SNAP-ChIP: A robust method for determining histone antibody specificity in ChIP

Barcoded synthetic nucleosomes serve as ChIP internal controls.

Histones are the core protein components of nucleosomes, which package DNA into the fundamental repeating units of the eukaryotic chromosome. These highly alkaline proteins are decorated with posttranslational modifications (PTMs) that serve as epigenetic signatures for gene expression and chromatin structure regulation; individual PTMs also play a significant role in processes such as DNA compaction, transcription, translation, genome integrity, and cell cycle. Distinguishing between PTMs is essential for accurate data interpretation and is often achieved using antibodies. However, determining the specificity of antibodies for particular histone PTMs is challenging because these antibodies need to recognize the difference between highly similar modifications such as mono-, di-, or trimethylation of a single histone residue.

In recent years, the assay for determining the specificity of antibodies that recognize histone PTMs has been the peptide array [1-3], a reliable method for screening an antibody's ability to distinguish its target PTM from similar modifications and in the context of neighboring modifications. However, peptide arrays use denaturing conditions and are therefore likely to be most useful in reporting an antibody's ability to recognize and distinguish its intended histone PTM in applications such as western blotting, in which linear epitopes are displayed under denaturing conditions.

Introducing SNAP-ChIP controls

The application of chromatin immunoprecipitation (ChIP) is critical to our understanding of histone PTM regulation and distribution in its native chromatin configuration. Unlike peptide arrays and western blotting, ChIP and ChIP-sequencing (ChIP-Seq) require that an antibody recognize its intended histone PTM in the context of a functional nucleosome engaged in varying levels of chromatin compaction. The distinct antibody requirements for these two different sets of applications were not fully appreciated until recently. The methods for validating histone PTM antibodies have advanced over the years but generally consisted of using dot blots or peptide arrays to infer antibody specificity for a PTM. These histone PTM antibodies were then employed in ChIP assays to demonstrate enrichment or depletion of a specific PTM at expected regions in the chromatin, leading to reports of histone occupancy in the literature that are based on antibody specificity validated by peptide arrays. However, a bead-based flow cytometry assay that mimics the immunoprecipitation step of ChIP using peptides containing histone PTMs revealed that antibody specificity as determined by peptide arrays does not always correlate with specificity determined by peptide immunoprecipitation [4].

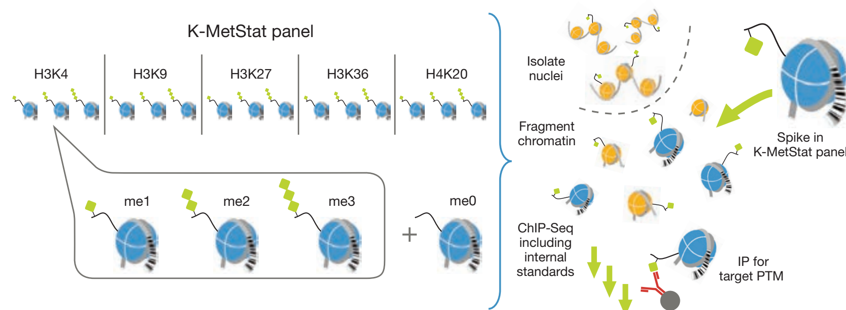

Figure 1. SNAP-ChIP technique for assessing antibody specificity. In a SNAP-ChIP™ assay, a panel of semi-synthetic nucleosomes containing specific histone PTMs is spiked in during the normal ChIP workflow. Because these nucleosomes are wrapped with unique DNA barcodes, they can later be quantified by qPCR to determine how much of each histone PTM is immunoprecipitated in the ChIP reaction. The full K-MetStat panel of histone PTMs includes H3K4, H3K9, H3K27, H3K36, and H4K20, in unmethylated and mono-, di-, and trimethylated forms. Figure used with permission from EpiCypher Inc.

Recently, histone PTM antibody specificity in ChIP applications has been further challenged using the Internal Standard Calibrated ChIP (ICeChIP) assay, a method in which a synthetic nucleosome containing a specific histone PTM is wrapped with a unique DNA barcode and then spiked into the normal ChIP workflow [5]. ICeChIP, which has been commercialized by EpiCypher Inc. as SNAP-ChIP™ (Sample Normalization and Antibody Profiling for Chromatin Immunoprecipitation) reagents and assays (Figure 1), can be used to determine if the antibody is pulling down the intended modification, as well as if the antibody is pulling down any other modifications among a panel of histone PTMs, distinguished on the basis of their DNA barcodes.

