone methyltransferase activity, and it has been suggested that Wiz may also be involved in G9a-mediated transcriptional activation.

Reduced levels of Wiz are associated with gene silencing
In the mutagenesis screen, haploin-sufficiency for *Wiz (Wiz^{MD30/+})* resulted in silencing of both the targeted GFP transgene reporter under the control of erythroid promoter/enhancer sequences as well as an independent locus, the *Agouti viable yellow (A^{vy})* allele, known to be sensitive to levels of epigenetic modifiers, lending further evidence that Wiz is involved in regulation of G9a-mediated transcriptional

activation. Furthermore, a reduction in levels of G9a/GLP are associated with Kleefstra syndrome, a neurological disorder that manifests in a range of symptoms including anxiety. Because of the high levels of Wiz in the brain, the researchers postulated that reduced levels of Wiz might also have neurological effects. Utilizing a suite of molecular and bioinformatics tools, the researchers demonstrate that Wiz binds active promoters and they provide evidence that suggests Wiz functions as a transcriptional activator in neural tissue. Furthermore, they show that *Wiz* mutant mice present with behavioral abnormalities, revealing an anxiety-like phenotype similar to what is observed with reduced G9a/GLP levels.

Wiz binds promoters and shows high consensus overlap with CTCF

In order to determine where Wiz was bound within the genome with respect to active and inactive genes, cerebellums of adult *Wiz*$^{+/+}$ (WT, wild-type) mice were collected and submitted to Active Motif for chromatin immunoprecipitation followed by sequencing (ChIP-Seq) services. ChIP-Seq analysis confirmed that Wiz binds across the genome at promoters and showed strong enrich-
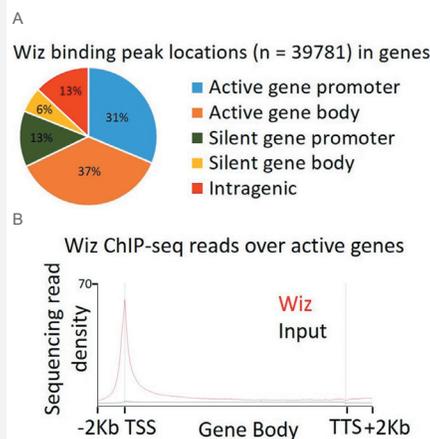

Figure 1: **Wiz binds across the genome and at promoter elements.** (A) Pie graph depicting the percentage of Wiz peaks that overlap with the promoter (up to 2 kb from a TSS) and genic sequence of active or silenced genes, according to RNA-Seq data mapped to Ensembl genes annotations. (B) Wiz ChIP-Seq occupancy over all Ensembl gene bodies is shown as deep sequencing read density along the transcription unit, including 2 kb up and downstream of the transcriptional start and stop site.
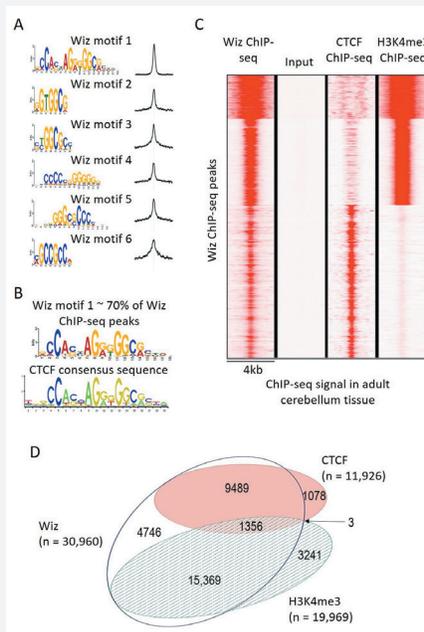

Figure 2: **Wiz binding consensus shows a high degree of overlap with CTCF.** (A) The six most enriched motifs in Wiz ChIP-Seq peaks (n = 39,781) and their distribution inside peaks are shown. (B) The most significant of these matches the CTCF-binding site consensus sequence (JASPAR CORE database - MA0139.1). (C) Read density for Wiz and the Encode CTCF and H3K4me3 ChIP-Seq datasets were calculated across a 4 kb region centered on the ~40,000 Wiz ChIP-Seq peaks. Input sequencing from the Wiz ChIP-Seq experiment is also shown. (D) Venn diagram showing the overlap for Wiz, CTCF and H3K4me3 ChIP-Seq peaks.

ment at the transcription start site (TSS) (Figure 1).

Motif analysis revealed that one of the common Wiz motifs showed correlation with the CTCF binding motifs (Figure 2A and 2B). A comparison of Wiz ChIP-Seq data with existing ENCODE ChIP-Seq data sets for CTCF and H3K4me3, a marker of active transcription, verified strong correlation with Wiz ChIP-Seq peaks, with almost all CTCF peaks overlapping with Wiz peaks (Figure 2C and 2D).

Wiz is associated with transcriptional activation in neural tissue

To identify sites within the genome where *Wiz* haploinsufficiency was causing differential gene expression, RNA-Seq was performed using brains from both E13.5 and adult *Wiz*$^{MD30/+}$ and *Wiz*$^{+/+}$ male and female mice. The results show that *Wiz* haploinsufficiency

causes reduced expression of a number of genes genome-wide. However, differential expression is observed mainly within gene clusters, in particular within the protocadherin β (mainly expressed in neurons) and cadherin 11-like clusters, and adjacent to telomeres, showing significant overlap with embryonic and adult expression patterns (Figure 3A). Telomeres are well-known hetero-chromatic regions, and protocadherin clusters exist as large tandem arrays important for gene silencing during heterochromatization, suggesting a role for Wiz in chromosome looping. Analysis of ChIP-Seq read density at the TSS of the differentially regulated genes in the *Wiz*$^{MD30/+}$ mice showed enrichment of Wiz at the TSS of genes with decreased expression, but not at the genes with increased expression, further lending evidence that Wiz acts as a transcriptional activator rather than repressor (Figure 3B).

Wiz haploinsufficiency in mice results in phenotypic alterations in behavior

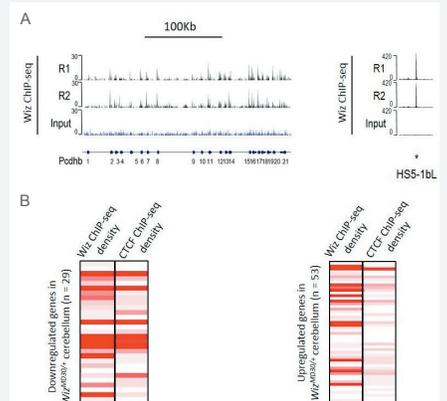To characterize any changes in behavior resulting from *Wiz* haploinsufficiency,

Figure 3: **Wiz binding at the protocadherin β locus in the adult cerebellum.** (A) Genome browser screenshots of the protocadherin β locus and the HS5-1bL enhancer (*indicated) located ~400 Kb downstream showing Wiz binding. (B) Shown is Wiz ChIP-Seq read density in adult cerebellum as a heatplot for the 29 genes that decreased and the 53 genes that increased in expression in the cerebellum of Wiz$^{MD30/+}$ mice. Also shown is CTCF ChIP-Seq read density. ChIP-Seq density was calculated for the 1 Kb surrounding the TSS of each gene showing that those with decreased expression showed high enrichment for Wiz and some for CTCF, whereas upregulated genes showed little enrichment for Wiz and no enrichment for CTCF.

the researchers conducted behavioral studies on cohorts of $Wiz^{MD30/+}$ and WT male mice. Their findings show various abnormalities in behavioral activity exhibited by the $Wiz^{MD30/+}$ mutant vs. WT mice, including increased displays of anxiety-like behavior.

Kdm5c-knockout mouse model of X-linked intellectual disability (XLID)

This article is a summary of the research published in *Cell Reports*.: Iwase *et al.*, A Mouse Model of X-linked Intellectual Disability Associated with Impaired Removal of Histone Methylation (2016), http://dx.doi.org/10.1016/j.celrep.2015.12.091.

