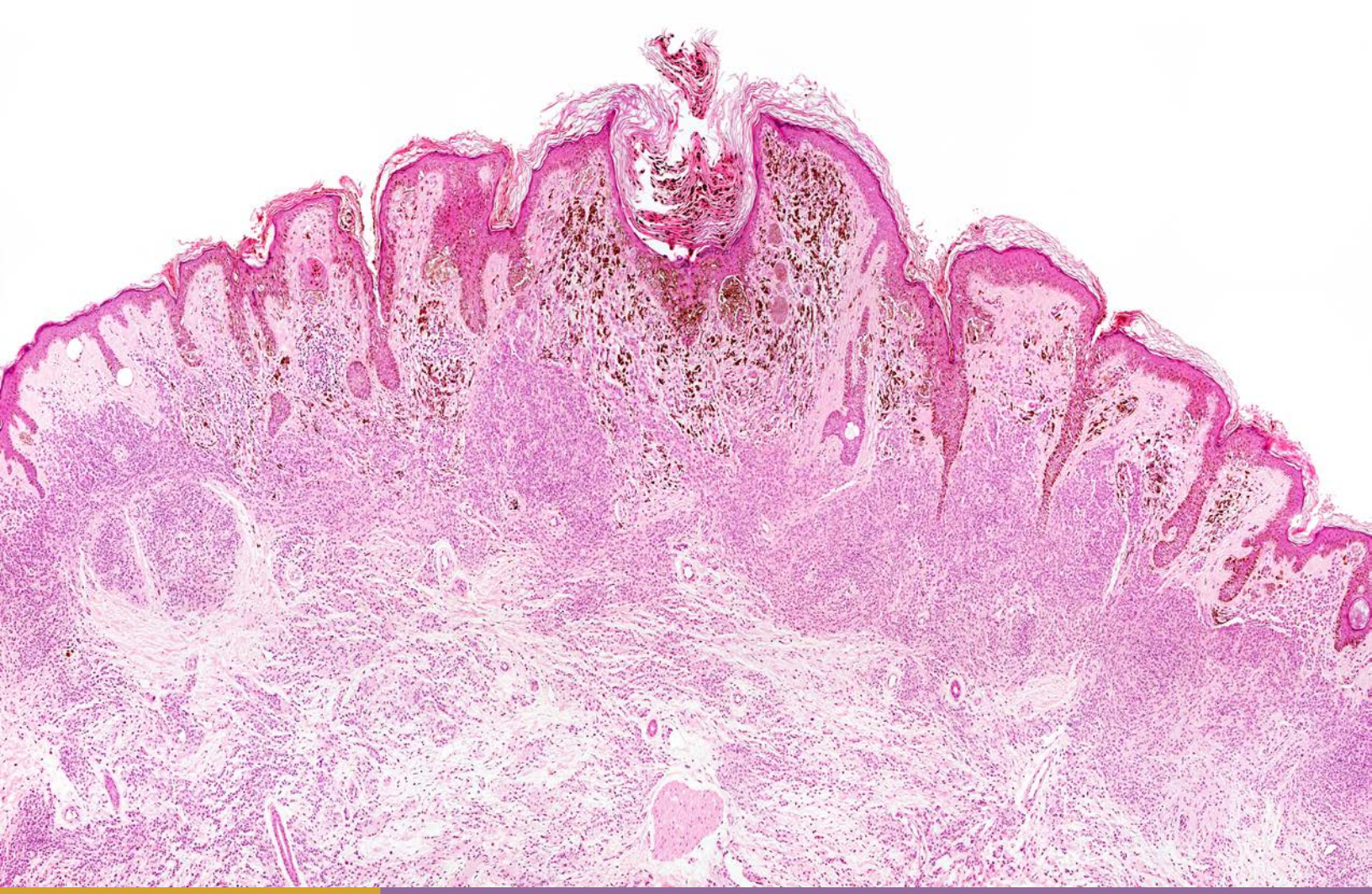


motif|vations

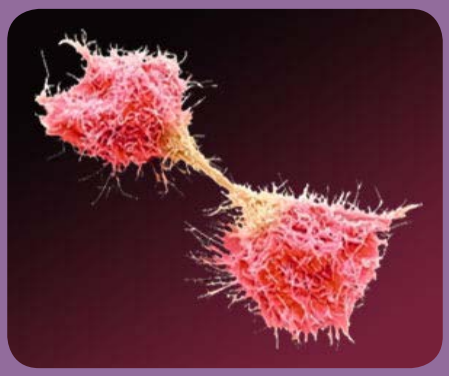
THE NEWSLETTER OF ACTIVE MOTIF

APRIL 2017 | VOLUME 18 NUMBER 1

special focus on cancer epigenetics

ACTIVE MOTIF®

Enabling Epigenetics Research



ACTIVE MOTIF – The Complicated Picture of Epigenetics in Cancer

The efforts of The Cancer Genome Atlas Project (TCGA), as well as those of the ENCODE and other consortia, have revealed a great deal of underappreciated biology by examining the combined genetics and epigenetics of patient tumors and cultured cells. These efforts yielded a large collection of new SNPs and translocations associated with misregulation of the tumor genome. Several clinical trials are underway, and the field of epigenetics has moved from an area of research to more of an applied tool.

IN THIS ISSUE

EPIGENETIC DRUGS & GENETICS OF EPIGENETICS

According to records available at www.clinicaltrials.gov, 340 studies are listed that contain the keyword "epigenetics" (completed, active or enrolling trials). These translational studies are diverse in the aspect of epigenetics explored and the diseases being investigated. In drug discovery, the families of deacetylases and DNA methyltransferases were the first to be targeted, with resulting drugs such as 5-azacytidine (DNMTi) and Vorinostat (HDACi) approved in 2004 and 2006 respectively for myelodysplastic syndrome and cutaneous T-cell lymphoma. While the DNMT protein family is relatively small (including DNMT1, 3a, and 3b), the pleitropic systemic effects of these compounds resulted in toxicity challenges. For the HDACi compounds, specifically targeting individual HDACs (10 family members) was not a possibility at the time, and direct targets of their individual biological actions were largely unknown.

Genetic mutations, including SNPs and translocations, that impact epigenetic proteins are now implicated in a wide spectrum of cancer types. As such, some of these proteins are now considered therapeutic targets for drug discovery (see table). This table lists five therapeutic targets that have been studied over the last decade. Epigenetic proteins remain challenging targets for drug discovery for multiple reasons. These proteins typically function in multiprotein complexes, and their enzymology is just now being elucidated with crystallographic studies. Collectively, activities of these epigenetic modifiers contribute to the "chromatin state" of a cell. Chromatin state can be determined by measuring certain histone modifications. It indicates regulatory regions within our genome, and whether a gene is active or repressed.

