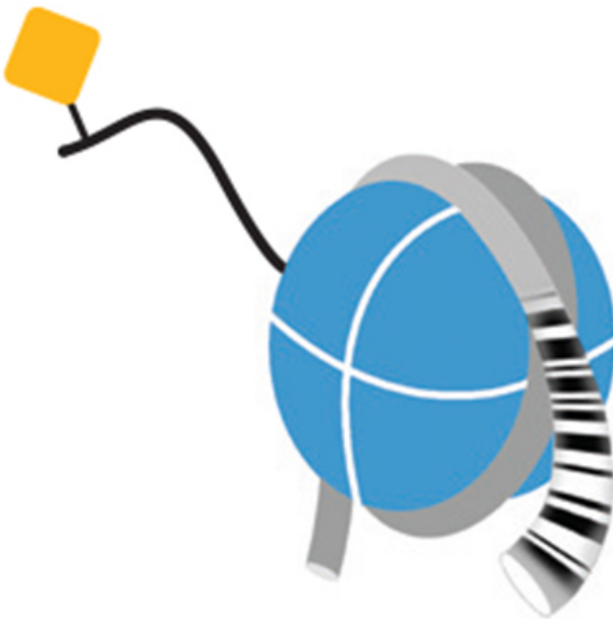


SNAP-ChIP[®]

K-AcylStat[™] Panel

Panel Version 1.0



SNAP-ChIP K-AcylStat Panel

Panel Version 1.0

Catalog No. 19-3001 (10 ChIP Reactions)

Catalog No. 19-3100 (100 ChIP Reactions)

Store at -20°C Upon Receipt

User Manual Version 1.0

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Description

SNAP-ChIP (Sample Normalization and Antibody Profiling Chromatin ImmunoPrecipitation) uses DNA-barcoded designer nucleosomes (dNucs) bearing distinct post-translational modifications (PTMs) as next-generation spike-in controls for ChIP. EpiCypher's SNAP-ChIP panels are directly compatible with your current ChIP workflow, with semi-synthetic nucleosomes bearing the PTM of interest immunoprecipitated and processed alongside sample chromatin (**Figure 1**). Recovery of the associated DNA barcodes is deciphered by quantitative PCR (ChIP-qPCR) or Next-Generation Sequencing (ChIP-seq). SNAP-ChIP provides defined standards to evaluate antibody performance and monitor technical variability in ChIP, setting it apart from other spike-in controls.

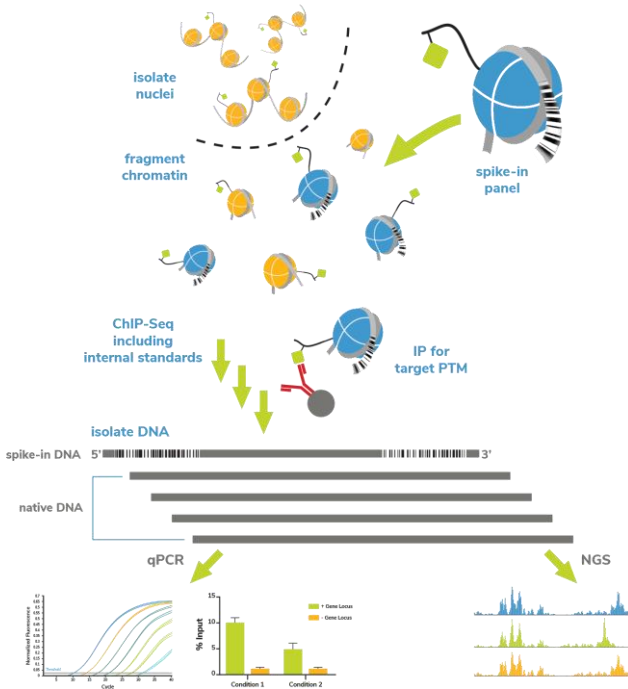


Figure 1. Overview of SNAP-ChIP (adapted from ICeChIP technology¹). A pool of recombinant dNucs with defined PTMs identified by unique DNA barcodes is added to sample chromatin prior to immunoprecipitation (IP). Capture of the barcoded nucleosomes (on- and off-target) allows the user to assess antibody performance (specificity and target enrichment) and monitor technical variability.