Intellectual disability (ID), which manifests in limited adaptive behavior and intellectual functioning, is a prevalent neurological disorder affecting 1-2% of the total population. Several genetic variations have been associated with different forms of ID, including mutations in the X-linked (XL) *KDM5C* histone H3K4 di- and tri-methyl (H3K4me2/3)-specific demethylase gene which account for close to 3% of all XLID cases. KDM5C is ubiquitously expressed, although at higher levels in brain and skeletal muscle, and has been implicated in various other neurological disorders, including autism spectrum disorder, Huntington's disease and cerebral palsy, suggesting it has a critical role in the development of the central nervous system.

Mouse models have been instrumental in investigating brain disorders caused by mutations, including those of chromatin modifiers such as *Crebbp* and *Ehmt1* methyltransferases. However, there have never been any mouse models of mutations of 'erasers' of methylation to investigate their contribution to brain function. To test the hypothesis that loss-of-function of *Kdm5c* is causal to XLID, researchers from the laboratory of Dr. Yang Shi at Harvard created a *Kdm5c*-knockout (KO) as a mouse model of XLID and collaborated with Active Motif's Epigenetic Services team to gain a better understanding of the role of regulation of histone modifications in normal and diseased brains.
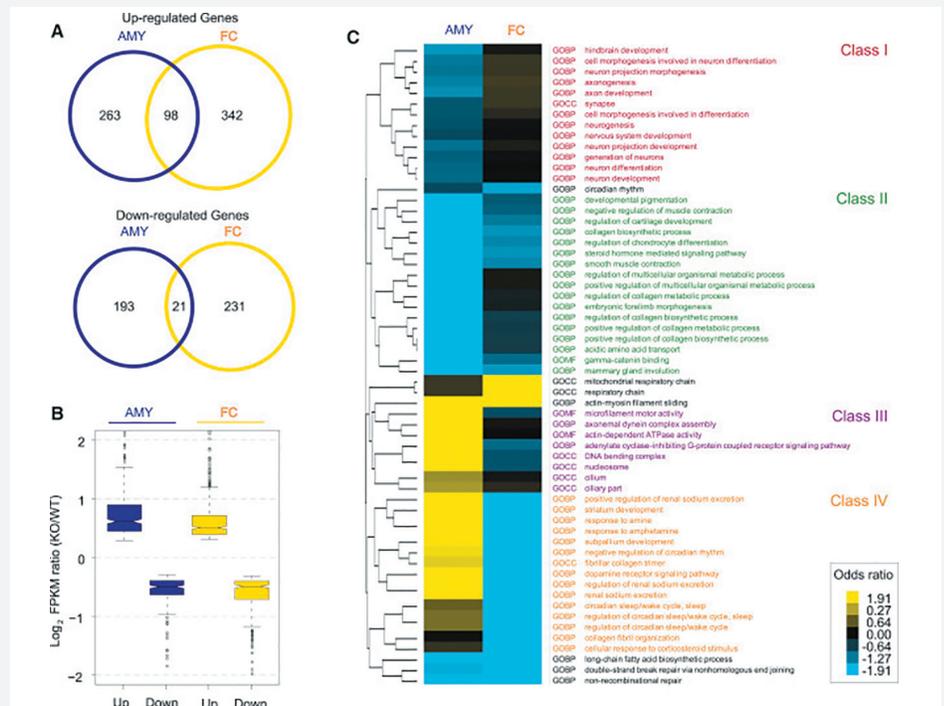

Figure 4: Altered transcription profiles in *Kdm5c*-KO brain tissues. RNA-Seq data were obtained from the amygdala (AMY) and the frontal cortex (FC) of three adult mice for each genotype. (A) Differentially expressed (DE) genes between WT and Kdm5c-KO brain. AMY and FC show a modest overlap. (B) KO/WT ratio of expression levels of DE genes. (C) LR path analysis of misregulated GO categories followed by clustering. The odds ratio for enrichment is shown. Yellow and blue indicate upregulation and downregulation in KO compared with WT, respectively. Four major classes (I to IV) represent differential alteration patterns between AMY and FC.

Kdm5c-KO mice exhibit abnormal social, adaptive and cognitive behaviors

To determine whether *Kdm5c*-KO mice displayed abnormalities observed in human XLID patients, the investigators performed a series of tests to assess the physical and behavioral variations between *Kdm5c*-KO (-/y, KO) and wild-type (+/y, WT) male littermates. Physically, the KO mice were reduced in size and body weight, also observed in XLID patients, but were otherwise in good health and fertile. The KO mice exhibited increased aggressive behavior, anxiety and hyperactivity, and impairments in cognitive and spatial learning and memory, which are also observed in XLID patients. In addition, KO mice displayed higher, although marginally non-significant, testosterone levels. Furthermore, KO mice displayed a lower interest in social interaction which is consistent with autistic behaviors that manifest in some individuals with *KDM5C* mutations.

Kdm5c loss affects both cellular phenotypes and gene expression in the brain

Abnormal dendritic arborization and spine morphology have been implicated as a cellular basis for ID. Previous *in vitro* studies have shown that *Kdm5c* is required for dendritic arborization in rat neurons in culture. However, no *in vivo* evidence exists to show a connection between Kdm5c and brain architecture. Therefore, the researchers examined whether the behavioral alterations observed in *Kdm5c*-KO mice is due to alterations in brain morphology. Their analysis revealed reduced dendritic arborization in the basolateral amygdala (BLA), an area critical for perception and emotional control, including fear and aggression. They also observed reduced spine density, abnormalities which have been previously linked to anxiety and cognitive defects, in the *Kdm5c*-KO vs. WT mice. In the cortex, a slight reduction in spine density was observed and dendritic arborization was found to be unaffected.

To determine the genome-wide impact of Kdm5c loss on the brain, the researchers performed RNA-Seq analysis of the amygdala (AMY) and frontal cortex (FC) transcriptomes of adult mice. They found a comparable number of differentially regulated genes in both brain areas, with a larger number of upregulated genes in KO vs. WT mice, consistent with the function of Kdm5c in removal of the H3K4me2/3 active chromatin mark (Figure 4A and 4B). Differential gene analysis showed that differentially regulated genes in the AMY and FC regions are associated with neural development and differentiation, synaptic pathways and behavior (Figure 4C). Interestingly, they also observed a statistically significant enrichment of misregulated androgen-responsive genes in the AMY and FC, consistent with higher testosterone levels observed in brains of *Kdm5c*-KO mice. Taken together, the data suggest these cellular alterations caused by *Kdm5c* loss-of-function may

potentially contribute to the behavioral phenotypes observed in *XLID*.

Molecular analysis of Kdm5c regulation of neuronal function

The researchers performed integrated functional genomic analysis of cortical neurons to investigate gene regulation by Kdm5c in a relatively homogenous cell population. ChIP-Seq analysis of Kdm5c data sets generated by Active Motif revealed that Kdm5c is mainly enriched at promoter and enhancer functional elements (Figure 5A). 93% of Kdm5c peaks were found within DNase hypersensitivity sites, a marker of open chromatin (Figure 5B). At promoters, a pronounced Kdm5c peak was observed at the transcription start site, and peaks often coincide with CpG islands, indicating that Kdm5c modulated transcription of active genes (Figure 5B and C). CpG islands often correlate with high H3K4me3 and mRNA levels, and Kdm5c-bound genes were found to coincide with

