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A simple method for improving the specificity of anti-methyl histone antibodies

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Antibodies differentiating between the mono-, di- and trimethylated forms of specific histone lysine residues are a critical tool in epigenome research, but show variable specificity, potentially limiting comparisons across studies and between samples. Using trimethyl histone H3 lysine 4 (H3K4me3)—a mark enriched at transcription start sites (TSS) of active genes—as an example, we describe how simple co-incubation with synthetic peptide of the K4me2 modification leads to increased specificity for K4me3 and a much sharper peak distribution proximal to TSS following chromatin immunoprecipitation and massively parallel sequencing (ChIP-Seq).

Chromatin-immunoprecipitation (ChIP) with site- and modification-specific anti-histone antibodies is a key approach in the field of epigenetics. Enrichment of a given histone modification can subsequently be measured at a defined gene locus with PCR (ChIP-PCR) or, more recently, on a genome-wide scale with massively parallel sequencing (ChIP-Seq).[1] In particular, methylation of specific histone lysine residues—including H3K4, H3K36, H3K79; H3K9, H3K27 and H4K20—represents one of the most highly regulated types of chromatin modifications.[2] Importantly, these lysine side chains can carry up to three methyl groups, and their mono-, di- and trimethylated forms are differentially localized within the genome and exert distinct effects on transcription. In the case of H3K4, the trimethylated form (H3K4me3) is primarily associated with transcription start sites, while the monomethylated form, H3K4me1, defines enhancer sequences and other regulatory elements further removed from proximal promoters.[3,4] Finally, the dimethyl mark, H3K4me2, appears to be more broadly distributed with open chromatin primarily around the 5' portion of genes.[3,4] Given that the functional effects of each of the three marks is very different, it is critical to perform ChIP with antibodies with selective recognition for either the mono-, di- or trimethylated forms.

Many of the commercially available modification-specific antibodies are polyclonal, reflecting a perception of the field that these preparations provide better signal-to-noise as compared to monoclonals. However, we noticed that—while most antibodies are highly selective for a specific histone lysine residue—there can be considerable lot-to-lot variability in me1/2/3 specificity. In particular, cross-reactivity for the H3K4me2 mark was frequently detected with anti-H3K4me3 antibodies (**Fig. 1A and B**). This represents a serious problem, as each of the different methylation marks confers specific effects on gene transcription.[2,5] Numerous

methods to improve antibody specificity, including affinity purification and pre-incubation with non-specific immunoglobulin, already exist. However, these methods are not directed towards removal of remaining antibodies with non-specific immunoreactivity for a particular antigen, in our case, me2. Here, we describe a simple, but effective, procedure for improving antibody specificity for the H3K4me3 mark.