Chromatin interrogation tools - a protein toolbox

Exploration of this interesting and complex biology has centered around chromatin immunoprecipitation (ChIP) using antibodies specific to various histone modifications. These post-translational modifications (*e.g.* acetylation and methylation) result from the activities of epigenetic "writers" (HATs and

KMTs) and "erasers" (HDACs and KDMs). Early in the discovery process, biochemical assays were established using synthetic peptides as substrates for these enzymes in *in vitro* enzymatic assays. However, not all enzymes were active for these peptides, and the enzyme activity *in vitro* did not always match reports of what was observed in cells or animal experiments. In some cases, enzymes could not be shown to recognize peptides as substrates, and were thought to be inactive due to missing components in multiprotein complexes. A need for more "native" substrates emerged, meaning fully assembled nucleosomes containing both DNA and 8 polypeptides from the histones H2A, H2B, H3, and H4.

When we consider what we now know about the targets in the table, challenges remain. We now appreciate the BET inhibitor target BRD4 contains protein recognition domains for 3 distinct modifications on a native nucleosome. It requires three modifications (H3K9ac, H3S10ph, and H4K16ac) to be engaged by its bromodomains before it can stabilize transcriptional elongation, and this combinatorial mechanism was not obvious from peptide binding studies. On the other hand, EZH2 and its protein partners have multiple allosteric mechanisms that impose regulation on whether H3K27 is methylated, and to what extent. The drug discovery research field requires protein substrates that more closely resemble what these enzymes utilize in the cell, and often the enzymes being studied must be co-expressed with other proteins to be sufficiently active. Hence, recombinant naked and modified nucleosomes are all part of the toolbox now. This protein complexity is difficult to recapitulate in a screening environment, and it remains somewhat of a barrier to progress in drug discovery. BET inhibitors, targeting bromodomain containing proteins, are being widely applied in clinical trials. Yet we do not understand fully the biology of the various members of this 8 member protein family, and in certain cases, unexpected findings have been observed in clinical trials.

The future

Our toolbox will evolve to accommodate the needs of these applied researchers. Both in DNA methylation assays, which can be diagnostic or predictive for disease progression, as well as in trying to therapeutically target the complex machines that make up the core of cellular epigenetic machinery. Today, in addition to the histone modification antibodies that applied researchers need for assay development, we offer full length and modified histones, recombinant and modified nucleosomes (see page 15). We will continue to develop products and services to help facilitate progress towards epitherapeutic development.

EPIGENETIC CANCER TARGETS

TARGET	SUMMARY
P300 (EP300)	Lysine acetyltransferase associated with several blood and solid tumor types. Multiple mutations including nonsense, missense, amplification, deletion, and translocation. Promiscuous activity on histone and non histone lysines, *e.g.* H3K14, H3K18, H3K23, H4K5, H4K8, H4K12, and H4K16.
EZH2 (KMT6)	Lysine methyltransferase, enzymatic component of PRC2 complex, targeted in myelomas and overexpressed in a variety of tumor types. Exhibits both loss and gain of function phenotypes. Specific for H3.1 and H3.3 K27, mono, di, and tri methylation.
DOT1L (KMT4)	Lysine methyltransferase, DOT1L has minimal somatic mutations but interacts with MLL translocation and fusion proteins. These fusion proteins are associated with various leukemias. Specific for H3K79 mono, di, and tri methylation.
LSD1 (KMD1A)	Lysine demethylase, overexpressed in prostate cancer. Genetic lesions in non-cancer contexts, it is targeted in MLL rearranged leukemias. Non-competitive inhibitors that disrupt it's important protein-protein interactions offer a therapeutic opportunity in certain genetic contexts. Demethylates H3K4 mono and di methylation, H3K9 mono and di methylation, and other non-histone proteins.
BRD4	Bromodomain, translocation and fusion partner of NUT in midline carcinoma. Both BRD3 and BRD4 have fusion partners that promote tumor growth in this disease. BRD4 dependency has been shown in certain leukemias. Many applications for BET inhibition are being evaluated in numerous cancer and non-cancer disease related studies. Its recognition of acetyl lysines is promiscuous depending on assay, may recognize H3K9ac, H3S10ph, and H4K16ac simultaneously.

To learn more about our active enzymes, histone substrates, and detection antibodies are available at www.activemotif.com/drugdiscovery

NEW

High Quality ChIP-Seq Data from as Few as 1,000 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 requirement is often not feasible for researchers working with primary cells, FACS sorted cells, or other rare sample types. To enable studies of the complex 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 1,000 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 sample 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 1,000 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 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.

Low Cell Number ChIP-Seq Kit

- Reproducible ChIP-Seq data from as little as 1,000 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

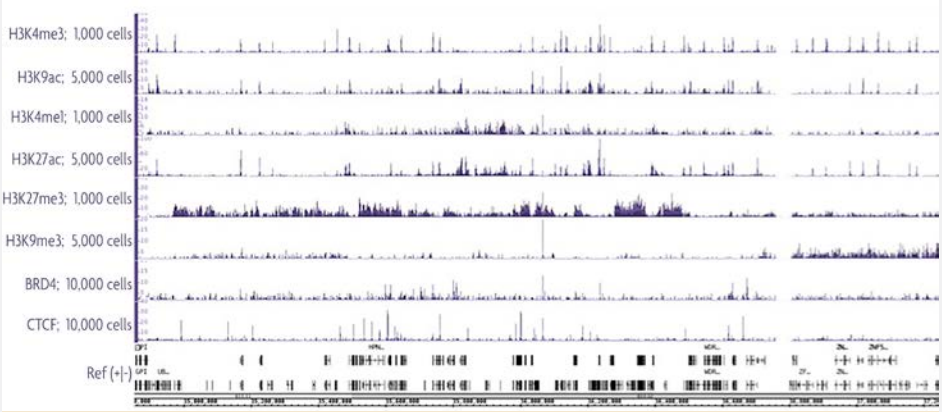


Figure 1: **Low Cell ChIP Seq works wtih 1,000 cells.** The Low Cell ChIP-Seq Kit was used to immunoprecipitate both robust and low abundance proteins and both active and repressive histone modifications using the input cell number listed in the y-axis. Nice peaks are observed above background from as little as 1,000 cells for histone marks H3K4me3, H3K4me1 and H3K27me3. Lower abundance proteins BRD4 and insulator protein CTCF show nice ChIP-Seq signal with 10,000 cells. Results shown represent a collection of individual Low Cell ChIP-Seq reactions from several cell types. Data has been reproducibly generated across multiple experiments.

