Hitting the mark: specificity analysis of histone antibodies

Introduction

The nucleosome, composed of the histones H2A, H2B, H3, and H4, is the fundamental repeating unit of the chromosome, and functions to package DNA in the nucleus. Histones are well known for their remarkable number of posttranslational modifications (PTMs), which include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, and ribosylation [1]. Because of the crucial role histone PTMs play in gene expression, packaging, and DNA repair, there is great interest in elucidating the various histone modifications (also termed "the histone code") under a given condition, for a given organism. However, due to the large number of residues on histones and the additional complexity that comes from the different methylation states of lysine or arginine residues, studying the epigenome is not a trivial task [1].

The availability of antibodies against histones and their PTMs has greatly facilitated our understanding of chromatin structure and function. Histone PTM–specific antibodies are essential reagents in a variety of experimental techniques such as chromatin immunoprecipitation (ChIP), western blotting, immunofluorescence, and immunohistochemistry. ChIP is used extensively to assess protein–DNA interactions and the occupancy of chromatin modifications on a genome-wide scale, and is therefore a crucial application for anti-histone antibodies. Certain histone modifications may display similar DNA-binding patterns, which could lead to a false-positive ChIP result. Therefore, the accuracy of a ChIP experiment depends upon the specificity of the antibody and its ability to distinguish between different PTMs such as dimethylation versus trimethylation states. Several recent studies aimed at testing the quality of commercially available histone PTM antibodies have raised concerns regarding their specificity, which could contribute to misinformed conclusions and a reproducibility crisis. As histone modifications are associated with several disease conditions, the specificity and performance of these antibodies is of paramount importance [1-5]. Thus, a rigorous specificity analysis and functional validation* of histone PTM antibodies is needed.

We have systematically analyzed histone PTM–specific Invitrogen™ antibodies, which include our proprietary rabbit recombinant antibodies, using peptide array, ChIP, and ELISA. We have identified several antibodies that are highly specific to the intended modifications and perform better than the corresponding highly cited antibodies from other suppliers. Here we highlight the important criteria that researchers should consider when choosing a histone PTM–specific antibody.

Methods and results

Strategy for benchmarking exercise

To compare the specificity of antibodies to a particular PTM, we tested Invitrogen antibodies and antibodies from other suppliers using histone peptide arrays, which contain 384 peptides from the N-terminal tails of histones, featuring 59 posttranslational modifications. When a peptide array did not contain a particular modification, we performed an indirect ELISA comparing unmodified peptides, target PTMs, and closely related PTMs. This analysis was followed up by testing antibody performance using ChIP. Antibodies from other suppliers were chosen based on citations and their usability in peptide arrays or dot blots, ChIP or ChIP-Seq, and additional applications like western blotting or immunocytochemistry (ICC).

Criteria for specificity: peptide arrays

To compare the specificity of antibodies to a particular PTM, we tested our antibodies using histone peptide arrays. Representative peptide arrays for two specific Invitrogen antibodies and two nonspecific antibodies from other suppliers are shown in Figure 1. The antibodies against H3K4me2 and H3K9ac (Cat. No. 710796 and 710293, respectively) are highly specific to their intended modification targets, since the signal is observed only from peptides that contain this modification. These data can also be represented as a graph of "specificity factor" for each modification, which is a ratio of the average intensity of all spots containing a particular PTM to the average intensity of all spots lacking that PTM on the peptide array (Figure 1A and 1C, middle panel). In contrast, antibodies from other suppliers against these same modifications (Figures 1B and 1D) are nonspecific, since they interact with peptides with modifications other than the intended one, as highlighted by the <2-fold difference in the specificity factors for binding at the target sites compared to the best nontarget sites (Figure 1B and 1D, middle panel). In the rest of the study, we display only the specificity factors derived from these peptide arrays for each antibody tested.