We describe two slightly different, equally effective protocols for improvement of antibody specificity, both of which include pre-incubation of antibody with peptide of the histone modification for which the antibody demonstrates non-specific immunoreactivity. In the first method, 4 µg rabbit polyclonal antibody against trimethylated histone H3 lysine 4 (H3K4me3; Millipore, 07-473) was incubated with 9 µg of dimethylated H3K4 peptide (H3K4me2; Abcam, ab7768) spotted onto a 0.5 cm^2 piece of nitrocellulose membrane in 500 µl 1x FSB (frozen storage buffer; 5 mM EDTA, 20 mM Tris-HCl and 50 mM NaCl, pH 7.5) for 1 hour at room temperature. The synthetic peptide was derived from amino acids 1–100 of human histone H3 (martkqtarkstggkaprkqlatkaarksapatggvkkphryrpgtvalreirryqkstellirklpfqrlvreiaqdf ktdlrfqssavmalqeaceay), dimethylated at lysine residue 4. In the second method, the H3K4me2 peptide was pipetted directly into the antibody solution, incubated for 1 h at room temperature, and the resulting antibody-peptide solution used for ChIP. To assess the immunoreactivity of the anti-H3K4me3 antibody for the mono-, di- and trimethylated forms of H3K4, we performed dot blots in which peptide for each of the three forms of H3K4 were spotted onto a membrane and allowed to dry; subsequently, membranes were incubated with anti-H3K4me3 antibody. As shown in **Figure 1A and B**, the untreated anti-H3K4me3 antibody demonstrates significant non-specific immunoreactivity for the dimethyl H3K4
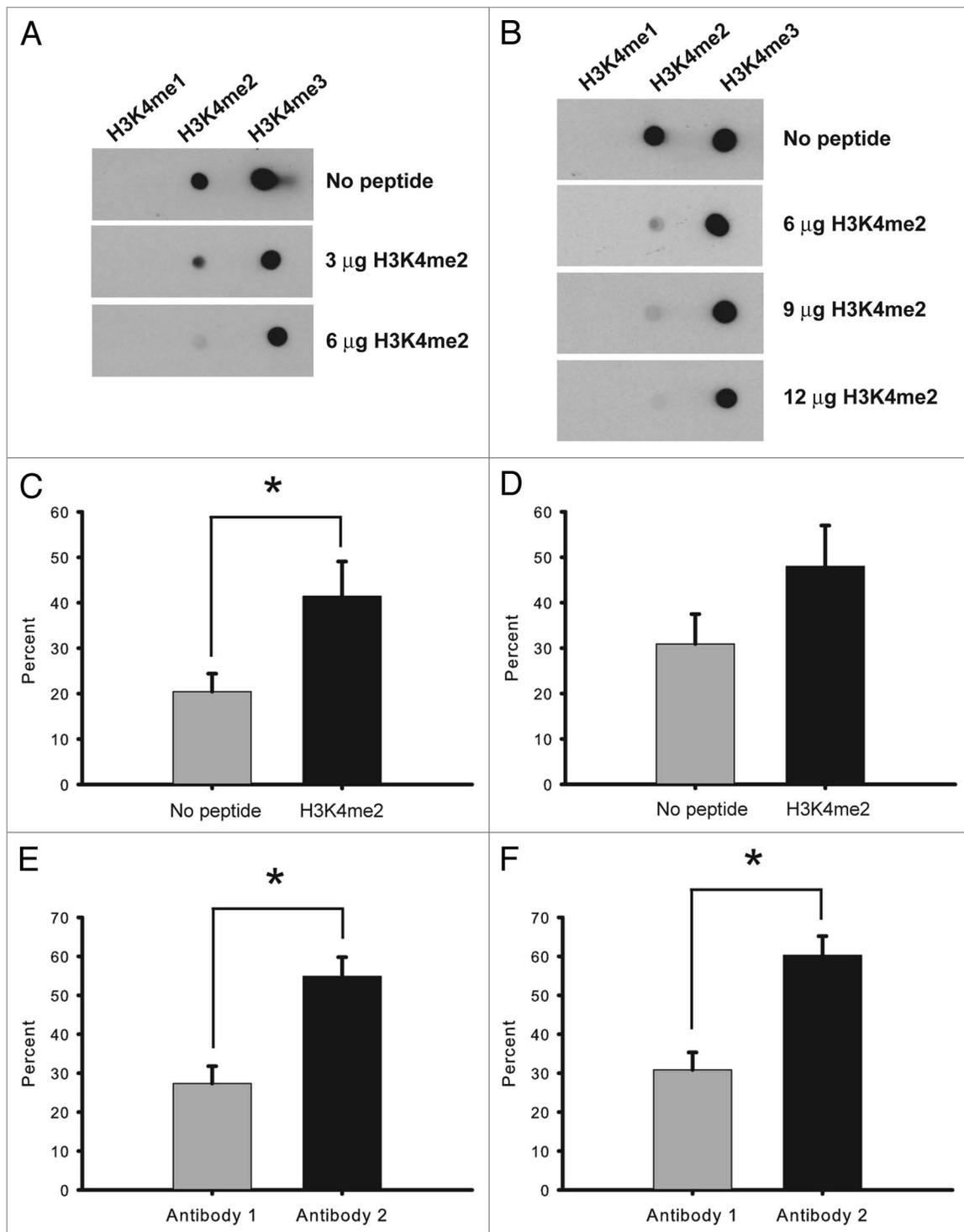
Figure 1. Preincubation of anti-H3K4me3 antibody with H3K4me2 peptide reduces nonspecific immunoreactivity and improves performance during ChIP. (A and B) Preincubation of anti-H3K4me3 antibody with increasing amounts of H3K4me2 peptide on membrane (A) or in solution (B) decreases immunoreactivity of the antibody for this modification on dot blot. (C and D) ChIP performed with anti-H3K3me3 antibody pre-incubated with 15 µg H3K4me2 peptide in solution results in significantly more sequences <1 kb from TSS compared to untreated antibody (C). There was no significant difference at <2 kb from TSS (D). (E and F) An anti-H3K4me3 antibody which demonstrates low immunoreactivity for H3K4me2 peptide on dot blot ("Antibody 2") results in significantly more sequences <1 kb (E) and <2 kb (F) from TSS post-ChIP compared to an anti-H3K4me3 antibody with high immunoreactivity for H3K4me2 peptide on dot blot ("Antibody 1").

peptide. However, pre-incubation of the antibody with H3K4me2 peptide visibly decreases this nonspecific immunoreactivity without altering immunoreactivity for its intended antigen.