increased expression and H3K4me3 levels, suggesting CpG islands and H3K4me3 serve as primary signatures for recruitment of Kdm5c (Figure 5D). Because the enzymatic function of Kdm5c is to demethylate the H3K4me2/3 active mark, it was postulated that Kdm5c functions as a transcriptional repressor at H3K4me3 promoters. Combined RNA-Seq and ChIP-Seq analysis of WT and KO mice showed that Kdm5c is not a global regulator of H3K4 methylation, but rather acts on a subset of actively transcribed promoters (Figure 5E). Combined RNA-Seq and H3K4me1 and H3K4me3 ChIP-Seq data from WT and KO mice showed that low, but not middle or high expressed Kdm5c targets were upregulated in knockout mice and showed increased H3K4me3 levels at promoters. Since this was only observed in low expressing genes, the researchers concluded that, although Kdm5c functions as a transcriptional repressor, it mainly acts to fine-tune expression of more sensitive low-expressing targets. RNA-Seq analysis demonstrated significant overlap in the differentially regulated genes identified using cultured neurons vs. KO mouse models for Kdm5c. In both cases, the changes observed were modest and upregulation and downregulation of expression in KO mice correlated with increase or decrease in H3K4me3 levels at promoters, respectively. Although the changes were modest, the researchers conclude that the additive effects of these changes collectively contribute to the observed behavioral and cellular abnormalities characteristic of ID.
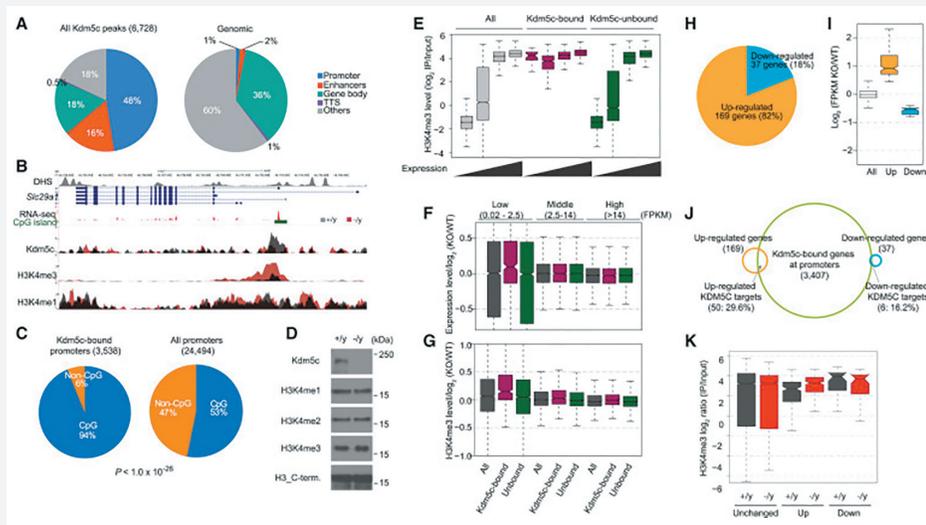

Figure 5: **Kdm5c is recruited to CpG promoters with high H3K4me3 in post-mitotic neurons and represses transcription.** ChIP-Seq and RNA-Seq were performed on two biological replicates of WT and *Kdm5c*-KO neurons. (A) Enrichment of Kdm5c peaks in promoter and enhancer regions (left) compared with genomic fractions (right). (B) UCSC browser shot of Kdm5c, H3K4me3, and H3K4me1 across the Kdm5c-bound locus *Slc29a1* (gray, WT; red, KO). RNA-Seq in WT neurons is shown in red; CpG island position is indicated in green. (C) Most Kdm5c-bound promoters harbor CpG islands (left), which represents strong enrichment over the genomic average (right). (D) Western blot showing global H3K4me levels are not altered in KO neurons. (E) WT neurons were used to divide genes into four groups according to expression level. Kdm5c-bound promoters with low expression levels show significantly higher H3K4me3 levels than unbound genes with the same expression levels. (F and G) Expression and H3K4me3 changes in KO neurons. WT neurons were used to divide genes into three groups according to expression level. Low-expressed Kdm5c-target genes showed the most noticeable increase in expression and H3K4me3 in KO neurons. (H) 206 differentially expressed (DE) genes in *Kdm5c*-KO neurons identified by RNA-Seq. (I) Expression level fold change of unchanged and DE genes in KO neurons compared with WT. (J) Overlap of Kdm5c-bound promoters with DE genes from KO/WT. (K) Upregulated and downregulated genes showed significantly higher or lower H3K4me3 levels in Kdm5c-KO neurons, respectively.

NEW
High Quality ChIP-Seq Data from as Few as 5,000 Cells

ChIP-Seq analysis typically requires millions of cells per immunoprecipitation (IP) reaction in order to obtain meaningful information about global changes across a large population of cells. This is often unattainable for researchers working with primary cells or other challenging sample types. To enable studies of the complexities of protein-DNA interactions from limited sample material, improvements to the traditional ChIP-Seq protocol are needed. Active Motif's Low Cell ChIP-Seq Kit addresses this need by providing a complete, optimized low cell ChIP-Seq workflow that enables generation of genome-wide binding profiles from as few as 5,000 cells.

Expect more from your low cell ChIP
Active Motif's Low Cell ChIP-Seq Kit is designed for highly sensitive chromatin immunoprecipitation from limited amounts of cell or tissue material. Active Motif has utilized its extensive expertise in ChIP-Seq to optimize chromatin preparation and immunoprecipitation procedures to lower the input requirements for detection of histone and transcription factor protein-DNA binding interactions. Chromatin can be prepared from as few as 5,000 cells or small tissue biopsies.

Low Cell ChIP-Seq not only reduces sample input requirements, but also resolves the issues often associated with low-cell ChIP, including poor signal-to-noise, inefficient library amplification and high duplication rates. Low background
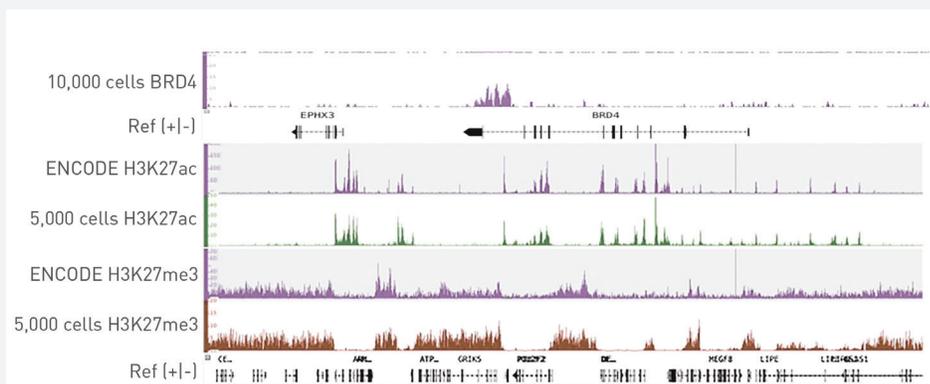

Figure 1: **The Low Cell ChIP-Seq Kit was used to immunoprecipitate 10,000 MDA-MB-468 cells with low abundance BRD4, or 5,000 GM12878 cells with either active histone mark H3K27ac or repressive histone mark H3K27me3.** Following ChIP, Illumina-compatible sequencing libraries were prepared using the included Next Gen DNA Library and Next Gen Indexing Kits. Samples were sequenced on the NextSeq 500. Results show high quality ChIP-Seq peaks can be obtained using as little as 5,000 cells for both active and repressive histone modifications and as low as 10,000 cells for low abundance BRD4.

Low Cell Number ChIP-Seq Kit

- Reproducible ChIP-Seq data from as little as 5,000 cells

- Works with histones and transcription factor targets

- Includes reagents to prepare a high complexity NGS library for use with Illumina® platforms

- Multiplex up to 16 samples on the same sequencing flow cell

Protein G agarose beads and blockers are used to minimize non-specific binding during the IP, while filtration columns provide a fast, easy and consistent solution for wash steps. Illumina-compatible sequencing libraries can be generated from as little as 10 pg ChIP DNA. Additionally, molecular identifiers (MIDs) are added with the P5 adapter during library preparation to distinguish PCR duplicates from fragmentation duplicates, thereby increasing the number of unique alignments.

What's in the box?
Each kit contains enough reagents to perform 16 Low Cell ChIP-Seq reactions. This includes reagents for chromatin preparation, immunoprecipitation, purification, as well as Active Motif's Next Gen DNA Library Kit and Next Gen Indexing Kit (see page 7) to make 16 unique NGS libraries for use on Illumina platforms.

To learn more on Low Cell ChIP-Seq, visit www.activemotif.com/chip-lowcell.