Analysis in relevant applications: ChIP assays

Next, these antibodies were evaluated for their performance using ChIP (Figure 1, lower panel). The specific Invitrogen antibodies against H3K4me2 and H3K9ac showed the expected enrichment of these modifications on the promoter regions of transcriptionally active genes (*PABCP1* and *cFOS* for Cat. No. 710796, and *CCND2* and *cFOS* for Cat. No. 710293) where these PTMs are known to be enriched, but not in the transcriptionally silent heterochromatin regions (*SAT2* and *αSAT* for Cat. No. 710796, and *SAT2* for Cat. No. 710293) that lack these modifications. However, regardless of their specificity score on the peptide array, all of these antibodies pulled down chromatin, which is routinely used as the only determinant for choosing a ChIP antibody. The data shown here suggest that in addition to showing expected enrichment on positive and negative genomic regions, antibodies intended for use in ChIP should also be tested for their specificity on peptide arrays, or they need to be verified by additional biological methods that give confidence that the antibody does not cross-react with unintended targets.

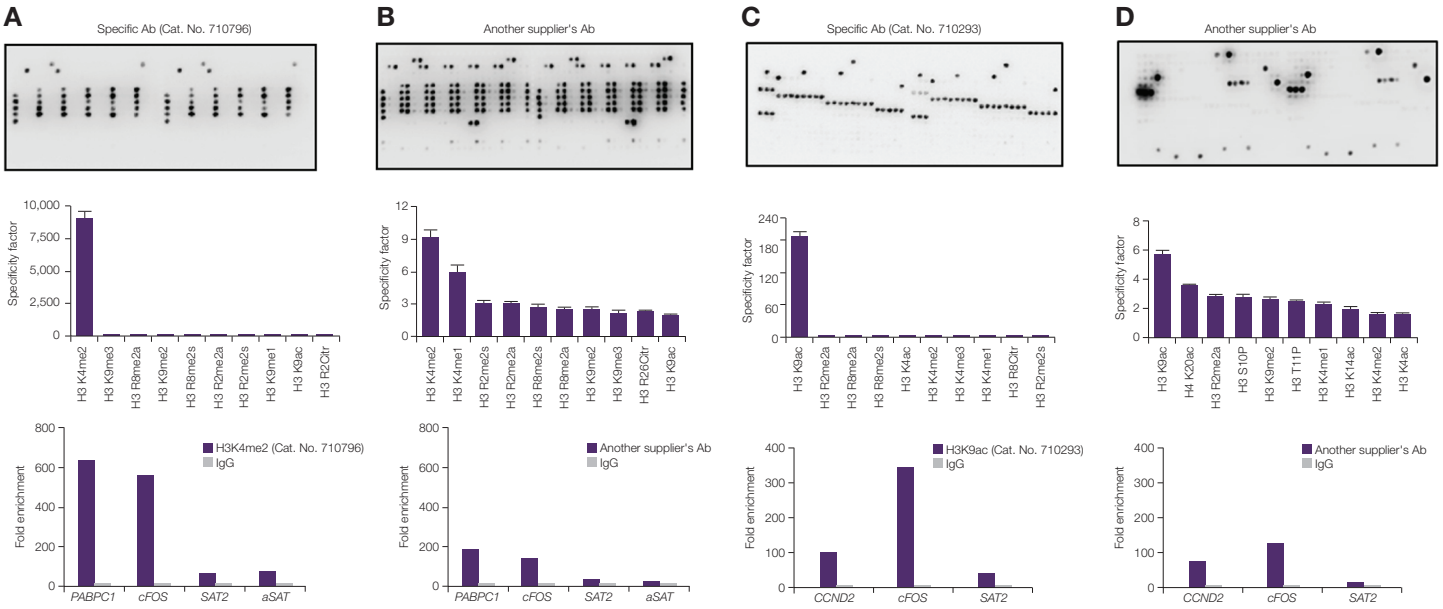

Figure 1. Specificity analysis and functional validation of specific (A, C) and corresponding nonspecific (B, D) antibodies raised against H3K4me2 (Cat. No. 710796) and H3K9ac (Cat. No. 710293) using ChIP. Top panels: Specificity of the four antibodies was determined by histone peptide arrays as described in the methods and results. Middle panels: The results of the peptide array analysis are also shown as specificity factors, which represent the ratio of the average intensity of all spots containing a particular modification divided by the average intensity of all spots lacking that modification. Bottom panels: ChIP was performed on sheared chromatin from 2 million HeLa cells using the Applied Biosystems™ MAGnify™ ChIP System (Cat. No. 49-2024). Normal rabbit IgG was used as a negative IP control. The purified DNA was analyzed using the Applied Biosystems™ 7500 Fast qPCR system (Cat. No. 4351106) with optimized PCR primer pairs for the promoters of the active *PABPC1*, *cFOS*, and *CCND1* genes, used as positive controls, and for the region of the inactive *SAT2* and *αSAT* satellite repeats, used as negative controls. Data are presented as fold enrichment of the antibody signal versus the negative control IgG, using the comparative C_t method.