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 5) 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 (NGS) 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 (Figure 1). Libraries can be generated from as little as 10 pg dsDNA, or from as low as 100 ng DNA if preparing PCR-free libraries. 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 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 resulting in more robust data.

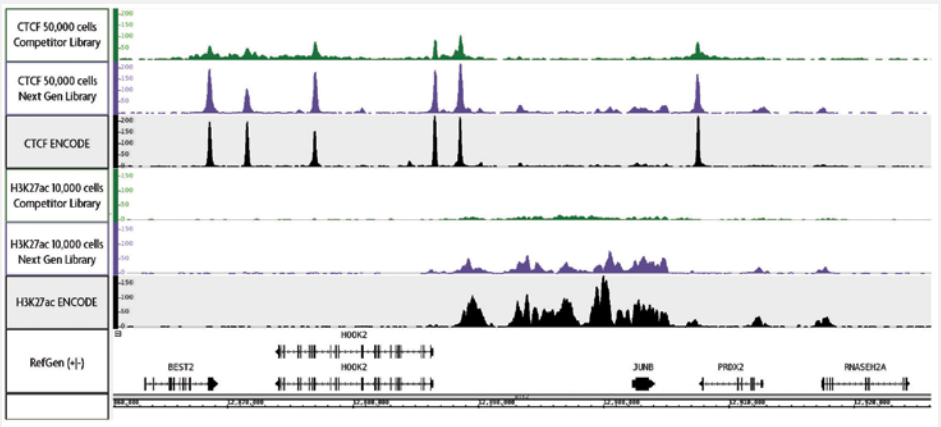
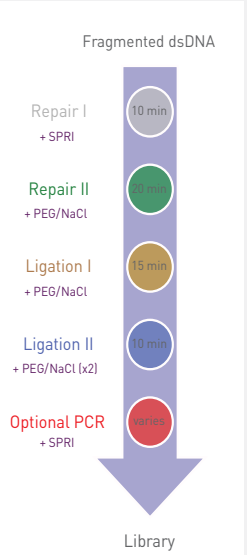


Figure 1: **Next Gen DNA Library Kit outperforms the competition.** Active Motif's Low Cell ChIP-Seq protocol was used to prepare chromatin from GM12878 cells using the number of cell equivalents listed. ChIP-Seq libraries were prepared using either the Next Gen DNA Library Kit or a competitor kit. Results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

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 ds-DNA. The Next Gen Indexing Kit contains 16 unique indices. Indices may be used individually or in combination to multiplex samples within the same sequencing reaction.



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

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 is 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.

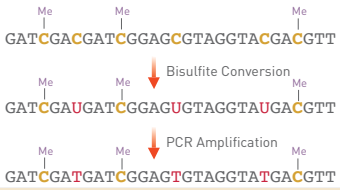


Figure 1: **Schematic of bisulfite conversion.** The bisulfite conversion reaction only modifies unmethylated cytosine residues. Methylated cytosines are unchanged.

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 DNA methylation. The conversion reaction occurs as a 3-step deamination of cytosine residues into uracil. As only unmethylated cytosine residues are susceptible to bisulfite conversion, the original methylation state of the DNA can be determined.

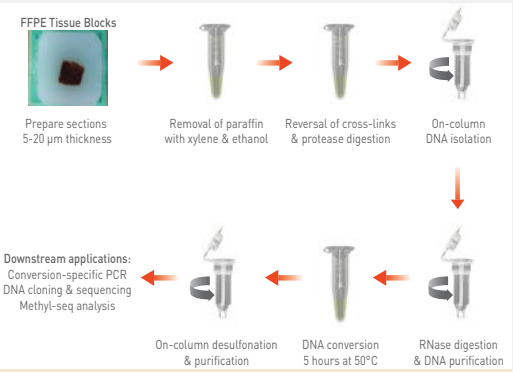


Figure 2: **Flow chart of FFPE Bisulfite Conversion Kit.**

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% bisulfite 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). To learn more, visit www.activemotif.com/bis-conv-ffpe.

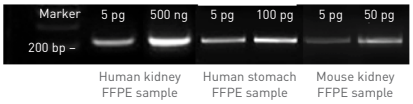


Figure 3: **Bisulfite conversion from as low as 5 pg DNA.** Active Motif's FFPE Bisulfite Conversion Kit was used to isolate genomic DNA from human kidney, human stomach, and mouse kidney FFPE tissue sections. Bisulfite conversion was performed using the input quantities listed. Results show a 220 bp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

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

Obtain Whole-Genome Profiles from Clinical Samples with ChIP-IT FFPE

Active Motif has utilized its years of experience and expertise to develop the first chromatin immunoprecipitation (ChIP) kit designed specifically for use with formalin-fixed, paraffin-embedded (FFPE) samples. The ChIP-IT® FFPE Chromatin Preparation Kit can extract high-quality chromatin from FFPE slides or tissue blocks, while the ChIP-IT® FFPE kit is sensitive enough to enrich histone and transcription factor targets with minimal background signal. To learn more about ChIP-IT FFPE, please visit us at www.activemotif.com/ffpechip.

First of its kind assay

FFPE tissue serves as the "gold standard" for the preservation of pathology samples, and represents an opportunity to study clinical outcomes of disease and treatment conditions. Traditionally, FFPE samples have not been useful in chromatin immunoprecipitation due to the limited size of the samples and the fact that the formalin fixation process often causes degradation and loss of antigenicity.

Active Motif's ChIP-IT FFPE Kit is the first ChIP Kit available that can extract high-quality chromatin from extremely limited starting material, while producing minimal background signal in ChIP, thereby enabling specific detection of the target protein of interest. The assay is sensitive enough to detect histone and transcription factor targets using qPCR or Next-generation sequencing (NGS).

ChIP-IT FFPE advantages

- Use with slides or tissue blocks
- Sensitive assay detects histone and transcription factor targets
- Positive controls are included to validate results at each step
- Compatible with downstream NGS and qPCR analysis

What's in the box?

ChIP-IT FFPE Chromatin Preparation Kit provides specially formulated reagents and protocol guidelines to extract chromatin from histological slides or tissue sections. While the quality of the extracted chromatin is dependent upon sample fixation and storage conditions, we have successfully extracted ChIP-grade chromatin from normal and tumor human colon FFPE blocks that were stored for more than 10 years under less than ideal conditions (Figure 1).

