

HistonePath™ ChIP-Seq and ChIP-on-chip

# Comprehensive discovery of histone modifications across whole genomes

Modifications of histone proteins constitute an important mechanism of gene regulation. Both discrete and global changes in histone modifications are associated with a wide variety of biological processes including cell differentiation, drug responsiveness and disease. Active Motif offers assay services and analysis for studying these important epigenetic changes with its chromatin immunoprecipitation (ChIP)-based HistonePath™ assays. These assays provide a high-quality, rapid solution for genome-wide mapping and quantitation of histone modifications. Active Motif's ChIP protocols offer higher sensitivity and lower background binding compared with other ChIP methods in general use, and thus Active Motif's HistonePath results in more informative ChIP-Seq and ChIP-on-chip data, including the detection of sites containing lower levels of modification. Active Motif also includes quality-control steps throughout the procedure to monitor chromatin preparation, ChIP enrichment and DNA amplification/library generation. Other advantages include qualification of all antibodies for ChIP, and comprehensive analysis of genome-wide data that includes multi-sample comparisons. HistonePath is a highly valuable assay for determining mechanistic changes underlying differential gene expression and is in use for a variety of applications, including the identification of histone modifications potentially useful as biomarkers in diseases such as cancer<sup>1</sup>.

Gene expression is governed by complex mechanisms including transcription factor binding to DNA and coordinated changes in chromatin structure. The primary protein components of chromatin are the histones, which are assembled along with DNA into larger complexes known as nucleosomes. Each nucleosome contains two copies of the core histones—H2A, H2B, H3 and H4—each of which has an accessible amino terminal tail with a high proportion of lysines and arginines. These amino acids can be modified by the addition or removal of methyl, acetyl and phosphoryl groups<sup>2</sup>. Combinations of these modifications make up the “histone code” that is “read” by other proteins, resulting in changes in chromatin structure and gene regulation.

The availability of a wide variety of antibodies that recognize site-specific modifications on histone tails has facilitated the generation of extensive data and a better understanding of the histone code. One particularly valuable technique to investigate histone modifications is ChIP. This technique, when used with histone modification-specific antibodies, identifies and quantifies those genomic regions containing the targeted

histone modifications. ChIP is performed by first covalently crosslinking the histone/DNA interactions using formaldehyde. Cells are lysed, chromatin (protein/DNA complexes) is isolated, the mixture is sonicated to shear the DNA into short fragments, and an antibody is used to immunoprecipitate the histones containing the specific modification of interest and its associated DNA. Following reversal of the crosslinks, the DNA is isolated and characterized using one of several downstream methods. Studies performed with genome-wide platforms such as next-generation sequencing (ChIP-Seq) and microarrays (ChIP-on-chip) have revealed that certain modifications are generally associated with either transcriptional activation or repression (Table 1)<sup>3-5</sup>.

### Gene-Specific Histone Changes Detected with HistonePath qPCR

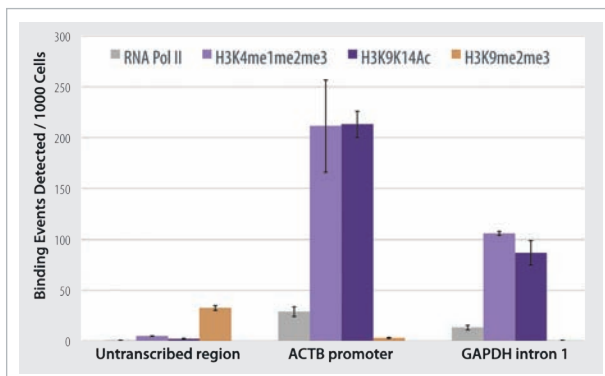
To illustrate detection and quantitation of activating and repressing histone modifications, ChIP was performed with chromatin from a human B cell line. Antibodies used were against two activating modifications 1) histone H3 mono-

Table 1. H3 and H4 histone modifications used in this application note and their associations with gene regulation.