Evaluation of histone methylation–specific antibodies using peptide arrays or ELISA

Methylation of lysine residues of histones is associated with diverse cellular functions, including genome organization and the transcriptional state of a gene. In addition, a lysine residue can be mono-, di-, or tri-methylated, and a methylation-specific antibody has to distinguish the type of methylation on a specific lysine residue. Figure 2 shows the specificity factors derived from peptide arrays of Invitrogen antibodies against mono-, di-, or tri-methylated lysines in histones H3 and H4. Where a peptide array was unavailable (H3K79me2, Cat. No. 710802), indirect cross-reactivity ELISA against unmodified peptides and those with target PTMs as well as other closely related PTMs was performed to test specificity. In all, 12 different antibodies against different methylations of H3 and H4 (Figure 2 and Table 1) showed 4- to 190-fold higher specificity factors for binding at the target site than for the best nontarget site, indicating higher specificity compared to other suppliers' antibodies for their respective target PTMs.

Evaluation of histone acetylation–specific antibodies by peptide array or ELISA

Acetylation of lysine residues on histones leads to chromatin relaxation and transcriptional activation, and is one of the most widely studied histone modifications. Like methylation, aberrant histone acetylation has been implicated in a variety of pathological conditions, including cancer, infection, and multiple sclerosis. Figure 3 shows the result of the specificity analysis of specific antibodies against acetylated lysines of histones H3 and H4. For H3K79ac (Cat. No. 710805) ELISA was performed to determine specificity, as this modification is absent on peptide arrays used herein. Together, these data highlight the specificity of Invitrogen™ antibodies against acetyl-histone. Please refer to Table 1 for the full list of histone acetylation–specific antibodies.