In an unprecedented study of over 50 commercially available antibodies to H3K4 methylation states, barcoded nucleosome spike-in ChIP controls revealed previously unrecognized cross-reactivity for numerous widely used antibodies that confounded data interpretation². To address this problem, EpiCypher is developing multiple SNAP-ChIP panels of related PTMs (e.g. lysine methylation, lysine acylation, arginine methylation, lysine ubiquitylation) to empower end users to directly evaluate antibody performance in ChIP, improving the likelihood of achieving high quality data and accurately ascribing biological functions to distinct PTM states.

EpiCypher’s lysine acylation status panel, or “K-AcylStat” Panel, is the third product in our SNAP-ChIP line. The K-AcylStat Panel consists of a pool of nucleosomes carrying twenty-two well-studied, disease relevant lysine acylation marks on histones H2A, H3, and H4 plus an unmodified control (**Figure 2**). A single spike-in of the panel allows users to check antibody specificity by examining the post-IP recovery of on- versus off-target SNAP-ChIP nucleosomes. Other available SNAP-ChIP Panels include the K-MetStat™ Panel (EpiCypher #19-1001) and the OncoStat™ Panel (EpiCypher #19-2001). Additional modification-specific panels (e.g. arginine methylation, lysine ubiquitylation) are currently in development.

K-AcylStat Panel

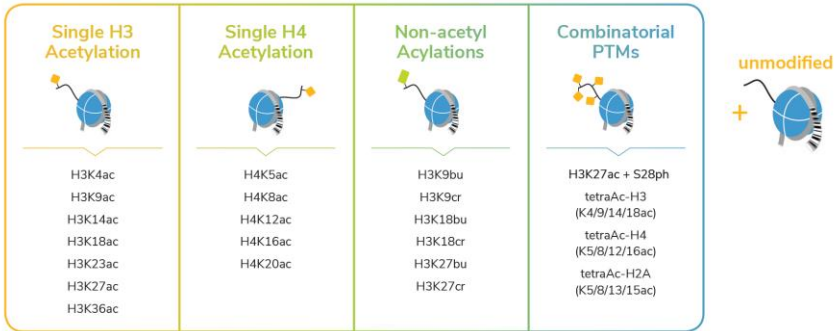


Figure 2. Schematic depicting the 22 dNucs plus an unmodified control included in the K-AcylStat Panel. Each nucleosome is wrapped by two unique DNA barcodes, providing an internal technical replicate for each PTM of interest.

SNAP-ChIP Advantages

- Homogenous, fully defined standards that faithfully represent target mononucleosomes in the experimental sample
- EpiCypher dNucs are subjected to rigorous quality control for lot-to-lot consistency
- Unique DNA barcodes can be distinguished from experimental sample genomes (confirmed human, mouse, *Drosophila* and *Saccharomyces cerevisiae*)
- Spike-ins provide a direct readout of antibody performance:
 - Panels contain a pool of related PTMs that allow users to determine antibody specificity (recovery of on- vs. off-target PTMs in the panel)
 - Determination of antibody enrichment (amount of target PTM immunoprecipitated relative to Input)
- Ability to monitor technical variability between samples
- Analysis of DNA barcodes (via qPCR) provides useful STOP / GO capability before advancing to NGS

SNAP-ChIP DNA Barcoding

Each recombinant nucleosome in the SNAP-ChIP panel is wrapped by a 147 bp nucleosome positioning sequence (NPS, derived from the Widom 601 clone³; **Figure 3**). Embedded within the NPS are two 22 bp DNA “barcode” regions. The 5' Forward Barcode is unique to each panel (e.g. K-MetStat, K-AcylStat, R-MetStat, K-UbStat). The 3' Reverse Barcode is unique to each dNuc within a panel (H3K4ac, H3K9ac, H3K27ac, etc.). Further, each recombinant nucleosome represented in the panel is wrapped by two distinct barcodes, providing an internal technical replicate for every experiment.