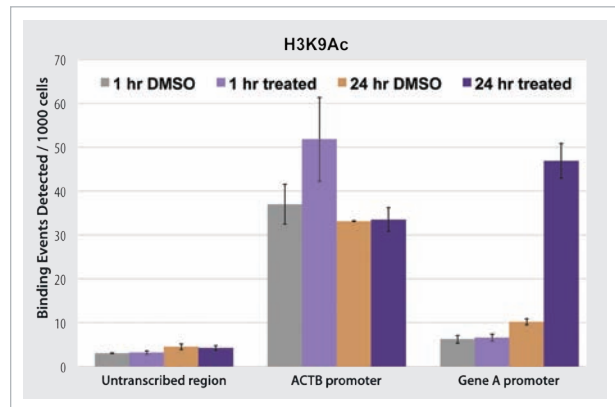
Methylation								
	H3K4	H3K9	H3K27					
Monomethylation	activation	activation	activation					
Dimethylation	activation	repression	repression					
Trimethylation	activation	repression	repression					
Acetylation								
	H3K4	H3K9	H3K14	H3K27	H4K5	H4K8	H4K12	H4K16
	activation	activation	activation	activation	activation	activation	activation	activation

di- and tri-methylated on lysine 4 (H3K4me1me2me3) and 2) histone H3 acetylated on lysines 9 and 14 (H3K9K14Ac), plus one repressing modification 3) histone H3 di- and tri-methylated on lysine 9 (H3K9me2me3). ChIP using an antibody against RNA Pol II was also performed to monitor transcriptional activity (Active Motif's TranscriptionPath™ Assay). ChIP DNAs were analyzed using qPCR with primers that amplify the promoter or first intron of the constitutively expressed housekeeping genes ACTB and GAPDH, or a silent genomic region > 10 kb away from any known gene (denoted “untranscribed region”). In Figure 1, ACTB and GAPDH were shown to be transcriptionally active by the presence of RNA Pol II on these genes. In contrast, RNA Pol II was not associated with the untranscribed region. ChIP for the activating modifications H3K4me1me2me3 and H3K9K14Ac showed significant signal associated with the transcriptionally active ACTB and GAPDH genes. In contrast, signal from the repressive modification was highest at the untranscribed region.

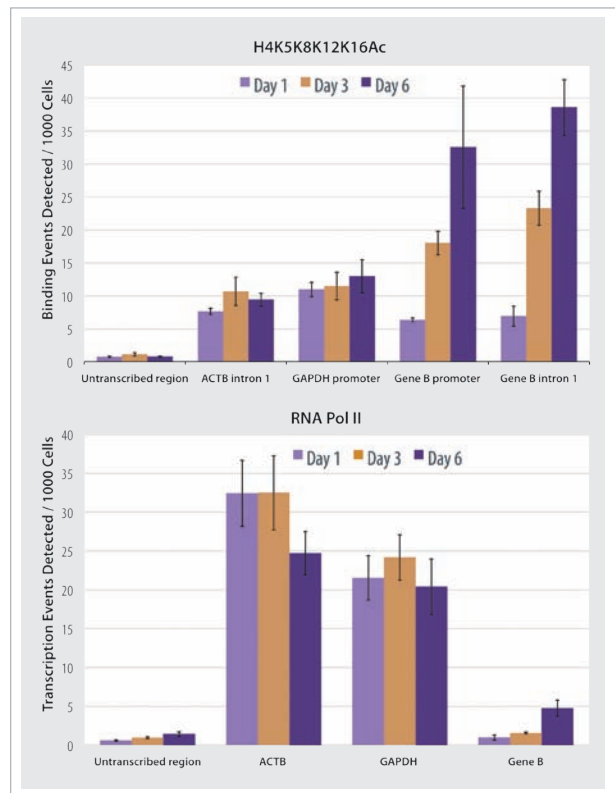
The differential expression of specific genes during biological processes such as cell division, embryonic development, and response to treatment has also been associated with alterations in histone modifications<sup>6-9</sup>. In Figure 2, HistonePath was performed in human cells to demonstrate gene-specific changes in histone modifications in response to drug treatment. ChIP was performed using an antibody against the activating modification H3K9Ac, and the resulting ChIP DNA was analyzed using qPCR. Signals in the constitutively active ACTB gene were high in vehicle-treated cells and remained at similar levels upon drug treatment. In contrast, the H3K9Ac level was low in the Gene A promoter in untreated cells and cells treated for 1 hour, but significantly increased after a 24-hour treatment. Similarly, as shown in Figure 3, HistonePath was used to detect gene-specific changes in histone modifications during cell differentiation. CD34+ mesenchymal stem cells differentiated to