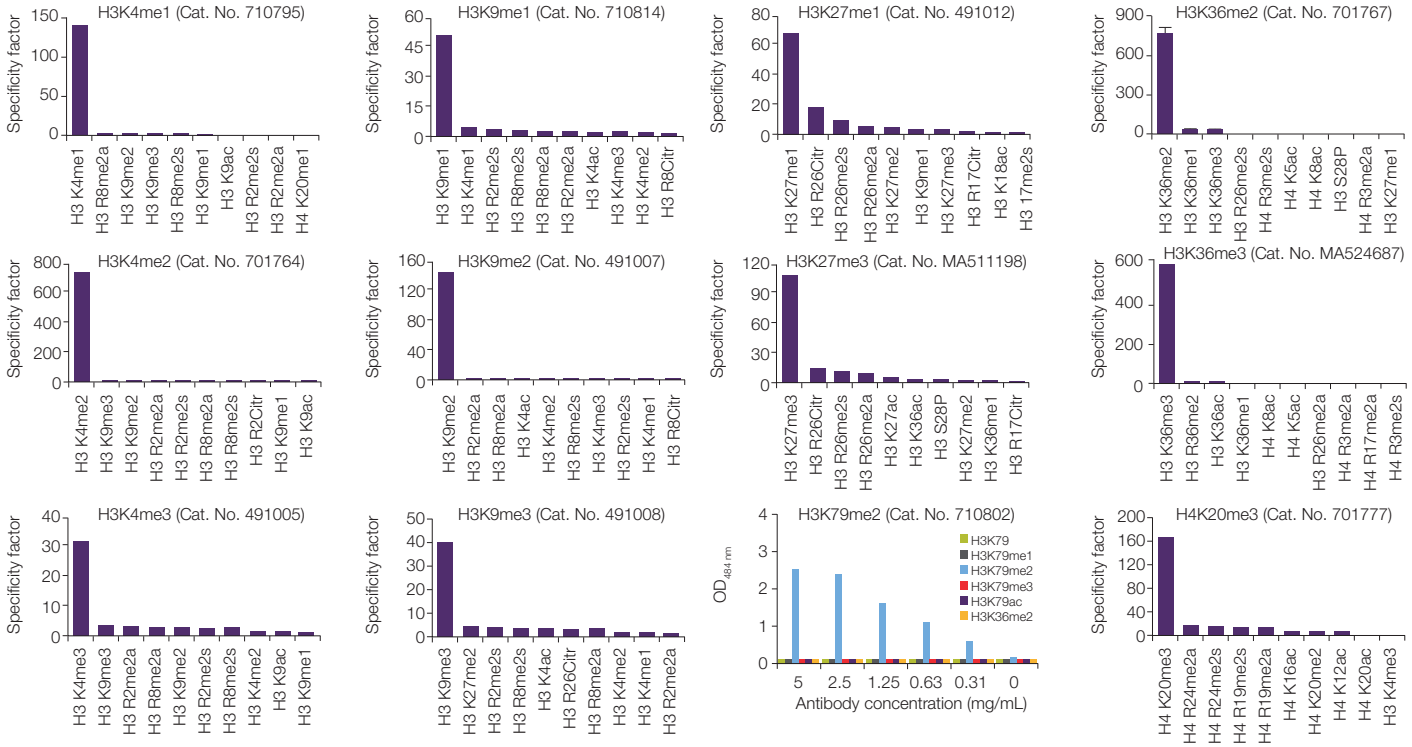

Figure 2. Specificity of Invitrogen antibodies raised against methylated lysine residues of histone H3 and H4 as determined by histone peptide arrays. The results of the peptide array analysis are shown as specificity factors, which represent the ratio of the average intensity of all spots containing a particular PTM divided by the average intensity of all spots lacking that PTM. For H3K79me2, ELISA was performed with various titrations of the H3 dimethyl peptide as well as competing peptides to demonstrate specificity of this antibody (Cat. No. 710802).