ChIP-IT FFPE includes our highly sensitive immunoprecipitation and DNA purification reagents, blockers, protease inhibitors and protein G agarose beads. Positive controls, such as a Histone H3K4me3 ChIP-Seq-validated antibody and human PCR primer sets, are included to confirm success at each step of the process. For downstream applications such as NGS, the ChIP-IT qPCR Analysis Kit is recommended to evaluate the quality of the ChIP-enriched DNA prior to sequencing.

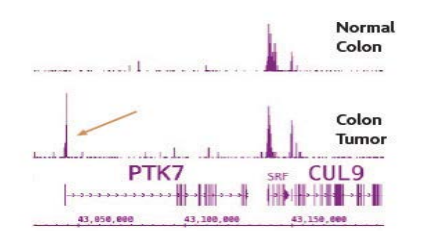


Figure 1: **ChIP-Seq on normal and tumor FFPE samples.** Chromatin was extracted from 10-year-old histological sections of a human colon tumor and matched normal colon for use in H3K4me3 ChIP-Seq. A subset of the genome-wide profile shows nearly equal H3K4me3 occupancy at SRF and CUL9 promoters. However, H3K4me3 is present at the promoter of the PTK7 gene, a gene known to be up-regulated in colon cancer, only in the tumor sample.

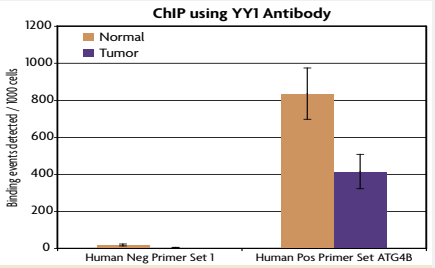


Figure 2: **qPCR analysis of normal and tumor colon samples assayed using ChIP-IT FFPE.** Chromatin was extracted from five 20 µm sections of matched normal and tumor human colon FFPE blocks and assayed with the ChIP-IT FFPE kit using an antibody for transcription factor YY1. The quality of the ChIP DNA was validated with the ChIP-IT qPCR Analysis Kit. qPCR results show the sensitivity and specificity of ChIP-IT FFPE.

Product	Format	Catalog No.
ChIP-IT® FFPE Chromatin Preparation Kit	5 rxns	53030
ChIP-IT® FFPE	16 rxns	53045
ChIP-IT® qPCR Analysis Kit	60 rxns	53029

NEW

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

Formalin-fixed, paraffin-embedded (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 have not been fully realized because of the difficulty these sample types present for use in conventional epigenetic techniques such as chromatin immunoprecipitation (ChIP). Challenges arise due to the limited size of FFPE materials 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 research areas, such as epigenetics where aberrant histone and DNA modifications have been linked to 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.

Sample types:

- FFPE blocks, sections, or curls

Targets:

- H3K4me1, H3K4me2, H3K4me3
- H3K9ac, H3K14ac
- H3K27ac, H3K27me3
- H3K36me3
- CTCF, *and more...*

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 require for optimization. Active Motif's new FFPE ChIP-Seq Service takes the burden of optimization out of your hands and 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 our end-to-end FFPE ChIP-Seq Service, visit us a www.activemotif.com/services-ffpechip.

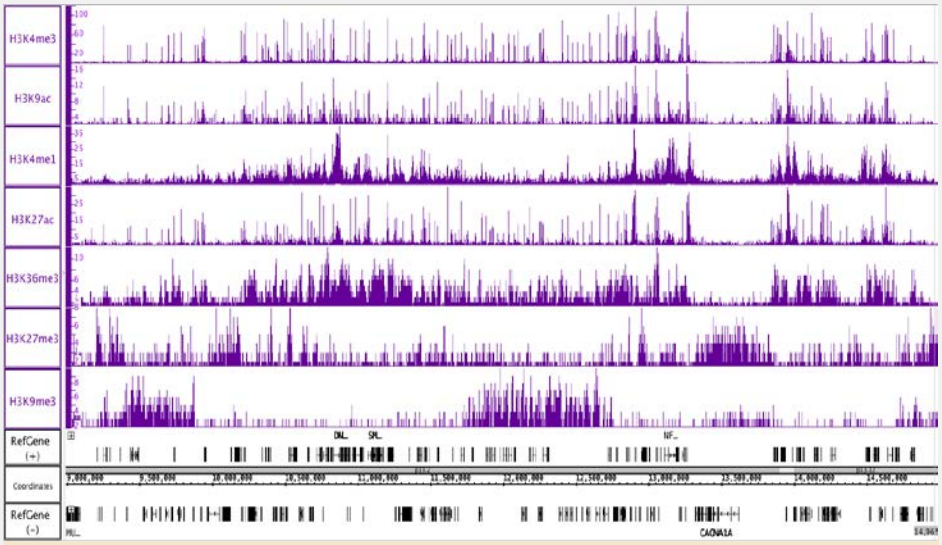


Figure 1: **ChIP-seq from an FFPE preserved colon tumor.** 10µm sections were taken from a FFPE preserved colon tumor followed by chromatin extraction and ChIP-Seq. Each 10µm section yielded enough chromatin for a single histone PTM ChIP-Seq experiment. The seven histone modification ChIP-Seq data sets presented above show the expected genomic localization patterns.

NEW

Epigenomic Profiling of Tumors

Epigenetic landscapes, like other molecular profiles such as gene expression, can be used to stratify patients into different therapeutic groups. As such, epigenomic biomarker development will be important to identify patients that will respond favorably to a given treatment. In recent years, researchers have begun to explore epigenetic modifications as biomarkers, an approach that has been met with success as related to DNA methylation. Histone modifications, on the other hand, have not been widely used or widely accepted as biomarkers, in part due to the challenges associated with the methods used to reliably measure histone modification patterns in patient samples. Advances in ChIP and ChIP-Seq at Active Motif now enable tumor profiling and biomarker discovery from tumor biopsies.

Epigenetic biomarkers in medulloblastoma

Medulloblastoma is a highly malignant pediatric brain tumor that accounts for 20% of all childhood brain tumors. These tumors are classified into four biologically and clinically distinct subgroups. Treatment choice is based on the molecular subgroup and treatment options include: surgery, radiation, and chemotherapy. Molecular subgroups can be identified using genome-wide profiles of H3K27ac as illustrated in a recent publication in *Cell* Reports: Lin *et al.* Active medulloblastoma enhancers reveal subgroup-specific cellular origins (2016) (Figure 1).

Fresh frozen biopsies are well suited for ChIP-Seq since molecular interactions are preserved during freezing and are maintained during storage. Frozen samples can immediately flow into the preparative step of formaldehyde fixation needed for ChIP. However, the vast majority of tumor samples are stored as FFPE blocks, not frozen samples. FFPE samples are more challenging due to extensive crosslinking which hampers chromatin extraction. Recent advances at Active Motif now enable the routine generation of high quality ChIP-Seq data sets from limited amounts of FFPE preserved tumors (Figure 2).