The barcode sequences are designed to be absent from the human, mouse, fruit fly and budding yeast reference genomes, and thus easily distinguished in paired-end sequencing¹. The full sequence of each barcoded DNA is available from the SNAP-ChIP product page (www.epicypher.com).

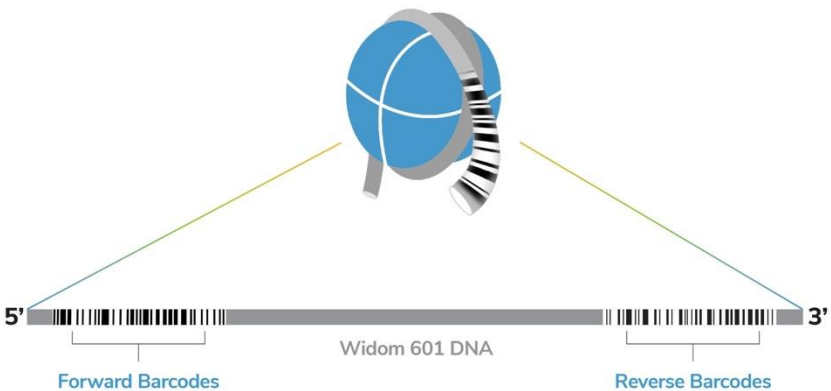


Figure 3. Schematic depicting the DNA barcoding scheme in each SNAP-ChIP panel. Two 22 bp barcode regions are embedded within the 147 bp nucleosome positioning sequence (gray). The Forward Barcode is unique to each panel, while the Reverse Barcode is unique to each nucleosome within a panel. Further, each nucleosome in a panel is wrapped by two distinct reverse barcodes, providing an internal technical replicate.

Materials Required but not Supplied

SNAP-ChIP K-AcylStat Panel contains enough reagent to perform 10 (Catalog #19-3001) or 100 (Catalog #19-3100) ChIP reactions (each from ~10⁶ cell equivalents) in the user-preferred workflow.

ChIP-seq

- Standard ChIP and ligation-based library prep reagents (user specific)

ChIP-qPCR

NOTE: TaqMan™ qPCR is recommended for greater sensitivity; however, SNAP-ChIP is also compatible with SYBR Green qPCR.

NOTE: Bio-Rad qPCR reagents referenced; however, the protocol can be adapted to alternate platforms.

- Standard ChIP reagents (user specific)
- qPCR Master Mix: e.g. Bio-Rad iTaq™ Universal Probes Supermix
- qPCR Plates & Seals: e.g. Bio-Rad HSP9601, Bio-Rad MSB1001
- qPCR Machine: e.g. Bio-Rad CFX Connect™
- SNAP-ChIP Dual Labeled Hydrolysis Probe*: EpiCypher #18-6001
- qPCR Primers[‡]:
 - K-AcylStat Forward Primer (universal to all barcoded nucleosomes in the K-AcylStat panel):
5'-CGT CGA ACG CGC GAT AT-3'
 - K-AcylStat Reverse Primers (specific to each individual barcoded nucleosome in the K-AcylStat panel): the full list of primer sequences is available on the SNAP-ChIP product page at www.epicypher.com

*Full TaqMan probe sequence for ordering from 3rd party vendor (e.g. IDT Technologies):
5'-/56-FAM/TCT AGC ACC GCT TAA ACG CAC GTA/3IABkFQ/-3'

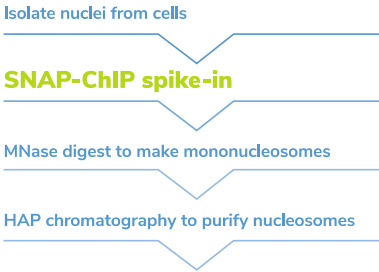
[‡]qPCR primers can also be ordered from a 3rd party vendor (e.g. IDT Technologies). Sequences are available from the SNAP-ChIP product page (www.epicypher.com).