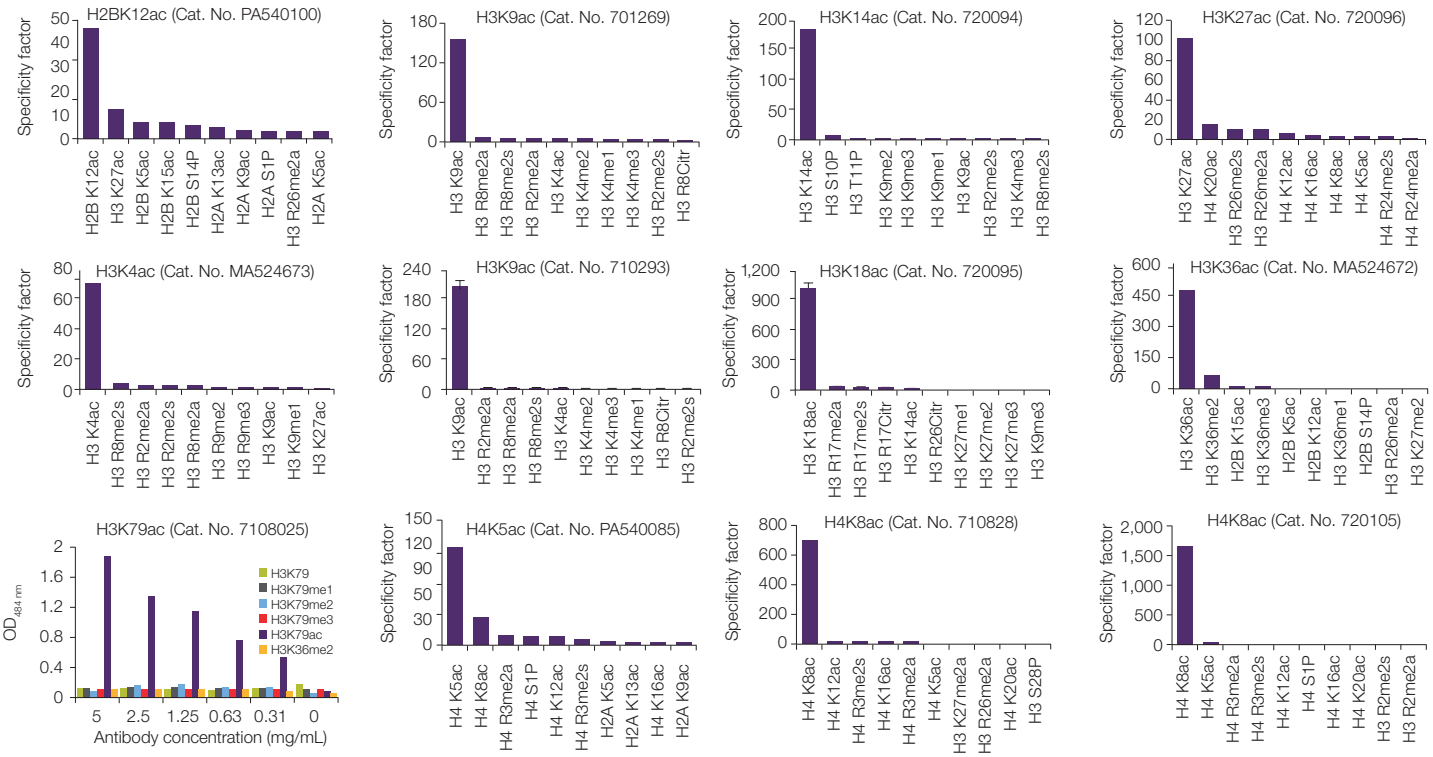

Figure 3. Specificity of Invitrogen antibodies raised against acetylated lysine residues of histone H3 and H4 as determined by histone peptide arrays. The results of the peptide array analysis are shown as specificity factors, which represent the ratio of the average intensity of all spots containing a particular PTM divided by the average intensity of all spots lacking that PTM. For H3K79ac, ELISA was performed with various titrations of the H3 acetyl peptide as well as competing peptides to demonstrate specificity of this antibody (Cat. No. 7108025).

Comparison of histone phosphorylation–specific antibodies using peptide arrays

Phosphorylation of the nucleosome core, as well as linker histones, is associated with chromosome condensation during the cell cycle, transcriptional regulation, and DNA damage repair. Phosphorylation of histones leads not only to the binding of specific reader proteins but also to changes in the affinity for reader or writer proteins of other histone modifications, thereby mediating crosstalk among different histone modifications. Figure 4 displays the results of peptide array analysis of some H3 and H4 phosphorylation–specific antibodies. The antibodies against H3pS10 (Cat. No. 701258), H3pS28 (Cat. No. 720099, PA540140), and H4pS1 (Cat. No. 720100) are highly specific for their intended PTM targets.