Active Motif provides end-to-end FFPE ChIP-Seq services as well as tumor profiling on frozen biopsies. To learn more about our custom services, or to get a quote, please visit us at **www.activemotif.com/epigenetic-services-info.**

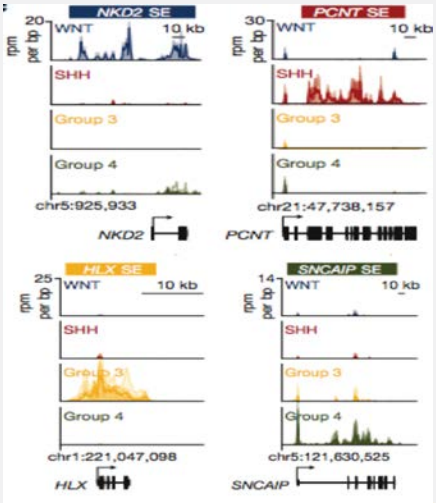


Figure 1: **H3K27ac profiles characterize medulloblastoma subgroups.** ChIP-Seq was performed at Active Motif using frozen biopsies from each of the four medulloblastoma subgroups: WNT (n=3), SHH (n=5), Group 3 (n=9) and Group 4 (n=11). Profiles from all samples within a group were overlayed for visualization. Subgroup-specific H3K27ac patterns were identified.

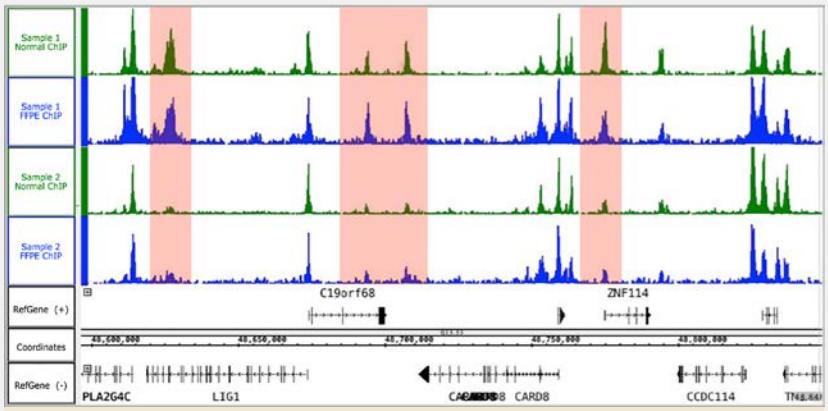


Figure 2: **H3K27ac ChIP-Seq comparison between fresh/frozen and FFPE preserved tumors.** ChIP was performed using chromatin from two human medulloblastoma biopsies. Biopsies were provided as both fresh/frozen (green) and FFPE (blue). ChIP-Seq data sets were similar with correlation coefficients of 0.889 and 0.826 between FFPE and fresh/frozen samples. Tumor-specific H3K27ac occupancy was detectable in the FFPE and fresh/frozen data sets (highlighted in red).

NEW

AbFlex™ Recombinant Antibodies

Batch to batch variability and poor characterization of antibodies is a concern within the research community and has led to calls for a better approach to antibody development. To address these concerns Active Motif has developed AbFlex™ recombinant antibodies using defined DNA sequences to produce highly specific, reproducible antibodies. Each AbFlex™ antibody contains tag sequences allowing attachment of a variety of labels directly to the antibody.

The problem with antibody variability

A 2008 study found fewer than half of 6,000 routinely used commercial antibodies recognized their specific targets exclusively (Berglund *et. al.*, 2008). In 2011, looking explicitly at histone modification state specific antibodies, another group found that among 246 antibodies, one-quarter failed tests for specificity (Egelhofer, *et. al.* 2011). Additionally, lot-to-lot variability in the commercial products often plague scientists who want to reproduce results from within their own lab or others. These and other concerns were summarized in two *Nature* comments articles written by two researchers with over 100 co-signatories. These articles expressed that new standards were needed to circumvent several issues involved in generating these reagents in animals (Bradbury, *et.al. Nature* 2015). These new standards included a call for all antibodies to be derived from defined sequences, and for those sequences to be available to the researchers.

Introducing AbFlex™ recombinant antibodies

In response to these requests, Active Motif has created a new type of antibody tool that will enable reproducible results across antibody batches. Active Motif's AbFlex™ recombinant antibodies, address the need for recombinant antibodies and includes value-added features for easy and flexible conjugation options. AbFlex™ recombinant antibodies are derived from high value parent antibodies (monoclonals or recombinants from a variety of species), and they have been engineered to contain a tripartite "tag" of functional molecular features (Figures 1 & 2).

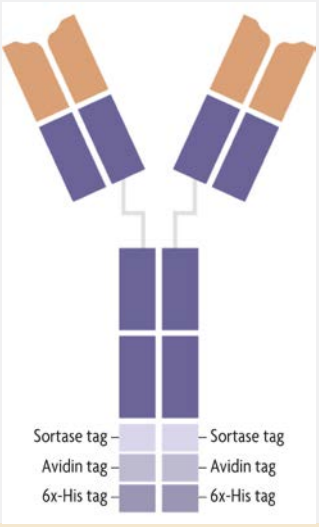


Figure 1: **Schematic diagram of an AbFlex™ recombinant antibody.** Heavy and light chains are shown with the cloned variable regions indicated in copper. Antibodies are expressed and purified with the molecular tags on the carboxyl terminus of the heavy chain as shown.

Molecularly cloned variable regions are combined with various heavy and light chain frameworks (*e.g.* murine IgG2a) to reconstitute the binding capabilities.

The tripartite tag specifies the following features on the carboxyl terminus of the heavy chain molecules:

- Avidin Tag for site directed, efficient biotinylation
- Sortase Tag, a LPETG motif for site directed conjugation via an optimized transpeptidase
- 6X-His Tag for standard affinity purification by nickel-based chromatography

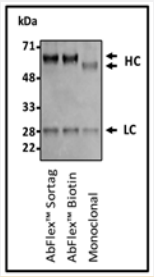


Figure 2: **SDS-PAGE analysis of AbFlex™ antibodies compared to a standard monoclonal.** AbFlex™ antibodies were heavy and light chains are indicated for the various antibodies.