The K-MetStat panel (EpiCypher SNAP-ChIP™ Spike-In Controls) is currently available for testing antibody specificity in SNAP-ChIP assays and includes unmethylated and mono-, di-, and trimethylated H3K4, H3K9, H3K27, H3K36, and H4K20, each with a unique DNA barcode

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that can be analyzed by either qPCR or DNA sequencing. Figure 2 shows the use of the K-MetStat panel for testing the specificity of an Invitrogen™ anti-H3K27me3 monoclonal antibody (Cat. No. MA5-11198). Antibody specificity was assessed by spiking HEK293 cell lysates with the K-MetStat panel and proceeding with a standard ChIP workflow. The amount of each of the spiked-in nucleosomes in the resulting immunoprecipitate was then quantified using qPCR. The data show that this Invitrogen antibody exhibited high specificity for H3K27me3 nucleosomes, with less than 15% cross-reactivity across the K-MetStat panel, and high efficiency of IP, with ~12% of the target nucleosome immunoprecipitated relative to the input.

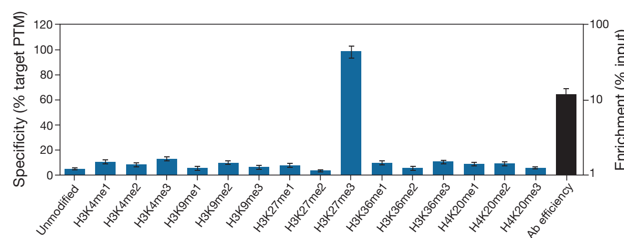

Figure 2. Histone PTM specificity analysis of an Invitrogen anti-H3K27me3 antibody. A SNAP-ChIP™ assay using an Invitrogen™ anti-H3K27me3 monoclonal antibody (Cat. No. MA5-11198) shows that it exhibits specificity to its target PTM. The antibody was tested in a SNAP-ChIP assay using chromatin isolated from HEK293 cell lysates, with the K-MetStat panel spiked in. Specificity (left y-axis, blue bars show mean ± SEM) was determined by qPCR for each modified nucleosome in the K-MetStat panel (x-axis) from six independent ChIP experiments. The black bar is antibody efficiency (right y-axis, log scale) and indicates % target nucleosome immunoprecipitated relative to input. Data used with permission from EpiCypher Inc.

Using SNAP-ChIP controls for antibody validation

The K-MetStat panel was used in a study of 54 commercially available antibodies, and no correlation was found between antibody peptide array specificity and antibody ICeChIP specificity [6]. The consequences of the lack of antibody specificity are significant. Using several H3K4 antibodies, Shah et al. performed ChIP-Seq, in which the ChIP protocol is followed by DNA sequencing to identify the histone occupancy. When the antibodies had similarly high specificity (>85% specific for the intended target), the histone occupancy (ChIP-Seq tracks) looked similar. In contrast, when a highly specific antibody was compared to one that was only 60% specific, the tracks looked different and the less specific antibody had additional peaks suggesting it recognized other histone PTMs and not just the intended target modification. This lack of antibody specificity can lead to incorrect assignment of histone occupancy and ultimately a misunderstanding of the biological role of a histone PTM.

Comparing ChIP-Seq data to previously published data is not always the best practice for validating a histone PTM antibody for ChIP because some less specific ChIP antibodies tested by Shah et al. had originally been used by the ENCODE project and others. Antibody specificity is of the utmost importance, particularly when performing ChIP for histone PTMs, and Thermo Fisher Scientific has begun employing SNAP-ChIP assays to validate Invitrogen histone PTM antibodies for ChIP. The SNAP-ChIP workflow can be used to determine both the efficiency and specificity of the antibody. Consistent with the literature, our own portfolio has revealed histone antibodies that are either concordant or discordant when tested in peptide array and SNAP-ChIP assays (Table 1).

These findings demonstrate the necessity of antibody specificity testing at an application level. As the histone PTM panels expand beyond the currently available K-MetStat panel, antibody testing for other histone methylation and acylation modifications will commence. Our goal is to develop a comprehensive antibody portfolio evaluated in the appropriate specificity test for a given application, providing antibodies that are highly specific for western blotting and for ChIP. To learn more about our expanded testing methodology, visit **thermofisher.com/antibodyvalidation**. ■

Table 1. Recommended antibodies for western blot (WB) based on specificity by peptide array and for ChIP based on specificity by SNAP-ChIP assay.

Target	Recommended for WB	Recommended for ChIP
H3K4me1	710795	710795
H3K4me2	710796	710796
H3K4me3	PA5-17420 and MA5-11199	PA5-27029
H3K9me1	710814	720091
H3K9me2	720092 and 710815	
H3K9me3	49-1008 and PA5-31910	
H3K27me1	49-1012	
H3K27me2		
H3K27me3	MA5-11198	MA5-11198
H3K36me1	701766	49-1016
H3K36me2	701767	
H3K36me3	MA5-24687	MA5-24687
H4K20me1	PA5-17027	MA5-18067
H4K20me2	720085	
H4K20me3	701777	MA5-18074

References

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