To test whether this increased specificity in immunoreactivity translated to improved specificity of DNA sequences pulled down during chromatin immunoprecipitation, we performed ChIP on human and mouse chromatin isolated from brain tissue using untreated or pre-incubated anti-H3K4me3 antibody using a standard protocol for native ChIP.[6] Subsequently, immunoprecipitated DNA was processed for deep sequencing by ligating the Genomic Adaptor Oligo Mix (Illumina) to fragments, followed by PCR amplification and gel purification of fragments around 250 base pairs.

As an initial assessment of the DNA immunoprecipitated by treated versus untreated antibody, a fraction of the library was subcloned into the pDrive vector (Qiagen) and transformed into DH5α cells (Invitrogen). Twenty clones were sequenced, aligned with the mouse or human genome, and their proximity to TSS determined. As shown in **Figure 1C**, anti-H3K4me3 antibody pre-incubated with H3K4me2 peptide pulled down significantly more sequences less than 1 kb from annotated TSS as compared to untreated antibody (41% versus 20%; p = 0.031). This is consistent with the literature showing that trimethylated H3K4 is highly localized around TSS of actively transcribed genes.[4]

Extending the relationship between specificity of immunoreactivity on dot blot to performance of antibody during ChIP, we compared sequences from human brain immunoprecipitated with two commercially available anti-H3K4me3 antibodies: one of which demonstrated significant non-specific immunoreactivity for H3K4me2 peptide on dot blot ("Antibody 1"; Abcam, ab8580) and the other of which exhibited fairly specific immunoreactivity for H3K4me3 ("Antibody 2"; Upstate, 07-473). **Figure 1E and F** show that Antibody 2 pulled down significantly more sequences <1 kb from TSS (55% versus 27%; p = 0.0018) and <2 kb from TSS (60% versus 31%; p = 0.001). It must be noted that non-specific immunoreactivity for any given antibody can vary significantly from lot-to-lot, independent of the manufacturer.