**Figure 1.** HistonePath qPCR showing that activating modifications H3K4me1me2me3 and H3K9K14Ac were associated with genes undergoing transcription as demonstrated by the presence of RNA Pol II. In contrast, repressing modification H3K9me2me3 was associated with an untranscribed genomic region.



**Figure 2.** HistonePath qPCR demonstrating that activating modification H3K9Ac was specifically increased at the promoter of gene A in response to a 24-hour drug treatment.



**Figure 3.** (Upper) HistonePath qPCR showing that differentiation of mesenchymal cells to adipocytes resulted in increased activating modification H4K5K8K12K16Ac in the promoter and first intron of Gene B at days 3 and 6. (Lower) Gene B transcription, as measured by the presence of RNA Pol II, was increased only on day 6.

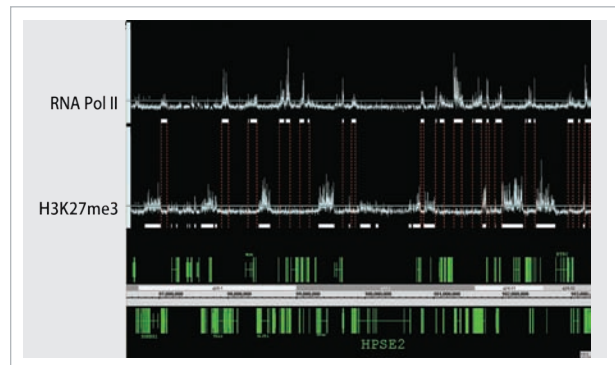
become adipocytes were assayed on days 1, 3 and 6 using an antibody against the histone modification H4K5K8K12K16Ac. H4 acetylation of ACTB and GAPDH did not change upon cell differentiation, whereas H4 acetylation in Gene B increased at the promoter and first intron at days 3 and 6. It is also evident that the increase in H4 acetylation preceded the activation of gene transcription because RNA Pol II association with Gene B was increased significantly only at day 6.

### HistonePath ChIP-on-chip Maps the Repressing Modification H3K27me3 Genome-Wide

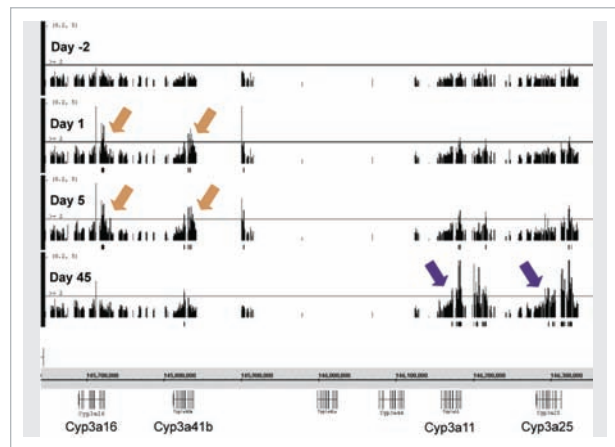
Genome-wide studies using ChIP-Seq and ChIP-on-chip with antibodies that recognize specific histone modifications yield a wealth of information about the role of each modification in gene regulation. In Figure 4, ChIP-on-chip using Affymetrix human tiling arrays was performed with an antibody against the repressing modification H3K27me3, which is associated with silenced genes and intergenic regions. ChIP-on-chip was also performed with an antibody against RNA Pol II phosphoserine 2 in order to measure gene transcription. As shown by a representative 9 Mb region of human chromosome 10, there was virtually no overlap between the presence of H3K27me3 and transcription by RNA Pol II, demonstrating the repressive nature of the H3K27me3 modification in this cell type. In addition, the data showed that H3K27me3 was present across extended stretches of DNA (in this view up to 300,000 consecutive bp), which is in contrast to most activating histone modifications that are associated with shorter regions of 2-3 Kb<sup>10</sup>.