Experimental Overview

Incorporate SNAP-ChIP into your CHIP experiments in three simple steps:

1. Spike the SNAP-ChIP Panel into samples at the earliest appropriate step in your CHIP protocol (see **Figure 4** or Experimental Protocol, Steps 1-2, p.8).
2. After immunoprecipitation and DNA isolation, use qPCR to assess whether CHIP has successfully (and specifically) enriched the target PTM. Use this information to decide whether to invest in NGS (STOP/GO capability). See Appendix 3 for considerations to determine antibody specificity in qPCR before sending for NGS.
3. NGS data will give further information regarding antibody specificity for all PTMs in the panel, identifying additional potential cross-reactivities. If all is as expected, users can feel confident to proceed with data analysis.

Native ChIP Workflow



Crosslinked ChIP Workflow

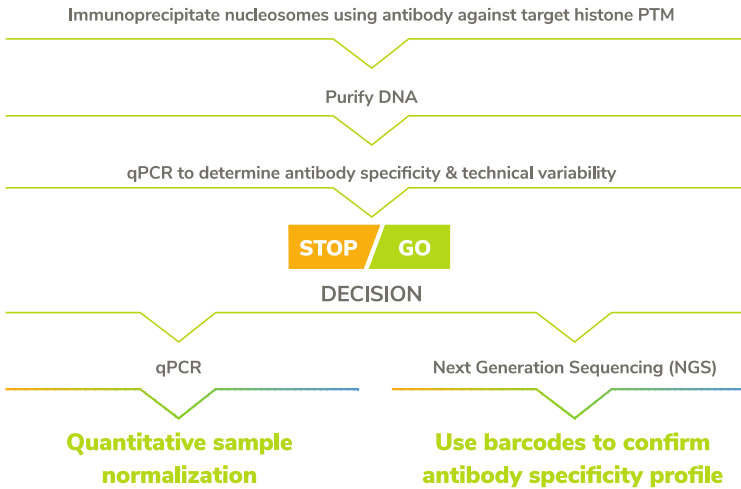
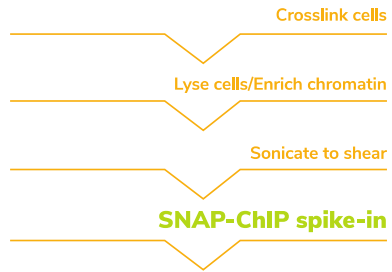


Figure 4. SNAP-ChIP is compatible with both native and crosslinked ChIP protocols, where a simple spike-in of the panel enables the assessment of antibody specificity and technical variability prior to investment in NGS (STOP/GO decision). SNAP-ChIP can also be used to normalize experimental data, improving quantitative comparisons in qPCR.

Experimental Protocol

1. Prepare samples using desired protocol (crosslinked or native).

NOTE: If using a crosslinked protocol, spike in SNAP-ChIP post-sonication. If using a native protocol, spike in prior to micrococcal nuclease digestion (Figure 4).

NOTE: SNAP-ChIP nucleosomes are compatible with standard crosslinked protocols containing up to 0.1% SDS in the ChIP buffer. For protocols with higher concentrations of ionic detergent during sample preparation, immunoprecipitation, and/or washing steps (nucleosomes should be intact), users should evaluate compatibility. However, 1% SDS in the elution step is fine (nucleosomes to be digested).