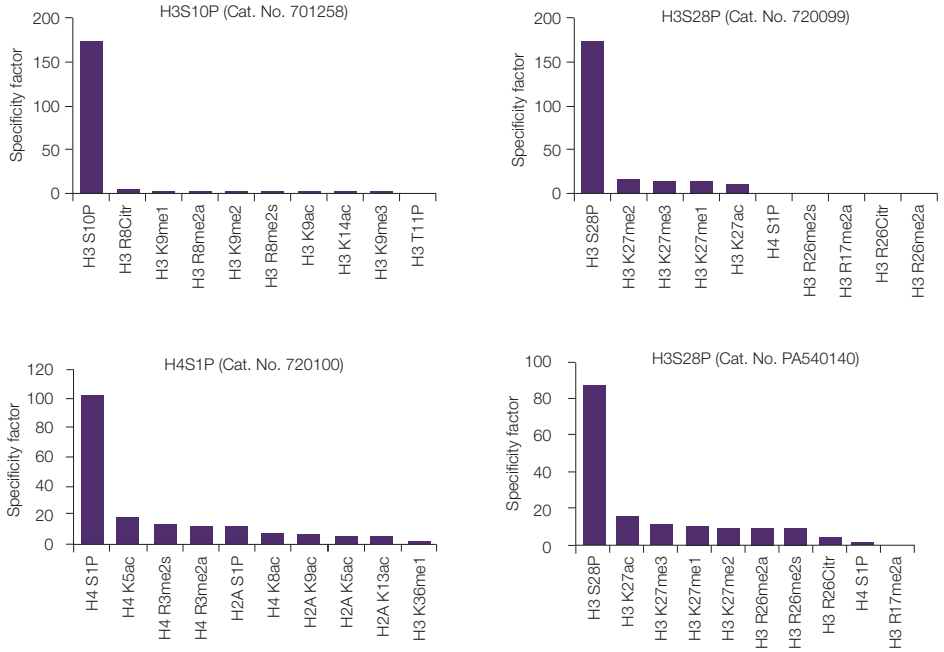

Figure 4. Specificity of Invitrogen antibodies raised against phosphorylated serine residues of histones H3 and H4 as determined by histone peptide arrays. The results of the peptide array analysis are shown as specificity factors, which represent the ratio of the average intensity of all spots containing a particular PTM divided by the average intensity of all spots lacking that PTM.

Conclusion

Stringent verification of specificity and functional verification using ChIP are the two major considerations when choosing a ChIP antibody. Because chromatin pull-down in ChIP is taken as evidence for the presence or absence of a histone PTM on a particular genomic locus, specificity determination of ChIP antibodies ensures that chromatin is not pulled down by nonspecific interactions. On the other hand, functional verifcation of an antibody in ChIP provides evidence that the target epitopes are accessible and that the antibody is binding to expected loci. (To find out more about our 2-part verification testing approach and how we have defined our specificity tests, go to **thermofisher.com/antibodyvalidation**.)

Recently, there has been an increasing concern on the lack of reproducibility of experiments using histone PTM antibodies [2-5]. To address this, we undertook extensive specificity testing and verification of all the histone PTM–specific antibodies in our portfolio, including a comparison of our antibodies to highly cited antibodies from other suppliers for a particular PTM. Table 1 identifies several Invitrogen antibodies that are more specific than the corresponding antibodies from other suppliers (marked with a double asterisk). Using peptide arrays and ELISA, we identified highly specific antibodies against methylated, acetylated, and phosphorylated residues in histones H3 and H4 (listed in Table 1). In addition, in the course of this validation exercise, we identified antibodies that perform well in ChIP. However, in the absence of supporting specificity data, ChIP alone could be misleading—and the results could look similar for specific and nonspecific antibodies. We have thus highlighted the controls that customers should consider utilizing when choosing an antibody for ChIP.

This comprehensive study underscores the breadth of histone PTM–specific Invitrogen antibodies and should serve as a useful guide to customers in choosing the right antibody for their research.

Table 1. Invitrogen histone modification antibodies validated by peptide array and ChIP, with performance comparison noted (last column indicates where Invitrogen antibody performance is equal or superior to antibodies from other suppliers).