Product	Format	Catalog No.
Low Cell ChIP-Seq Kit	16 rxns	53084

NEW
NGS DNA Library Preparation with Molecular Identifiers Eliminates Bias from PCR Duplicates

Improve the quality of your Next-generation sequencing data sets by including molecular identifiers (MIDs) into your DNA library preparation. Active Motif's Next Gen DNA Library Kit includes MIDs as part of the P5 adapter for accurate de-duplication of PCR replicates from single read sequencing. The ability to distinguish and selectively eliminate PCR duplicates from fragmentation duplicates improves the quality of data by increasing the number of unique alignments. The Next Gen DNA Library Kit can be used to prepare high complexity NGS libraries from double-stranded genomic DNA, ChIP DNA, FFPE DNA, or cell-free DNA (cfDNA).

High complexity NGS DNA Libraries
The Next Gen DNA Library Kit* is designed to generate high complexity DNA libraries for Next-generation sequencing for use with Illumina® platforms. Libraries can be generated from as little as 10 pg double-stranded DNA, or from as low as 100 ng DNA if preparing PCR-free libraries (Figure 1). The library kit is used in combination with the Next Gen Indexing Kit, which contains 16 unique index adapters that may be used to multiplex different samples together during cluster generation for co-sequencing on the same flow cell.

Molecular identifiers (MIDs)
The Next Gen DNA Library Kit offers the advantage of including molecular identifiers during library generation. The MID is a 9 base random N sequence that is added with the P5 adapter (Figure 2). Addition of the MID is strand-specific to enable accurate de-duplication from single read sequencing by distinguishing PCR duplicates from fragmentation duplicates. This increases the number of unique alignments from sequencing for more accurate data analysis.
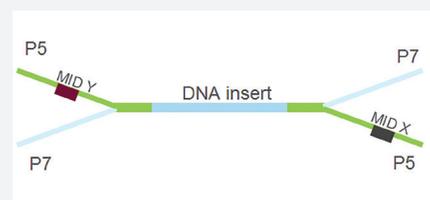

Figure 2: Schematic of a completed MID tagged and indexed DNA library molecule.

What's in the box?
The Next Gen DNA Library Kit contains the reagents needed to repair both 5′ and 3′ termini and sequentially attach Illumina adapter sequences to the ends of fragmented dsDNA. The Next Gen Indexing Kit contains 16 unique indices. Indices may be used individually or in combination to multiplex samples together within the same sequencing reaction.
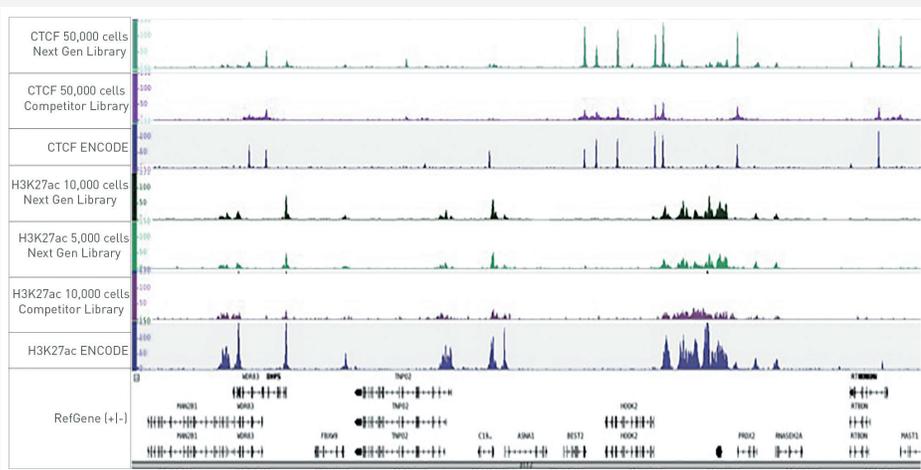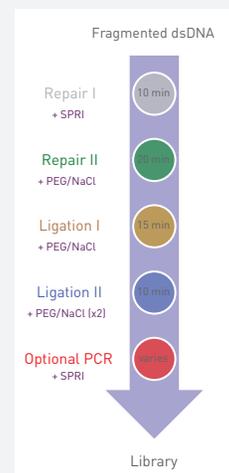

Figure 1: Active Motif's Low Cell ChIP-Seq protocol was used to prepare chromatin from GM12878 cells using the number of cell equivalents listed. ChIP-Seq libraries were prepared using either the Next Gen DNA Library Kit or a competitor kit. Results show strong peaks that match the ENCODE data sets for the Next Gen Libraries, even if starting from low cell numbers.

Product	Format	Catalog No.
Next Gen DNA Library Kit	16 rxns	53216
Next Gen Indexing Kit (16 indices)	64 rxns	53264

*The Next Gen DNA Library and Indexing Kits are Powered by Swift Biosciences.

NEW
Reveal Histone Modification Patterns in Clinical Samples with Active Motif's FFPE ChIP-Seq Service

FFPE tissue blocks and histology slides are a valuable resource for retrospective research on clinical samples. In the field of epigenetics, the benefits of this archived material has not been fully realized because of the difficulty these sample types present for use in conventional epigenetic techniques such as chromatin immunoprecipitation (ChIP) due to their limited size and the harsh conditions under which these samples have been prepared. Active Motif's new FFPE ChIP-Seq Service gives you access to breakthrough technology from our Epigenetic Services lab that now enables the generation of genome-wide histone modification profiles from FFPE samples.

Accessing epigenetic information from archived FFPE samples
The ability to study FFPE samples provides researchers with an opportunity to link FFPE data to disease, diagnosis and biomarker discovery. There is increasing interest in utilizing these clinical samples for studies in various research areas, such as epigenetics where aberrant histone and DNA modifications have been linked to various human pathologies, including neurological, immune and neoplastic disorders. ChIP is widely used in epigenetics research to determine global histone modification patterns. In some diseases, such as cancer, enzymes that regulate histone modification deposition and removal have been shown to be mutated. Research into cancer therapies aimed at restoring normal histone landscapes through small molecule inhibition are now underway and raise the possibility that histone modification occupancy profiles can inform treatments or predict patient outcome.

Active Motif's new FFPE ChIP-Seq Service performs the work for you!
A major challenge arises when attempting to extract epigenetic information from clinical samples. FFPE tissue is typically limited in size, lacks consistency in preparation, and often displays degradation and loss of antigenicity due to harsh fixation conditions or prolonged storage, presenting an obstacle for performing sensitive epigenetic techniques such as ChIP. These factors can increase the difficulty of chromatin preparation and often

lead to the need for optimization. Active Motif's new FFPE ChIP-Seq Service takes the burden of optimization out of your hands and now makes it possible to profile histone modification binding patterns or perform biomarker identification studies in FFPE patient samples with ease. Simply send us your FFPE blocks or sections and we will do the rest!

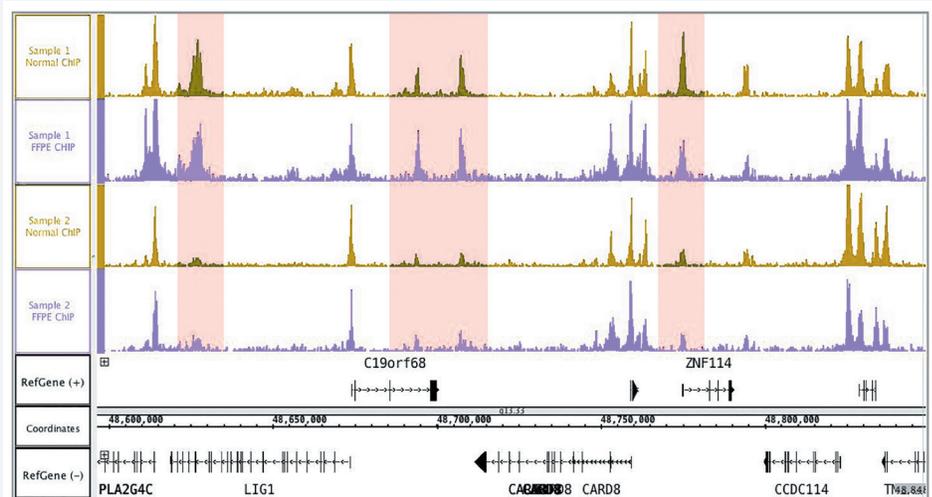To learn more about or end-to-end FFPE ChIP-Seq Service, visit us at www.activemotif.com/services-ffpechip.