Designed for flexibility

These engineered, AbFlex™ recombinant antibodies, are expressed from mammalian cells to ensure lot-to-lot uniformity, performance, and standardized presentation. When combined with the Sortase labeling* system, the molecular tags are compatible with the following tri-glycine dependent conjugation options:

- Biotin

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- TEV Biotin (for protease digestion to release from biotin-streptavidin matrices)
- Horseradish Peroxidase (HRP)
- Alkaline Phosphatase (AP)
- ATTO488, 535, 550, 647N

Quality is key

To ensure quality of AbFlex™ antibodies, each conversion is re-validated in applications that the parent antibody was validated in (Figures 3-5). Examples of applications testing (depending on the parent antibody's product claims) include:

- Western blot
- ChIP or ChIP-Seq
- Plate or bead based ELISA
- Immunofluorescence (with or without secondary amplification)

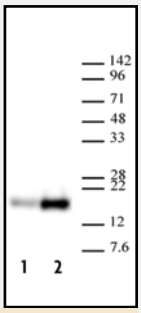


Figure 3: **AbFlex™ Histone H3K9ac recombinant antibody tested by Western blot.** HeLa nuclear extract (20 µg per lane) probed with AbFlex™ Histone H3K9ac antibody (2 µg/ml dilution).Lane 1: untreated cells. Lane 2: cells treated with HDAC inhibitor sodium butyrate.

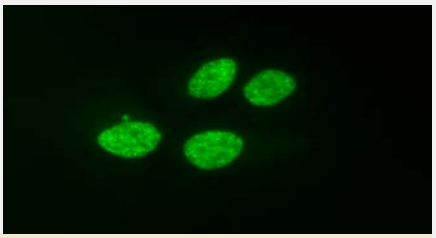


Figure 4: **AbFlex™ Histone H3K9ac recombinant antibody tested by immunofluorescence.** HeLa cell stained with 2 µg/mL of AbFlex™ Histone H3K9ac antibody followed by anti-mouse-IgG-488.

Functionality of the molecular features—The AbFlex™ advantage

New AbFlex™ antibodies will be added routinely, and currently include antibodies to histone modifications, tags, transcription factors, and epigenetic readers, writers, and erasers. The advantages offered by the tag features include two options for enzymatic addition of biotin or TEV Biotin in molar ratios of 1 label to 1 heavy chain for a maximum possible labeling of 2 labels per conjugation. Biotinylation is site directed and near the C-terminus, thus enabling consistent orientation in streptavidin bead or plate applications so that the antigen recognition site is uniformly presented on surfaces. This advantage, as well as the ability to prevent over-labeling, eliminates the need to re-validate an antibody's performance post labeling. This is particularly advantageous, as chemical labeling can often interfere with a given antibody's ability to recognize its antigen specifically post labeling. The enzymatic ligation is performed under mild conditions, and there is no risk of labeling conjugates on the antigen recognition portion of the antibody. The only restriction on coupling via Sortase requires the antibody to have the LPXTG is the antibody recognition sequence, and the molecule of interest requires a poly-glycine for conjugation purposes. AbFlex™ custom services is also available for customers interested in converting their own monoclonals into the AbFlex™ framework.

To learn more about our custom services, or to get a quote contact us at 877-222-9543 or visit us at www.activemotif.com/services.

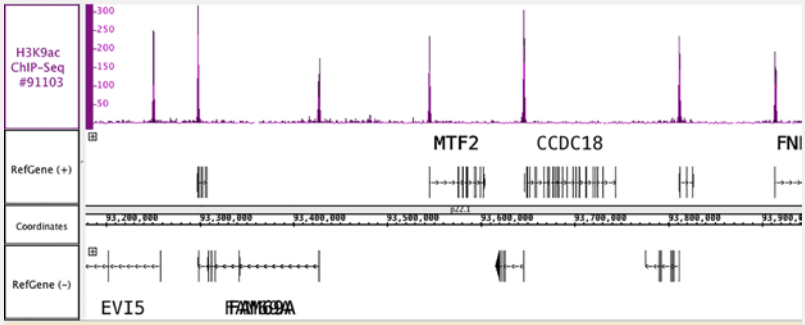


Figure 5: **AbFlex™ Histone H3K9ac recombinant antibody tested by ChIP-Seq.** ChIP was performed using the ChIP-IT® High Sensitivity Kit (Cat. No. 53040) with 30 µg of chromatin from PC9 cells with 4 µg of antibody. ChIP DNA was sequenced on the Illumina® HiSeq and 22 million sequence tags were mapped to identify histone H3K9ac binding sites. The image shows binding across a region of chromosome 1.

AbFlex™ Recombinant Antibodies

- Recombinant full length antibodies with heavy and light chains (*e.g.* mouse IgG2a)
- Three functional tags for purification, biotinylation, and protein ligation (6X-His Tag, AvidinTag, and Sortase Tag)
- Consistent, site directed-labeling

Product	Format	Catalog No.
AbFlex™ Histone H3K9ac antibody (rAb)	100 µg	91103
AbFlex™ Histone H4K20me3 antibody (rAb)	100 µg	91107
AbFlex™ Cas9 antibody (rAb)	100 µg	91123
AbFlex™ His Tag antibody (rAb)	100 µg	91183
AbFlex™ JMJD2A Antibody (rAb)	100 µg	91143
AbFlex™ EED antibody (rAb)	100 µg	91135

NEW

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-transcriptional 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 stemness, epithelial-to-mesenchymal transition, and metastasis (Sahu *et al.*, 2016).

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.

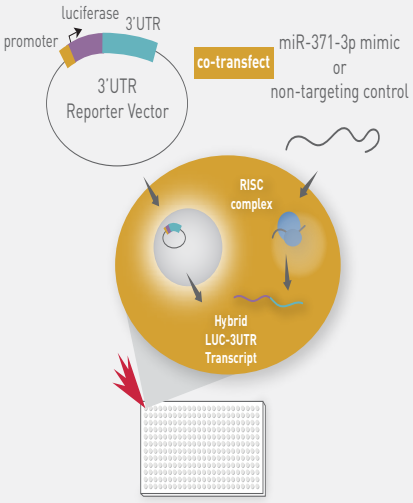


Figure 1: **Experimental design for miR-122 functional screen using the LightSwitch System.**

To enable researchers to leverage 3' untranslated region (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 highlyoptimized 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).

To learn more, please visit www.activemotif.com/ls-3utr.