2. Spike-in 2 μL SNAP-ChIP per 10 μg sample chromatin ($\sim 10^6$ cell equivalents per ChIP). If more or less chromatin is used, scale the spike-in volume accordingly (see FAQ's, p. 14 for more information).

NOTE: see Appendix 1 "Quantification of Sample Chromatin"

3. Proceed with IP using an antibody to the desired PTM.

NOTE: Be sure to saturate the IP resin (e.g. magnetic beads) with antibody: the lot-specific datasheet should contain bead capacity.

4. Purify immunoprecipitated DNA (e.g. Serapure, AMPure, Qiagen PCR Purification Kit, or equivalent).
5. Run qPCR to evaluate antibody performance and technical variability:

qPCR Reaction mix (per 10 μL reaction)		
Reagent	Final Concentration	Volume to add
1:10 diluted ChIP or Input DNA	User-specific	4.0 μL
2X TaqMan qPCR Master Mix	1X	5.0 μL
20X SNAP-ChIP Primer Mix	1X (250 nM for each primer)	0.5 μL
20X SNAP-ChIP Probe	1X (250 nM)	0.5 μL

6. Run qPCR using appropriate reaction conditions (e.g. BioRad iTaq Universal Probes Supermix = 95°C for 3 min followed by 40 cycles of 95°C for 5 sec plus 60°C for 30 sec).
7. Analyze data using standard $\Delta\Delta\text{Ct}$ calculation (see Data Analysis, p.10)
8. Evaluate whether IP was specific, and the experiment is thus of sufficient quality to invest in NGS. Proceed with library preparation.
9. Use the spike-in DNA barcode sequences (see product page at www.epicypher.com) to align read counts to SNAP-ChIP.

Data Analysis: Determining Antibody Specificity

Why do I need to assess antibody specificity in ChIP?

- Antibody cross-reactivity can lead to gross data misinterpretation, see Appendix 3 for further cross-reactivity considerations.
- It is essential to determine antibody capability in the application of interest. Commonly used methods for antibody validation (e.g. peptide arrays) are biophysically very different from ChIP; therefore, any cross-reactivity (or lack thereof) detected in such an approach may not be applicable in a ChIP context².
- Exclusive use of positive / negative genomic loci controls to monitor IP enrichment is invariably a lower resolution approach (and may be further compromised if the initial control loci were identified with cross-reactive reagents).
- SNAP-ChIP addresses these limitations by enabling platform-specific validation of antibody specificity within every ChIP experiment (**Figure 5**).

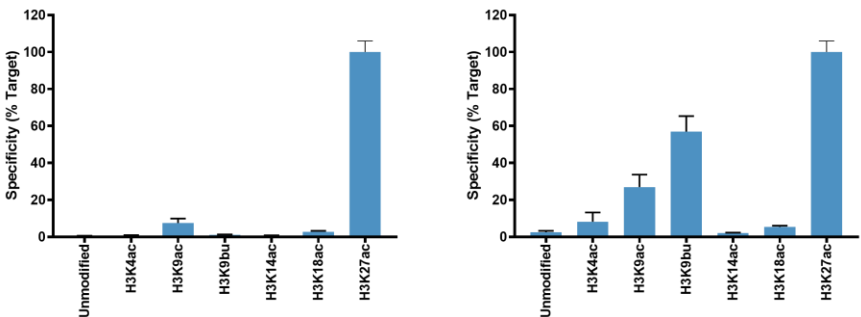


Figure 5. A SNAP-ChIP experiment using two H3K27ac antibodies. qPCR for the barcodes corresponding to the indicated acyl states shows that Antibody #1 (left) is highly specific for H3K27ac, exhibiting <10% cross-reactivity with off-target PTMs. In contrast, Antibody #2 (right) shows ~60% cross-reactivity with H3K9bu and ~30% cross-reactivity with H3K9ac, compromising any corresponding ChIP data.