Sample types:
• FFPE blocks or sections
Targets:
• H3K4me1, H3K4me2, H3K4me3
• H3K9ac, H3K14ac
• H3K27ac
• H3K36me3
• CTCF, *and more...*

Figure 1: ChIP was performed using chromatin from two human glioblastoma biopsies and an antibody against H3K27ac. Biopsies were provided as both FFPE prepared samples (purple) and fresh/frozen samples (copper). FFPE and fresh/frozen ChIP-Seq data sets were similar. Tumor-specific H3K27ac occupancy was detectable in the FFPE and fresh/frozen data sets (highlighted in red).

Easily Isolate Intact Histones for PTM Analysis

Histone post-translational modifications (PTMs) play a crucial role in the regulation of transcription and chromosome packaging. Epigenetic modifications such as histone methylation, acetylation, and phosphorylation help to recruit chromatin remodeling proteins that assist in unraveling the chromatin to allow access to the basal transcriptional machinery, or deposit repressive modifications which lead to chromatin condensation and gene silencing. Don't risk losing your histone PTMs during extraction. Isolate core histone proteins from cells or tissues using a protocol designed specifically to extract and retain histone PTMs.

Histone charge is key to isolation

The basic structural unit of chromatin is the nucleosome, which consists of DNA wrapped around an octamer of core histone proteins (H2A, H2B, H3 and H4). The interaction between histones and DNA occurs because histones are positively charged proteins that are attracted to the negatively charged DNA backbone.

The highly basic nature of histone proteins often precludes them from isolation using traditional methods for protein extraction. Instead, histones must be isolated under acidic conditions to retain solubility. The inclusion of inhibitors helps to preserve histone modifications such as phosphorylation, acetylation and methylation at specific amino acid residues on the N-terminal histone tails.

Active Motif's new Histone Extraction Kit provides a simple method to acid precipitate histones from cell culture, tissue (including brain), and primary cells (e.g. T-cells) while preserving histone modifications (Figure 1). For cultured cells, both a one-step and two-step extraction protocol are included for sample optimization. Lysates prepared using the Histone Extraction Kit are suitable for use in downstream applications such as Western blot, Histone Modification ELISAs or Active Motif's Histone H3 PTM Multiplex Kit.

For researchers looking for a more purified histone lysate, we recommend using one of Active Motif's Histone Purification Kits which include additional column purification and precipitation steps to remove non-histone proteins.

For more information on histone isolation or PTM analysis, visit us at www.activemotif.com/histone.
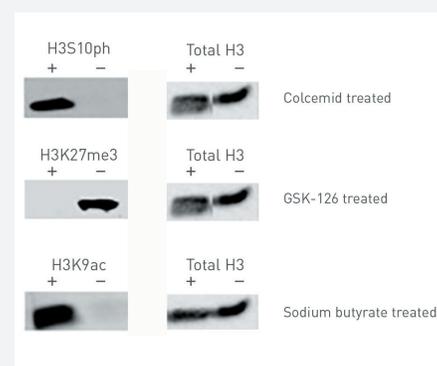

Figure 1: A Western blot confirms histone PTMs are preserved following use of the Histone Extraction Kit to isolate lysates from untreated (–) and treated (+) HeLa cells using the one-step extraction protocol.

What's in the box?

The Histone Extraction Kit contains enough reagents for 100 extractions from cultured cell lines and tissues, or two 96-well cell culture plates. Supplemental protocols are provided for working with primary cells.

Histone Extraction Kit Highlights

- Acid extraction protocol isolates highly basic histone proteins from cell culture, tissue, and primary cells (e.g. T-cells)

- Preserves histone acetylation, methylation and phosphorylation

- Compatible with downstream analysis by Western blot, ELISA and Active Motif's Histone H3 PTM Multiplex Kit

Product	Format	Catalog No.
Histone Extraction Kit	192 rxns	40028
Histone Purification Kit	10 rxns	40025
Histone Purification Mini Kit	20 rxns	40026
Histone H3 trimethyl Lys 4 ELISA	1 x 96 rxns	53113
Total Histone H3 ELISA	1 x 96 rxns	53110
Histone H3 PTM Multiplex Kit	96 rxns	33115

NEW

RRBS Service for Genome-scale DNA Methylation Profiling

Active Motif has now expanded it's suite of DNA methylation services with the introduction of our new RRBS (Reduced Representation Bisulfite Sequencing) Service. The addition of RRBS along with our existing MeDIP-Seq and Targeted Bisulfite Sequencing services provides a broad choice of applications for DNA methylation analysis. Our RRBS Service queries the methylation status of more than 4 million individual CpGs using only 1 µg of starting material. As with all of our services, customers only need to provide DNA, tissues or cells, and we will perform the entire experiment from sample preparation to bioinformatic analysis.

RRBS Service

Customers submit DNA, cell pellets or frozen tissues, then *we perform*:

1. DNA purification
2. DNA digestion
3. Sequencing adapter ligation
4. Bisulfite conversion
5. PCR amplification
6. Sequencing on Illumina® platform
7. Bioinformatic analysis

Why use RRBS for DNA methylation analysis?

DNA methylation is a stable and heritable epigenetic modification that is involved in the regulation of gene expression. DNA methylation patterns are cell-type specific and are indicative of cellular origin or fate. Alterations in DNA methylation patterns can be prognostic of disease and even provide information about the underlying mechanisms and cause of disease. Therefore, comparative analysis of DNA methylation patterns from normal and diseased or treated vs. untreated samples can be a productive strategy for the identification of biomarkers.

Advantage of RRBS for biomarker identification

RRBS is an ideal method for biomarker identification in that it increases the efficiency of genome-scale DNA methylation profiling without compromising the sensitivity achieved by conventional Whole Genome Bisulfite Sequencing (WGBS). RRBS significantly lessens the number of nucleotides analyzed by deep sequencing by enriching for regions of high CpG content within the genome.

This method dramatically reduces the scale and expense of WGBS yet still provides millions of data points focused at biologically relevant positions such as promoters and CpG islands. By utilizing Active Motif's new end-to-end RRBS Service, you can experience all of the benefits this method provides for whole-genome DNA methylation analysis including:

> Single base resolution
> Quantitation at each base
> Data at millions of locations across the genome
> Data enriched at promoters and CpG islands
> Significantly less expensive than Whole Genome Bisulfite Sequencing

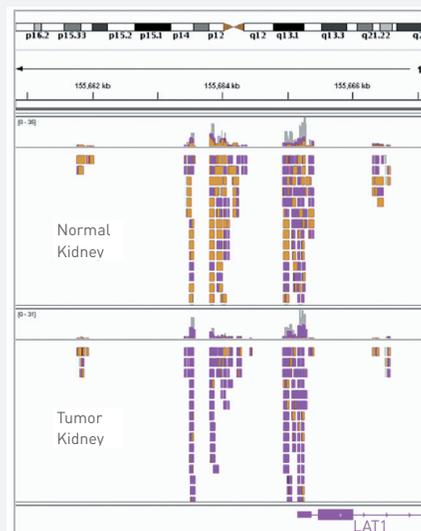For more information, please visit us at www.activemotif.com/services-rrbs.

Figure 1: **RRBS data using biopsied human kidney tumor and adjacent normal kidney.** The displayed region is a representative region from the genome-wide data set and shows differential DNA methylation at the promoter of the *LAT1* gene. Each block is a separate data point with copper representing a methylated cytosine and purple representing the unmethylated base.

RRBS Service Features

- Low input material requirements
  > 1 µg DNA, 500K cells or 25 mg tissue

- Data provided on > 4 million CpGs

- Data from biologically relevant regions
  > Promoters
  > CpG islands

Is DNA Methylation a Biomarker in Your FFPE Sample?

Formalin-fixed, paraffin-embedded (FFPE) tissues are a valuable resource for retrospective research on clinical samples because information about treatments and outcomes are often available. Data obtained from FFPE collections can be used to identify biomarkers for disease research. Active Motif's FFPE Bisulfite Conversion Kit enables you to obtain DNA methylation profiles at single base-pair resolution from FFPE core samples or tissue sections. Bisulfite converted DNA can be analyzed to identify locus-specific or genome-wide DNA methylation patterns.