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

In a recent study, Dr. Nisebita Sahu explores the potential role for miRNAs in transient drug tolerance. Acquired resistance to cancer drug therapies almost always occurs in advanced-stage patients, even following

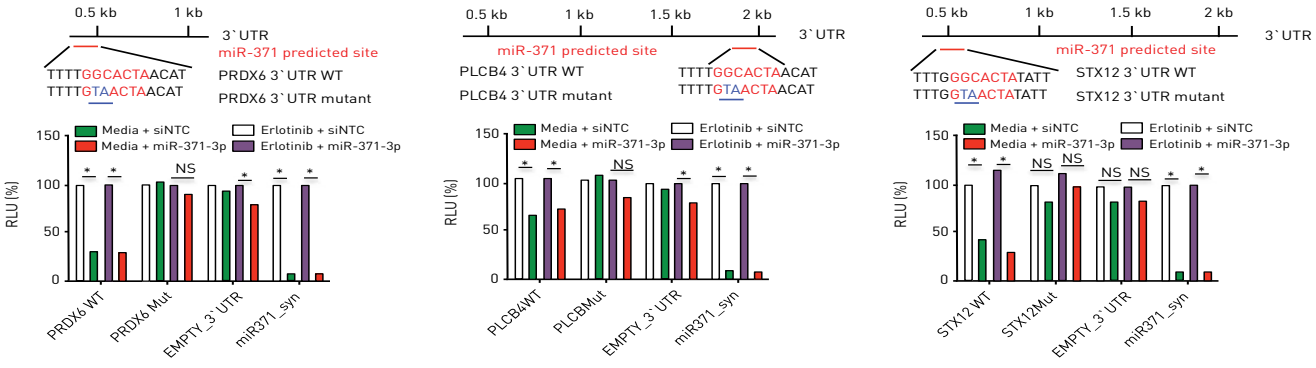


Figure 3: **Mutational validation of relevant miR-371-3p targets using the LightSwitch system.** Mutations of seed sequences of the putative miR-371-3p recognition element in target 3'UTRs prevented inhibition by miR-371-3.

a significant response to treatment. In addition to mutational mechanisms, various non-mutational resistance mechanisms have now been recognized.

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 drug-tolerant persisters (DTPs), did not display any (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, *KDM3B*, 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 *KDM3B*, suggesting that miR-371 does not regulate DTPs via *KDM3B*. 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 heterogeneous 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.

*This article is a summary of the research performed by Dr. Settleman at the Discovery Oncology department at Genentech, Inc,. published in *Nature Communications*: Sahu, N. *et al.* Functional screening implicates miR-371-3p and peroxiredoxin 6 in reversible tolerance to cancer drugs. *Nat. Commun.* 7:12351. DOI: 10.1038/ncomms12351 (2016).

To learn more about 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

ChIP Normalization Reagents Reduce Effects of Technical Variation and Reveal Biological Changes

ChIP is a multi-step process in which variations caused by sample loss during immunoprecipitation and library preparation, uneven sequencing read depths, or hand-to-hand differences make it difficult to compare data sets. To overcome this challenge, Active Motif has developed a Spike-in strategy that utilizes *Drosophila* chromatin and a *Drosophila*-specific antibody for normalization of technical variation and sample processing bias. Additionally, the normalization strategy can be used for detecting conditional effects, such as those induced by compound treatments or mutants.

How does it work?
Active Motif's Spike-in Normalization Strategy works with both ChIP-qPCR and ChIP-Seq analysis to eliminate bias and reveal biological changes in your samples (Figure 1). ChIP normalization can easily be implemented simply by integration of our Spike-in reagents into your standard ChIP protocol.

A standard ChIP reaction is set up using experimental chromatin (*e.g.* human) and an antibody of interest. In addition, *Drosophila melanogaster* Spike-in Chromatin is added, or "spiked-in", to each reaction as a minor fraction of total chromatin. An antibody that recognizes the *Drosophila*-specific histone variant, H2Av, is also added to the reaction. The Spike-in Antibody provides a mechanism to reliably pull down a small fraction of *Drosophila* chromatin that is consistent across all samples (Figure 2).

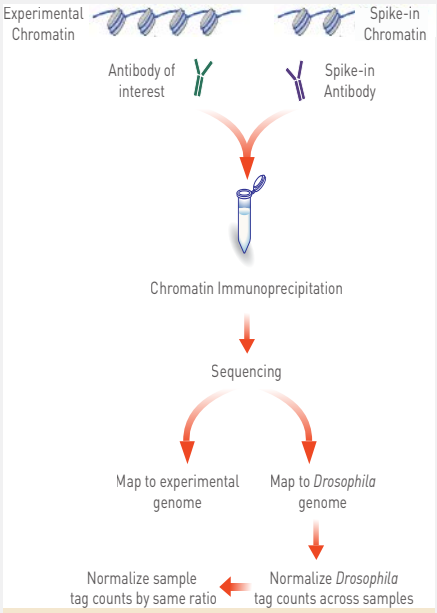


Figure 2: **ChIP-Seq Normalization Workflow.**

Figure 1: **ChIP-Seq Spike-in Normalization Strategy reveals changes in H3K27me3 levels following treatment with an EZH2 inhibitor.** Cells treated with a small molecule inhibitor of EZH2 methyltransferase have dramatic reductions in global H3K27me3 levels. However, H3K27me3 ChIP-Seq using standard ChIP-Seq protocols (-) does not detect these differences. Incorporation of Active Motif's ChIP-Seq Spike-in Strategy (+) reveals the expected decrease in H3K27me3 ChIP-Seq signal.

Since variation introduced during the ChIP procedure will also occur with the Spike-in Chromatin, a normalization factor can be created based on the *Drosophila* signal and applied to the sample genome.

To learn more about ChIP normalization, visit www.activemotif.com/spikein

Product	Format	Catalog No.
Spike-in Chromatin	15 rxns	53083
Spike-in Antibody	50 µg	61686
Drosophila Positive Control Primer Set Pbgs	96 rxns	71037
Drosophila Negative Control Primer Set 1	96 rxns	71028
Drosophila Negative Control Primer Set 3	96 rxns	71038

NEW

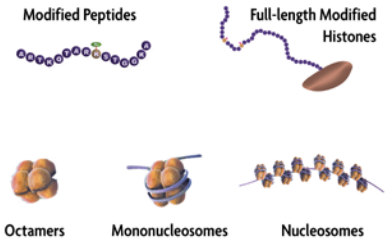
Assay-Ready Recombinant Proteins, Substrates, and Inhibitors

There is no need to waste valuable time and resources generating proteins and substrates for screening, drug discovery, and target validation programs. Active Motif continues to add to its collection of over 300 purified, assay-ready recombinant proteins and physiologically-relevant assay substrates for use in enzyme activity assays, inhibitor screens, and binding studies. We also offer a collection of activator and inhibitor compounds to modulate the activity of proteins that regulate DNA methylation, histones, and binding proteins.