How to assess antibody specificity using the SNAP-ChIP spike-in:

1. Perform SNAP-ChIP as in the Experimental Protocol section (p.8).
2. Run qPCR to check recovery of the barcodes corresponding to the on-target PTM compared to off-target PTMs.

NOTE: It is recommended at minimum to check antibody specificity against PTMs that present the most likely source of cross-reactivity, see Appendix 3. If an antibody exhibits specificity at this stage, the remaining PTMs in the panel can be actively checked by qPCR or will be passively captured in ChIP-seq.

3. Analyze data to calculate antibody specificity (off-target relative to on-target, **Table 1**) and enrichment (% on-target Input recovered, below).

NOTE: The enrichment score provides a measure of the amount of target recovered after IP. An antibody can exhibit high specificity for the target but have low enrichment, which could become problematic for experimental normalization due to noise in the data. Antibodies with enrichment >10% generally yield reproducible results. Antibodies with reduced enrichment (1-10%) may be usable if they exhibit high specificity. Antibodies with enrichment scores <1% often result in excessively variable data.

$$\% \text{ Enrichment} = \left(\frac{2^{Ct_{Input} - Ct_{IP}}}{x} \right) \times 100\%$$

where Ct_{Input} = qPCR cycle threshold for SNAP-ChIP on-target Input
 Ct_{IP} = qPCR cycle threshold for SNAP-ChIP on-target IP
 x = percent of Input sample that was set aside (e.g. 10%)

4. In ChIP-seq, all barcodes will be captured in the NGS data. Align the read counts to the barcodes. A SNAP-ChIP alignment algorithm for paired-end sequencing is available at www.basepairtech.com. FASTA-formatted sequences for developing custom analysis pipelines are also available on the product page at www.epicypher.com.

Ct Mean	ΔCt	$\Delta \Delta Ct$	RQ (Relative Quantification)	Specificity (% Target)
= Average of qPCR technical replicates	$= Ct_{IP} - Ct_{Input}$	$= \Delta Ct_{off-target} - \Delta Ct_{on-target}$	$= 2^{-\Delta \Delta Ct}$	$= RQ \times 100\%$

Table 1. Calculations for determining antibody specificity using the SNAP-ChIP on- and off-target barcodes, where “on-target” refers to the SNAP-ChIP dNuc immunoprecipitated by the ChIP antibody and “off-target” refers to any other dNuc in the SNAP-ChIP panel.

Data Analysis: Normalization

Why do I need to use SNAP-ChIP to normalize my experiments?

SNAP-ChIP provides a homogenous, defined spike-in control that can be used as a normalization factor to standardize experiments. This controls for unanticipated technical variability (**Figure 6**) and enables trans-experiment comparisons. The use of SNAP-ChIP to account for technical variation in the ChIP procedure reduces noise in the data, thereby improving detection of true biological changes.

How to normalize ChIP data using the SNAP-ChIP spike-in:

1. Perform SNAP-ChIP-qPCR as in the Experimental Protocol section (p.8), keeping the ratio of SNAP-ChIP to sample chromatin consistent across samples.

NOTE: It is essential to run an Input (pre-IP chromatin) control for every ChIP sample.

2. Check that the antibody did not cross-react with unintended targets in the SNAP-ChIP panel (see **Figure 6**, panel 1).

NOTE: Cross-reactivity prohibits normalization because any recovery of off-target PTMs contributes sample data which cannot be accounted in the normalization. As an example: the biological perturbation under study differentially impacts the antibody on-target (H3K27ac) and off-target (H3K9ac) signals in the sample chromatin: this cannot be corrected by the on-target spike-in normalization.