DNA methylation as a biomarker

FFPE samples are highly valuable for their ability to relate clinical outcomes to disease states and epigenetic profiles. A common biomarker observed in the cancer epigenome is altered DNA methylation patterns including global DNA hypomethylation and promoter-specific hypermethylation silencing tumor suppressor genes. Aberrant methylation profiles correlate with multiple developmental diseases and cancers. By analyzing DNA methylation profiles in FFPE tissues, a better understanding of disease progression and treatments can be obtained.
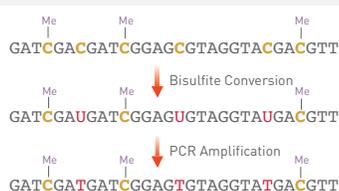

Figure 1: Schematic of bisulfite conversion illustrates the conversion reaction only modifies unmethylated cytosine residues. Methylated cytosines are unchanged.

Benefits of bisulfite conversion

Bisulfite conversion followed by DNA sequencing has long been considered the "gold standard" in site-specific DNA methylation analysis as it provides single base-pair resolution of the DNA methylation profile. The conversion reaction occurs as a 3-step deamination of cytosine residues into uracil. As only unmethylated cytosine
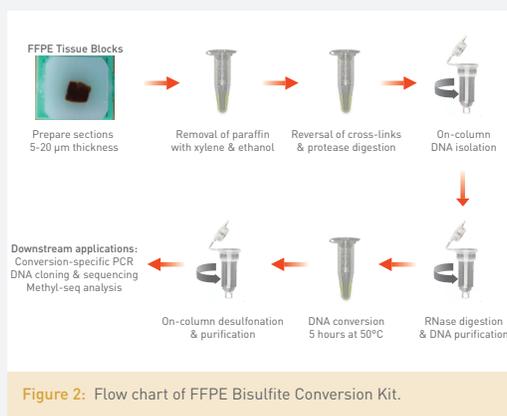

Figure 2: Flow chart of FFPE Bisulfite Conversion Kit.

residues are susceptible to bisulfite conversion, the original methylation state of the DNA can be determined.

FFPE challenges

FFPE samples are often stored for long periods of time under less than optimal conditions and the harsh chemicals needed to preserve tissue structure and prevent putrefaction often lead to highly fragmented and degraded DNA, making it challenging to obtain usable data. Active Motif's FFPE Bisulfite Conversion Kit has addressed this challenge by providing optimized reagents throughout the workflow to improve the recovery of high quality DNA, minimize degradation and provide greater than 99% conversion efficiency (Figure 2).

What's in the box?

Active Motif's FFPE Bisulfite Conversion Kit contains enough reagents to perform 40 DNA isolations from up to four 20 µm FFPE tissue sections or 35 mg of unsectioned, core samples per reaction. Bisulfite conversion reagents are included for 40 samples, with input DNA requirements ranging from 5 pg - 2 µg per reaction. A positive control conversion-specific PCR primer pair that is specific for bisulfite-converted human or mouse DNA is also included in the kit. Because the primer pair only produces a PCR product if conversion has occurred, you can confirm the procedure worked before starting downstream sequencing (Figure 3). For more information, visit www.activemotif.com/bis-conv-ffpe.
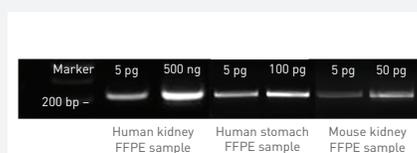

Figure 3: **Bisulfite conversion from as low as 5 pg DNA.** Active Motif's FFPE Bisulfite Conversion Kit was used to isolate genomic DNA from human kidney, human stomach and mouse kidney FFPE tissue sections. Bisulfite conversion was performed using the input quantities listed. Results show a 220 bp conversion-specific PCR amplicon that is present only in bisulfite converted DNA.

Product	Format	Catalog No.
FFPE Bisulfite Conversion Kit	40 rxns	55021

NEW
Simple High-throughput Screen to Evaluate Changes in Global DNA Methylation

Active Motif offers two simple-to-use solutions to screen for global changes in DNA methylation levels. The first utilizes the repetitive element, LINE-1, to quantify 5-methylcytosine (5-mC) levels in either human or murine samples. The second specifically detects global changes in the DNA methylation variant, 5-hydroxymethylcytosine (5-hmC). Both are provided as quantitative plate-based ELISA assays to enable easy, rapid high-throughput processing of samples in a single experiment.
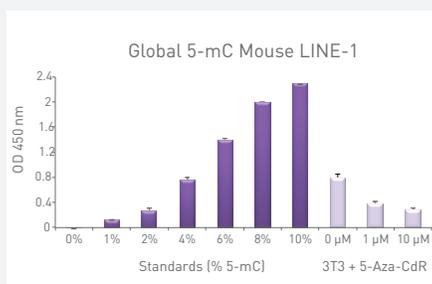

Figure 1: The Global DNA Methylation Mouse – LINE-1 Kit was used to compare the methylcytosine levels between two genomic DNA samples. Assay results show a decrease in 5-mC levels resulting from treatment of 3T3 cells with 5-Aza-2-deoxycytidine, a DNA methyltransferase inhibitor. The % 5-mC is calculated using the included DNA standards. Methylcytosine levels of the DNA standards are displayed as a percentage of total cytosine content.

Using global DNA methylation changes to study disease
Population studies have revealed a strong correlation between global DNA methylation levels and factors such as environmental exposures, lifestyle or clinical outcomes. The ability to screen patient samples for changes in global 5-mC or 5-hmC levels will provide much needed insight into our understanding of the relationship between DNA methylation and gene expression and its correlation with diseases such as cancer.

DNA methylation is characterized by a methyl group within the cytosine of a CpG dinucleotide. These CpG sites are often found within repetitive regions

and are normally methylated to repress transcription, while global hypomethylation of repetitive elements is observed in cancer. Likewise, the DNA methylation variant 5-hmC is known to play a role in transcriptional regulation and embryonic development and may also serve as a prognostic indicator in certain neurodegenerative disorders.

Global 5-mC quantification
Active Motif's simple, plate-based Global DNA Methylation – LINE-1 Kit uses a consensus sequence within the human or mouse repetitive Long Interspersed Nucleotide Element 1 (LINE-1) as a surrogate readout for global 5-mC levels. First, genomic DNA is enzymatically digested to create LINE-1 fragments. The DNA is then hybridized with a LINE-1 consensus probe. Following capture onto a 96-well plate, a 5-mC antibody and colorimetric detection reagents are used to generate a signal that is easily measured with a microplate reader. The assay includes DNA standards for 5-mC quantification and can detect as little as 0.5% methylcytosine (Figure 1).

Global 5-hmC quantification
The Global 5-hmC Quantification Kit uses a DNA binding agent to capture enzymatically digested DNA fragments to the plate and an antibody specific for 5-hmC for detection. This colorimetric assay works with as little as 20 ng of genomic DNA and includes DNA standards for quantification (Figure 2).
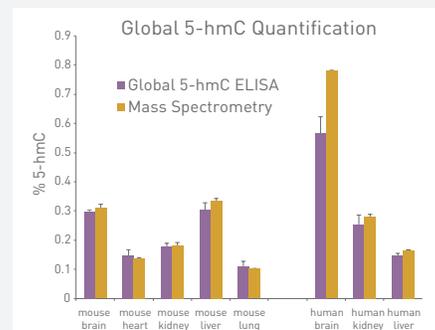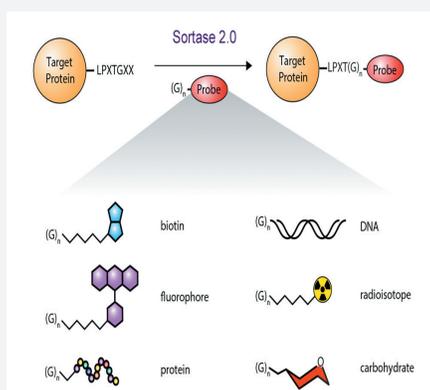

Figure 2: The Global 5-hmC Quantification Kit was used to determine the % 5-hmC in genomic DNA isolated from mouse and human tissues. Genomic DNA was tested at 20 ng/well for human brain and 50 ng/well for all other DNA samples. The % 5-hmC was determined using the included DNA standards. Results were compared to mass spectrometry data obtained from 500 ng of the same DNA samples. Results show the Global 5-hmC Kit provides equivalent quantification using only a fraction of the starting material.