### HistonePath ChIP-on-chip Detects Histone Modifications Associated with Liver Cyp3a Gene Expression

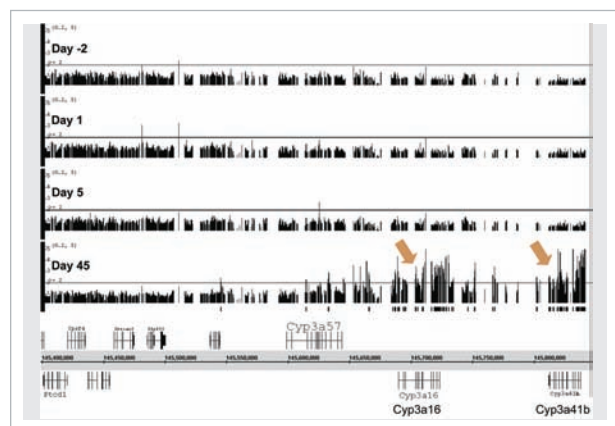
A second illustration of ChIP-on-chip involves the investigation of several histone modifications in regulating the Cyp3a genes in mouse liver<sup>11</sup>. Cyp3a16 and Cyp3a11 exhibit high and low expression, respectively, in the neonatal stages but then undergo a developmental switch. As mice mature, Cyp3a16 is turned off while Cyp3a11 is turned on and expressed in adults. Because these genes encode drug-metabolizing enzymes, it is important to understand the mechanisms controlling this developmental switch. Antibodies against the activating histone modification H3K4me2 and repressing histone modification H3K27me3 were used along with Affymetrix mouse tiling arrays. As shown in Figures 5 and 6, differences in the two histone modifications were identified at various genes within the Cyp3a locus on chromosome 5. For example, the Cyp3a16 and Cyp3a41b genes showed an increase in H3K4me2 at days 1 and 5 after birth (copper arrows), with the levels decreasing to close to baseline by day 45 (adult). In contrast, genes Cyp3a11 and Cyp3a25 showed a modest increase for the same histone modification at day 5 and a significant increase at day 45 (purple arrows). As expected, these changes correlated with increased RNA expression of the same genes at the same time



**Figure 4.** HistonePath ChIP-on-chip showing non-overlapping patterns of H3K27me3 and RNA Pol II phosphoserine 2. Repressing modification H3K27me3 was associated with silent genes and intergenic regions while RNA Pol II was associated with actively transcribed genes. In the 9 Mb shown using the Integrated Genome Browser, blocks below the peaks demarcate areas of association. Red lines were added to help demonstrate the non-overlapping patterns.



**Figure 5.** HistonePath ChIP-on-chip demonstrating changes in activating modification H3K4me2 during mouse liver development. Copper arrows indicate increased levels at the Cyp3A16 and Cyp3a41b genes at days 1 and 5. Purple arrows indicate increased levels at the Cyp3a11 and Cyp3a25 genes at day 45. The region shown was visualized using the Integrated Genome Browser.



**Figure 6.** HistonePath ChIP-on-chip showing areas of repressing modification H3K27me3 during mouse liver development. Copper arrows indicate increased H3K27me3 at the Cyp3a16 and Cyp3a41b genes at day 45. The region shown was visualized using the Integrated Genome Browser.