Target specificity	Short target name	Cat. No.	Higher specificity compared to other suppliers' products**
Acetyl-Histone H2A.Z (Lys7)	H2A.ZK7ac	MA524665	
Acetyl-Histone H2A.Z (Lys5, Lys7, Lys11)	H2A.Z(K5,K7,K11)ac	PA540095	
Acetyl-Histone H2B (Lys12)	H2BK12ac	PA540100	
Acetyl-Histone H2B (Lys20)	H2BK20ac	MA524698; PA517821	
Acetyl-Histone H3 (Lys4)	H3K4ac	MA524673; PA540093	
Acetyl-Histone H3 (Lys9)	H3K9ac	701269**; 710293**	+
Acetyl-Histone H3 (Lys14)	H3K14ac	MA524668; 720094**	+
Acetyl-Histone H3 (Lys18)	H3K18ac	720095**; MA524669; PA517801	+
Acetyl-Histone H3 (Lys27)	H3K27ac	720096; MA524671; PA540096	
Acetyl-Histone H3 (Lys36)	H3K36ac	MA524672	
Acetyl-Histone H3 (Lys56)	H3K56ac	MA524674; PA540101	
Acetyl-Histone H3 (Lys79)	H3K79ac	710805**; MA524675	+
Acetyl-Histone H4 (Lys5)	H4K5ac	PA540085	
Acetyl-Histone H4 (Lys8)	H4K8ac	701796; 710828**; 720105**; PA516182; PA540092	+
Acetyl-Histone H4 (Lys12)	H4K12ac	701797; 710829	
Acetyl-Histone H4 (Lys5, Lys8, Lys12)	H4(K5,K8,K12)ac	PA540083	
Acetyl-Histone H4 (Lys16)	H4K16ac	720083	

** Denotes antibodies that are more specific than the corresponding antibodies from other suppliers.

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Table 1. (Continued)

Target specificity	Short target name	Cat. No.	Higher specificity compared to other suppliers' products**
Acetyl-Histone H4 (Lys20)	H4K20ac	701778; 710810	
Methyl-Histone H3 (Lys4)	H3K4me1	701763**; 710795**; PA540087	+
Di-Methyl-Histone H3 (Lys4)	H3K4me2	701764**; 710796**; MA514977; PA531912	+
Tri-Methyl-Histone H3 (Lys4)	H3K4me3	MA511199; 491005; PA517420	
Methyl-Histone H3 (Lys9)	H3K9me1	710814**; PA511183	+
Di-Methyl-Histone H3 (Lys9)	H3K9me2	701783; 710815; 720092; PA516195	+
Tri-Methyl-Histone H3 (Lys9)	H3K9me3	701784; 710816; 491007**; 491008**; PA531910	+
Di-Methyl-Histone H3 (Lys23)	H3K23me2	MA524678	
Methyl-Histone H3 (Lys27)	H3K27me1	720097; 491012**	+
Tri-Methyl-Histone H3 (Lys27)	H3K27me3	A15024; MA511198**; PA531817	+
Di-Methyl-Histone H3 (Lys36)	H3K36me2	701767**; MA514867	+
Tri-Methyl-Histone H3 (Lys36)	H3K36me3	MA524687; PA517109	
Di-Methyl-Histone H3 (Lys79)	H3K79me2	710802**	+
Tri-Methyl-Histone H3 (Lys79)	H3K79me3	491020; MA524688	
Methyl-Histone H3 (Arg2)	H3R2me1	711734	
Methyl-Histone H4 (Lys20)	H4K20me1	PA517027	
Tri-Methyl-Histone H4 (Lys20)	H4K20me3	701777**; 710809; PA540089	+
Phospho-Histone H3 (Ser10)	H3pS10	701258**; 710282; PA512526; PA517869; PA540138	+
Phospho-Histone H3 (Ser28)	H3pS28	720099**; PA540140**	+
Phospho-Histone H4 (Ser1)	H4pS1	720100**	+

** Denotes antibodies that are more specific than the corresponding antibodies from other suppliers.

References

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* The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

Find out more at **thermofisher.com/antibodyvalidation**

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