Product	Format	Catalog No.
NEW: Global DNA Methylation Mouse – LINE-1	1 x 96 rxns	55022
Global DNA Methylation – LINE-1 Kit	1 x 96 rxns	55017
Global 5-hmC Quantification Kit	1 x 96 rxns	55018

NEW
Sortase 2.0 – A Versatile Platform for Rapid, Controlled, Site-specific Protein Modification

The proteome is diverse with many nuances surrounding structure and function, and post-translational modifications (PTMs) compound an already complex system. Identification, characterization and mapping of protein modifications enables insight into protein function, unlocking disease pathways and aiding in identification of drug targets. Currently, researchers have few options for modifying proteins for functional research, and each comes with its own set of limitations. To overcome these limitations, Active Motif now offers a proprietary labeling technology, Sortase 2.0.

Deciphering protein structure and function with better tools is vital to unlocking disease pathways or identifying novel drug targets. However, conventional techniques have their shortcomings. Genetic based approaches can alter a protein's biophysical properties and do not allow incorporation of synthetic modifications. Traditional chemical modification methods are not site-specific and may lead to a loss of protein function. Active Motif's Sortase 2.0 is a novel technology for protein modification and labeling that can achieve markedly higher activity in less time than the wild-type Sortase A while still providing site-specific control over the target modification.

Sortase is a transpeptidase found in Gram-positive bacteria that functions to anchor surface proteins to the bacterial cell wall. Sortase A modifies

Sortase 2.0 Advantage

- 10X higher activity than wild-type Sortase A
- Fast, simple site-directed labeling
- N- or C-terminal reaction
- Works with multiple expression systems

proteins by recognizing and cleaving a carboxy-terminal sorting signal. Sortase 2.0 is a proprietary engineered variant of Sortase A that specifically labels the C- or N-terminus of proteins at the site of the Sortase 2.0 recognition sequence (LP[X]TG). The activity of the Sortase 2.0 is roughly 10X greater than Sortase A and is calcium independent. Furthermore, the small size of the recognition sequence extension prevents its interference with protein function and trafficking. Proteins equipped with the C- or N-terminus LP[X]TG sequence can be labeled in solution and do not require extensive purification prior to labeling, as only proteins carrying the this sequence will be modified. Alternatively, proteins

carrying the LP[X]TG sequence can be labeled directly on the cell surface. Proteins that have been genetically engineered to contain the LP[X]TG motif can be labeled by Sortase 2.0 using a wide variety of tags, peptides, DNA, carbohydrates or fluorophores.

The Sortase 2.0 N- and C-Terminal Protein Labeling Kits provide sufficient materials to label up to 25 nmol of protein or 5×10^6 cells. Each kit contains a Sortag-ready control protein with a glycine attached at the respective terminus and a His6-tag along with a Sortase Buffer used to dilute substrate peptides or proteins to the desired concentration prior to Sortagging. The Sortase 2.0 enzyme provided in the kit is also modified with a His6-tag and can easily be removed by adsorption onto Ni-NTA resin.

For more information, please visit us at www.activemotif.com/sortase2.0.

COMING SOON:
ABFLEX RECOMBINANT ANTIBODIES
proprietary direct labeling technology

Product	Format	Catalog No.
Sortase 2.0	250 µg	13100
Sortase 2.0 N-Terminal Protein Labeling Kit	1 Kit	13102
Sortase 2.0 C-Terminal Protein Labeling Kit	1 Kit	13103

A Genome-wide 3'UTR Collection for High-throughput Functional Screening of miRNA Targets

Active Motif's LightSwitch™ Luciferase Assay System includes a genome-wide collection of 12,000 3'UTR reporters to enable researchers to better understand microRNA (miRNA) and 3'UTR interactions. In a recent study, the laboratory of Dr. Settleman performed a functional screen of 879 human miRNAs which identified miR-371-3p as a potent suppressor of drug tolerance. Using the LightSwitch system, they identified *PRDX6* (peroxiredoxin 6) as a key target of miR-371-3p in establishing drug tolerance, suggesting that co-targeting of *PRDX6* or modulating miR-371-3p expression together with targeted cancer therapies may delay or prevent acquired drug resistance.

LightSwitch™ 3'UTR Reporter Collection and Assay System

MicroRNAs are important regulators of gene expression involved in both normal and pathological processes. Accumulating evidence suggests a role for miRNAs in epigenetically regulating various phenotypic states in cancer cells. miRNAs can impact genetic programs through post-tran-scriptional silencing of target genes either by promoting degradation of target messenger RNAs (mRNAs) or by inhibiting their translation. miRNAs have been implicated in the regulation of various aspects of cancer biology, including drug resistance, cancer cell
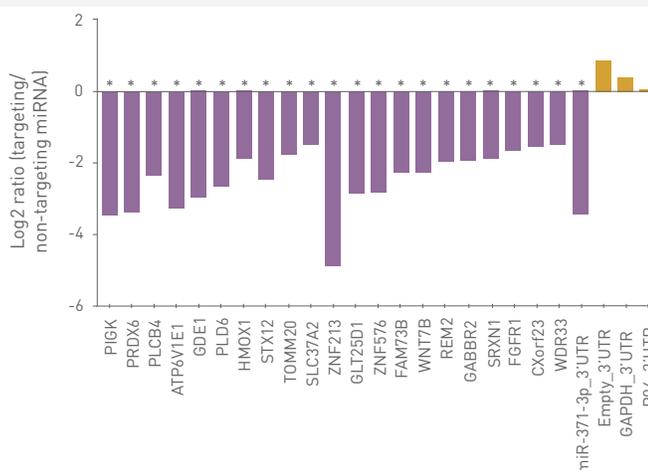
Figure 2: **Identification of relevant miR-371-3p targets using the LightSwitch luciferase reporter assay and 3'UTR target reporters.** Downregulation of RenSP luciferase by miR-371-3p upon erotinib treatment from 3'UTR LightSwitch reporters corresponding to candidate miR-371-3p target genes *p<0.05.
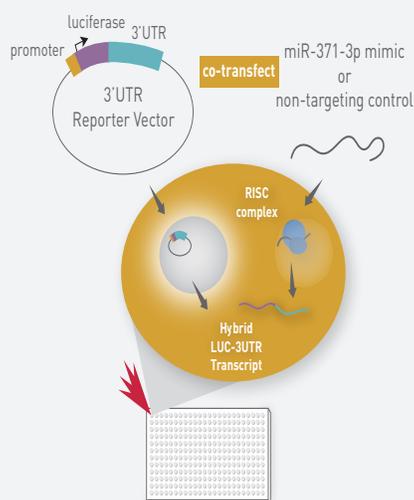
Figure 1: Experimental design for miR-122 functional screen using the LightSwitch System.

stemness, epithelial-to-mesenchymal transition and metastasis (Sahu *et al.*, 2016). To enable researchers to leverage 3'UTR reporter assays in their cancer research on a high-throughput scale, we have created a genome-wide collection of 12,000 human 3'UTR luciferase reporters in the highly optimized LightSwitch Luciferase Assay System. The Active Motif LightSwitch assay is ideal for performing miRNA target validation and assessing the functional impact of miRNA-3'UTR interactions. Combined with our large collection of miRNA mimics and inhibitors, you have everything needed to study miRNA-3'UTR interactions, validate miRNA targets, and to measure

RNA stability and the functional impact of miRNAs on a gene-by-gene basis (Figure 1).

For more information, please visit www.activemotif.com/ls-3utr.