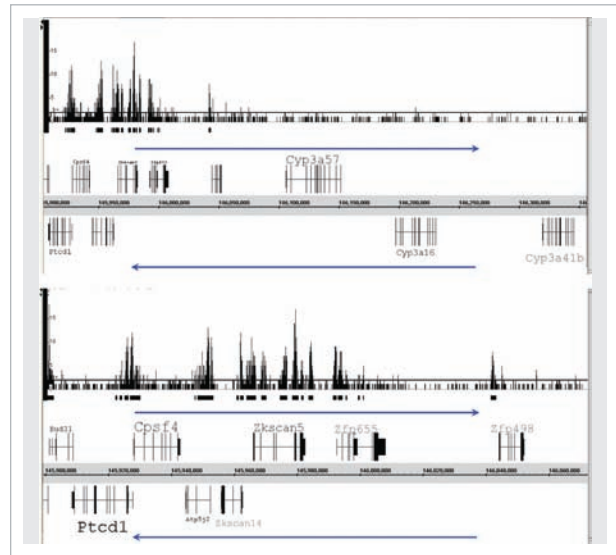
points (data not shown). Cyp3a16 and Cyp3a41b were found to exhibit a different pattern for the repressing histone modification H3K27me3. As shown in Figure 6, significant increases in this modification were observed only at day 45 (copper arrows), which correlated with the absence of Cyp3a16 and Cyp3a41b RNA in adult mice. These results suggest that changes in chromatin structure mediated through histone modifications participate mechanistically in transcriptional regulation of Cyp3a genes during liver maturation.

### HistonePath ChIP-Seq Detects Histone Modifications Genome-Wide

In a separate experiment, ChIP-Seq using an antibody against the activating histone modification H3K4me2 was performed with chromatin from mouse embryonic stem cells, which do not express members of the Cyp3a gene family. Figure 7 (Upper) displays ChIP-Seq data for part of the Cyp3a locus as well as adjacent genes. The absence of the H3K4me2 modification near the Cyp3a genes correlated with the absence of expression of these genes in this cell type. In contrast, the expressed genes located outside the Cyp3a locus exhibited significant levels of the H3K4me2 modification. A magnified view of several of the expressed genes (Figure 7, Lower) shows that the H3K4me2 modification was largely localized around the transcriptional start sites of these genes. However, as demonstrated by the Zkscan5 gene, H3K4me3 can also be present throughout genes or at the 3' ends.

### Conclusion

An understanding of how histone modifications and other epigenetic alterations affect chromatin structure and gene expression is rapidly advancing with the availability of tools for genome-wide studies. The ChIP methodology provides critical information needed for advancing studies of disease progression, stem cell research, drug efficacy and biomarker discovery, and can also lead to therapeutic approaches that target histones and/or histone-modifying enzymes. However, ChIP is complex and, when combined with next-generation sequencing or microarrays, requires numerous critical steps that, if not performed optimally, can result in inaccurate or incomplete results. Active Motif's HistonePath assays provide customers with a high-quality, reproducible solution for generating and analyzing whole-genome histone data that will put investigators on the fast track to answering questions pertaining to epigenetics and gene regulation in their cell systems.



**Figure 7.** (Upper) HistonePath ChIP-Seq demonstrating the absence of activating modification H3K4me2 in the Cyp3a locus of mouse stem cells (non-liver). Active genes outside the Cyp3a locus showed the presence of H3K4me2. The region illustrated was visualized using the Integrated Genome Browser. Arrows represent directions of gene transcription. (Lower) Magnified view showing that H3K4me2 was predominantly localized to transcription start sites.

### Overview of Active Motif Services

Active Motif services have wide utility in the discovery and development of targeted drugs, novel diagnostic biomarkers, and the identification of causal disease and pharmacological pathways.

We offer a complete suite of epigenetic services for understanding gene regulation and genetic pathways:

- **FactorPath™** for discovery of transcription factor binding sites
- **TranscriptionPath™** for discovery of genomic DNA sequences undergoing active transcription in cells
- **MethylPath™** and **HistonePath™** for discovery and analysis of epigenetic events such as DNA methylation and histone modification
- **Bisulfite Sequencing** for determining the methylation status of cytosines at specific CpGs
- **Antibody Qualification** to validate those antibodies that are suitable for use in ChIP, ChIP-Seq and ChIP-on-chip

All of our services include expert consultation on experimental design, quality controls at multiple steps and comprehensive data analysis. Please contact us with any questions you might have about initiating a project in your cell system.

## References

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