Assay-Ready Proteins

- **Histone modifying enzymes**
  HATs, HDACs, HMTs, & HDMs
- **Binding domains**
  Bromodomains, & SET domains
- **DNA modifying enzymes**
  DNMTs & TET
- **Transcription factors**
  NFκB, AP-1, p53, and more...
- **Bulk sizes available**

Assay-Ready Substrates



Epigenetic Activators & Inhibitors

- Lysine Demethylase & Methyltransferase
- Arginine Methyltransferase
- Histone Acetyltransferase & Deacetylase
- Sirtuins
- DNA Methylation
- Bromodomain

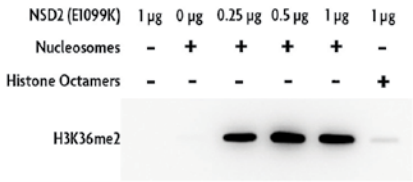
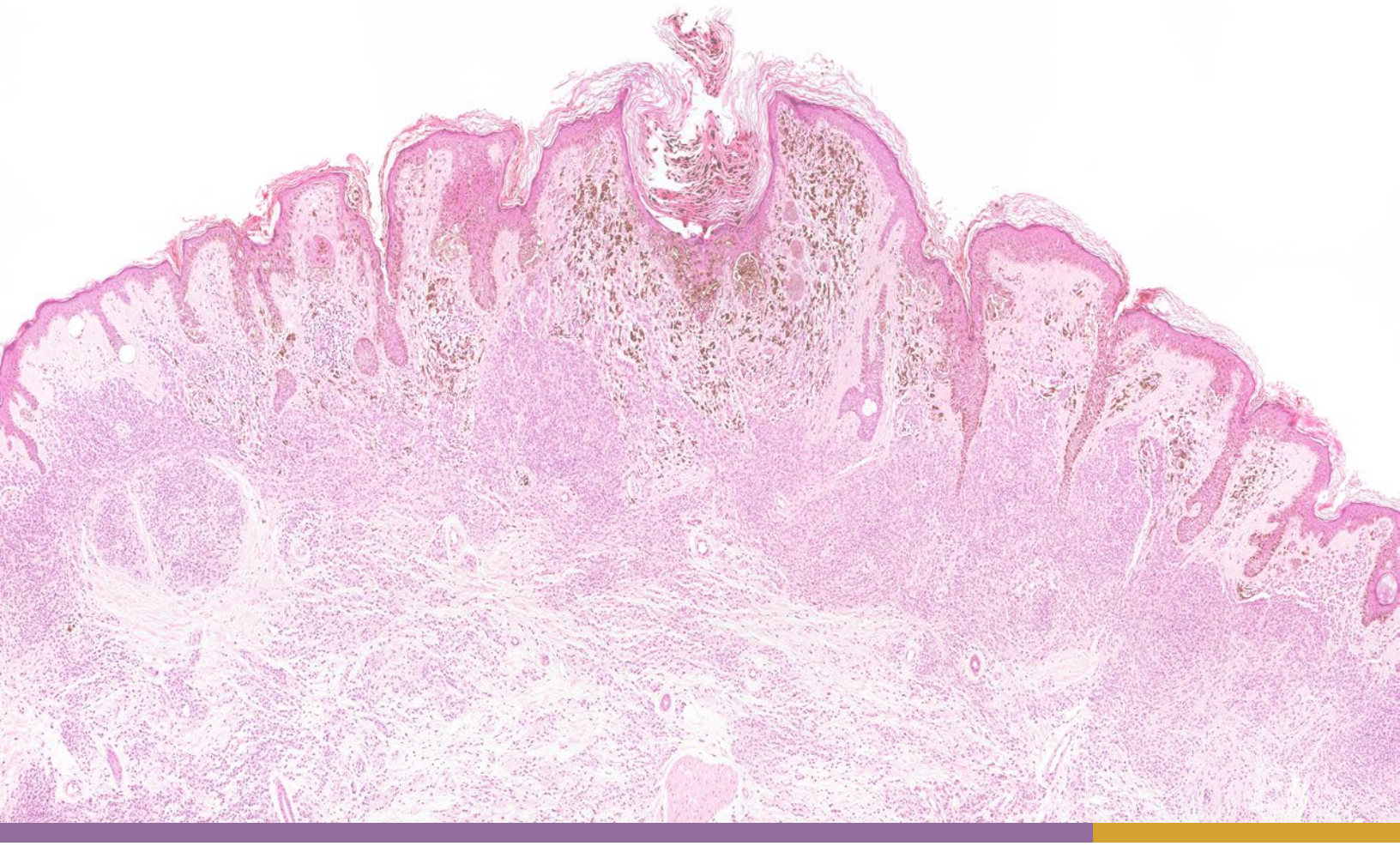


Figure 1: **Recombinant NSD2 (E1099K) activity assay.** Recombinant NSD2 (E1099K)was incubated with either 2 µg Recombinant Nucleosomes (H3.3) (Catalog No. 31468) or 2 µg Histone Octamer (H3.3) (Catalog No. 31472) at the concentrations shown above in reaction buffer for 3 hours at room temperature. Activity was detected by Western blot for H3K36me2. Results show the substrate preference of NSD2 (E1099K) for nucleosomes over octamers.

A few of the available proteins, substrates, and epigenetic modulators are shown in the table to the right.

To learn more, visit us at www.activemotif.com/proteins and www.activemotif.com/smallmol

Product	Format	Catalog No.
NEW: Recombinant Histone H2A.X	100 µg	31251
Recombinant Histone H3K4me3	50 µg	31210
Recombinant Histone H3K9ac	25 µg	31253
Recombinant Histone Octamer (H3.1)	100 µg	31470
Recombinant Histone Octamer (H3.1) - biotinylated	50 µg	31471
Recombinant Mononucleosomes (H3.1) - biotinylated	20 µg	31467
Recombinant Mononucleosomes (H3.3) - biotinylated	20 µg	31469
Recombinant Nucleosomes (H3.1)	20 µg	31466
Recombinant Nucleosomes (H3.3)	20 µg	31468
NEW: Recombinant AGO1 protein	20 µg	31522
NEW: Recombinant HDAC3 / NCOR2 Complex	20 µg	31526
NEW: Recombinant NSD2 (E1099K) protein	20 µg	31546
NEW: Recombinant SIRT1 (193-741) protein	100 µg	31533
NEW: SGC707 (PRMT3 inhibitor)	5 mg	14131
BML-278 (SIRT1 activator)	5 mg	14025
5´-Azacytidine (DNMT inhibitor)	50 mg	14103



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