Functional screening implicates miR-371-3p and PRDX6 in reversible tolerance to cancer drugs

Acquired resistance to cancer drug therapies almost always occurs in advanced-stage patients, even following a significant response to treatment. In addition to mutational mechanisms, various non-mutational resistance mechanisms have now been recognized. In a recent study, Dr. Nisebita Sahu
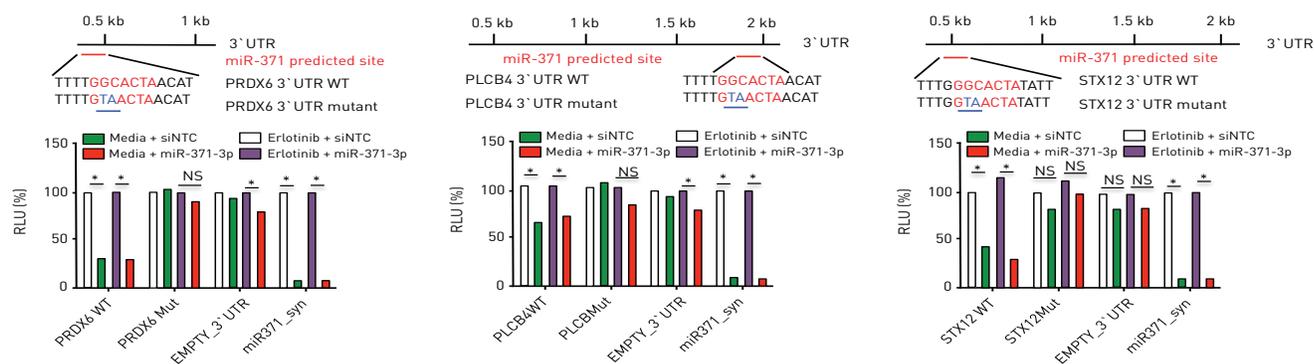
Figure 3: Mutational validation of relevant miR-371-3p targets using the LightSwitch system.
Mutations of seed sequences of the putative miR-371-3p recognition element in target 3'UTRs prevented inhibition by miR-371-3.

explores the potential role for miRNAs in transient drug tolerance.

To investigate whether miRNAs are required to engage or maintain the drug-tolerant state, Sahu et al. functionally screened 879 human miRNAs and identified miR-371-3p as a potent suppressor of drug tolerance.

Identification of miR-371-3p targets

Next, candidate gene targets of miR-371-3p were identified using the TargetScan prediction algorithm. The KDM5A and IGF1R genes, which encode proteins previously found to regulate DTPs (drug-tolerant persisters), did not display any 3′ untranslated regions (3′UTRs) that could be potentially targeted by miR-371. Direct regulation by miR-371-3p of the top 70 predicted targets was assessed using the Active Motif LightSwitch Luciferase Reporter Assay System following erlotinib treatment in the presence of miR-371-3p. In addition, KDM-3B, which displayed partial sequence homology in the 3′UTR with miR-371, was explored using the 3′UTR luciferase reporter assay. However, miR-371 was unable to target the 3′UTR of KDM-3B, suggesting that miR-371 does not regulate DTPs via KDM-3B. Significantly, 21 genes displayed reduced luciferase activity upon erlotinib treatment,

implicating these genes as bona fide targets of miR-371-3p (Figure 2).

To further validate the selective downregulation of specific gene targets by miR-371-3p, the single putative miR-371-3p recognition site within PRDX6, PLCβ4 and STX12 3′UTR sequences was mutated, which abolished the ability of miR-371-3p to inhibit luciferase reporter expression. These results confirm miR-371-3p as a direct regulator of these genes and suggest that a single recognition element is sufficient for their regulation by miR-371-3p (Figure 3).

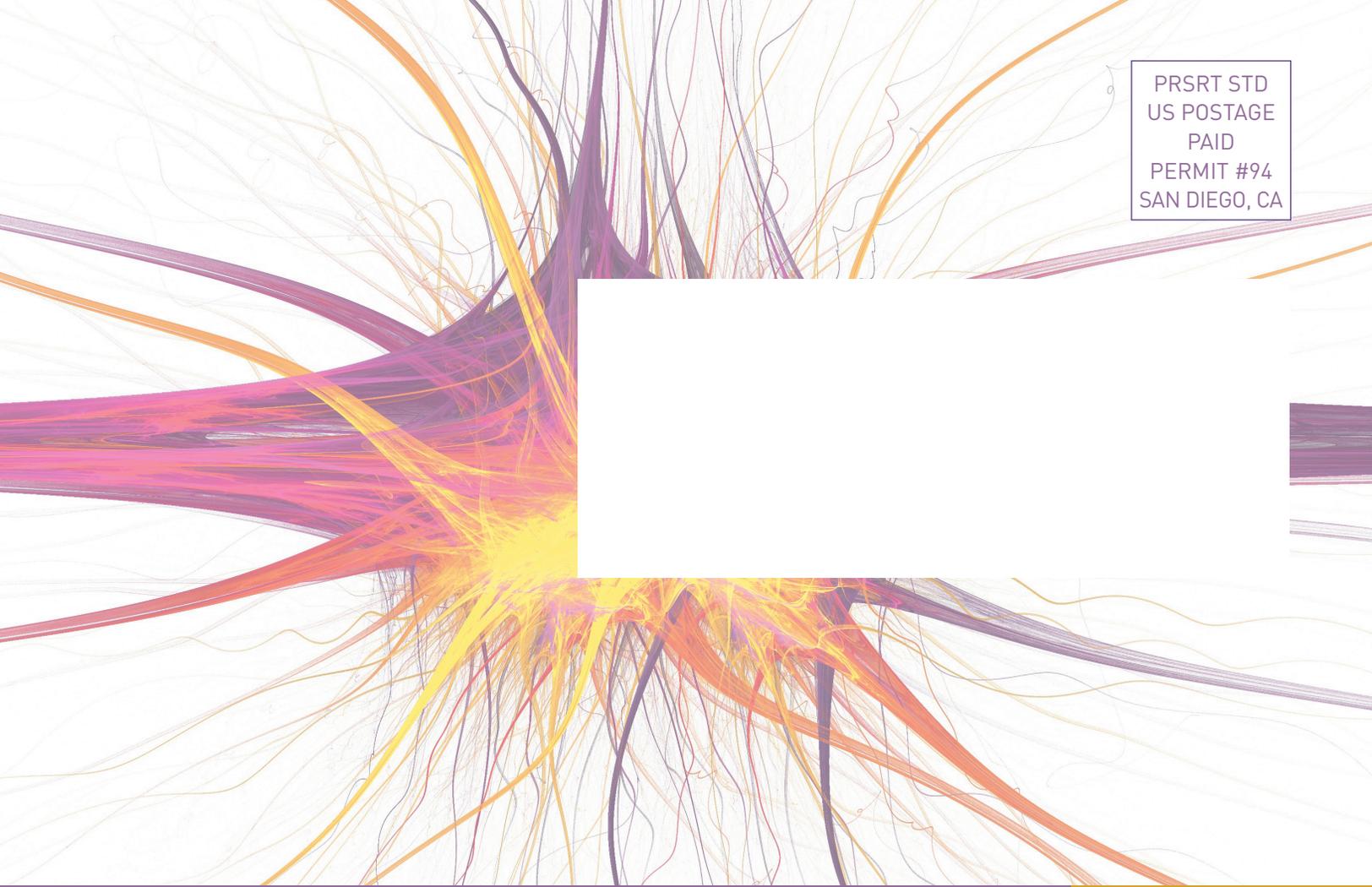### The LightSwitch Assay System in functional validation of miRNA targets

Taken together, The Active Motif LightSwitch™ miRNA Target Validation and Custom Mutagenesis Services were key components in enabling

Sahu et al. to reveal the miR-371-3p target gene PRDX6 as a key regulator of the reversible drug tolerance that frequently emerges within hetero-geneous cancer cell populations. In conclusion, the findings presented by the Settleman laboratory in this Nature Communications report reveal a major regulatory role for miRNAs in the emergence of reversible drug tolerance.

For more information LightSwitch products and services, visit us at www.activemotif.com/lightswitch.

Product	Format	Catalog No.
LightSwitch™ 3′UTR GoClone	5 µg	32011
LightSwitch™ Custom Cloning	5 µg	32051
LightSwitch™ Custom Mutagenesis	5 µg	32052
LightSwitch™ miRNA Mimics & Inhibitors	5 nmol	29000
LightSwitch™ Luciferase Assay Kit	100 assays	32031
LightSwitch™ Synthetic miRNA Target	5 µg	32003
LightSwitch™ miRNA Target Validation Service	Custom	32055

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