Next, we asked whether pre-incubation of anti-H3K4me3 antibody with H3K4me2 peptide led to increased percentage of sequences in close proximity to annotated TSS genome-wide. Four mouse H3K4me3 ChIP libraries were deep sequenced by an Illumina Genome Analyzer (GA II) and genomic regions containing a significantly large number of reads, called peaks, were detected with the MACS software.[7] Data from each sample were normalized for sequencing depth and the distribution of H3K4me3 peaks relative to TSS was determined. The percentages
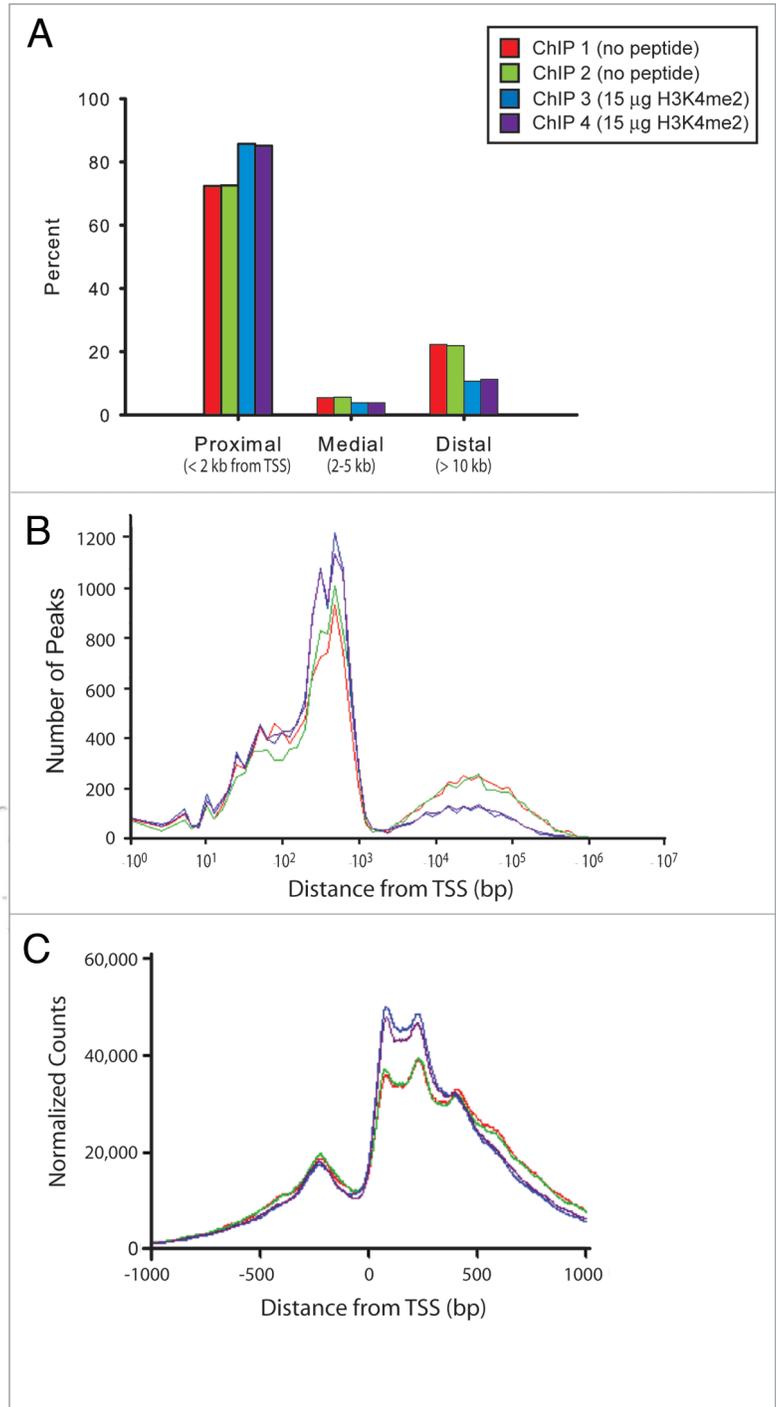

Figure 2. ChIP-Seq performed with pre-incubated anti-H3K4me3 antibody results in a greater percentage of proximal peaks. (A) ChIP performed with pre-incubated anti-H3K4me3 antibody results in a greater percentage of proximal peaks (~85% of total peaks) compared to untreated antibody (~72% of total peaks). (B) The two ChIP samples prepared with pre-incubated anti-H3K4me3 antibody show a higher proportion of peaks in close proximity to TSS and a proportional decrease in those distal to TSS. (C) The two ChIP samples prepared with pre-incubated anti-H3K4me3 antibody demonstrate a higher proportion of sequence tags directly downstream from TSS compared to samples prepared with untreated antibody. In addition, the number of tags in the pre-incubated samples decline more quickly with increasing distance from TSS.

of proximal (<2 kb from TSS), medial (2–5 kb) and distal (>10 kb) peaks are shown in **Figure 2A**. It is apparent that the two samples prepared with H3K4me3 antibody pre-incubated with 15 μg H3K4me2 peptide in solution contain a higher percentage of proximal peaks (~85%) compared to samples prepared with untreated antibody (~72%). The distribution of peaks relative to TSS is shown in **Figure 2B**. We performed Wilcoxon rank sum tests on the distances of the peaks to the nearest TSS between samples. Peaks in the two samples prepared with peptide (ChIP 3 and 4) were significantly closer to a TSS than peaks in the two samples prepared without peptide (ChIP 1 and 2): p-value $<8.9 \times 10^{-36}$ for all four pair-wise comparisons. Next, in order to characterize the detailed distribution of sequence tags around TSS, we used the GSA software[8] to produce an aggregation plot, shown in **Figure 2C**. The two samples prepared with pre-incubated antibody have much higher numbers of tags immediately downstream from the TSS, while a faster decline further downstream, compared with the two samples prepared with untreated antibody. As previously reported,[4,8] the periodic behavior of the aggregation plot indicates well-positioned nucleosomes.

In summary, we show that pre-incubation of commercially available anti-H3K4me3 antibody with a peptide of the H3K4me2 epitope results in significant improvement in antibody specificity and much sharper peaks around TSS genome-wide. These clean-up procedures are particularly important given that the three forms of methylated H3K4 possess different functions with regards to regulation of transcription.

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