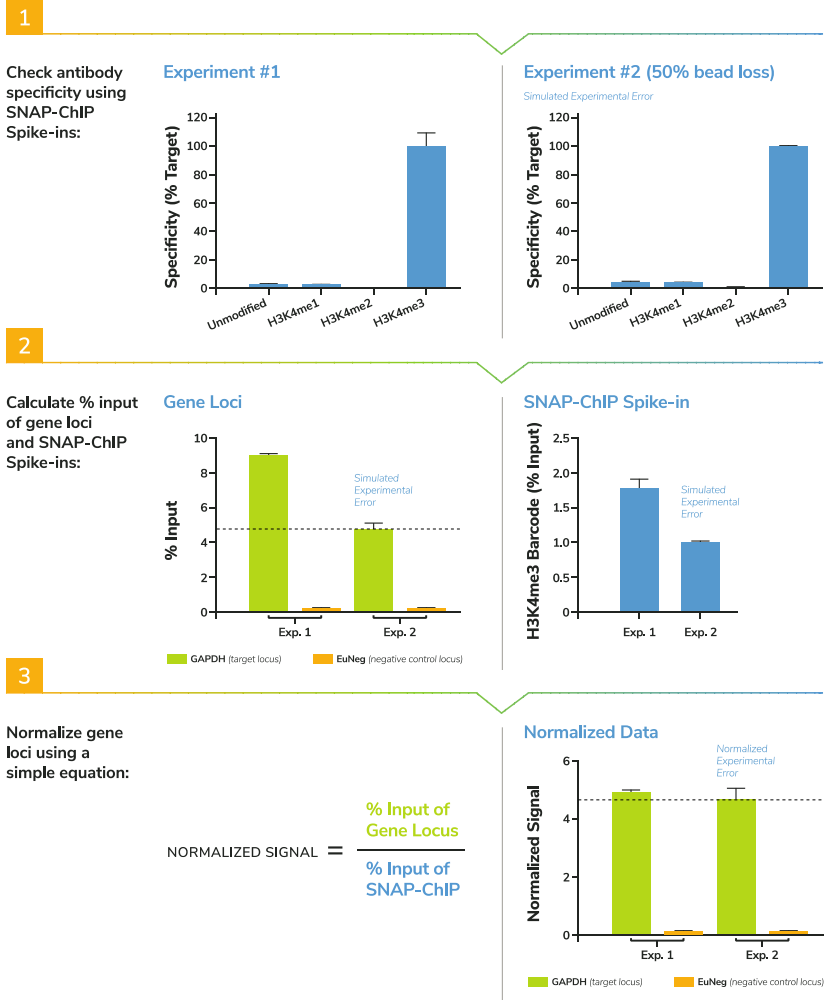
3. Calculate the % Input for each gene locus of interest (or genome-wide for ChIP-seq).
4. Calculate the % Input for the on-target SNAP-ChIP spike-in (e.g. in a H3K27ac ChIP experiment, use the barcodes corresponding to the H3K27ac dNuc in the SNAP-ChIP panel).

NOTE: See the % Enrichment calculation (p.10) to calculate the % Input for ChIP-qPCR. For ChIP-seq, simply divide the read counts for IP/Input.

5. Apply the formula:

$$\text{Normalized signal} = \frac{(\% \text{ Input of Gene Locus})}{(\% \text{ Input of SNAP ChIP})}$$

SNAP-ChIP Normalization Workflow



Appendix 1: Quantification of Sample Chromatin

NOTE: The guidelines below are for UV absorbance measurement of DNA. When working with small numbers of cells (<10⁶) below the sensitivity of standard spectrophotometers (~2 ng/μL) a more sensitive DNA quantitation tool (e.g. fluorometer) may be needed.

Native ChIP

1. After nuclei purification, remove a small aliquot of sample. Dilute 1:10 in 2 M NaCl to extract proteins (e.g. dilute 2 μL nuclei in 18 μL NaCl).
2. Vortex for 10 minutes or water bath sonicate to solubilize DNA.
3. Use a spectrophotometer to determine nucleic acid content by measuring the absorbance at 260 nm (OD₂₆₀).

NOTE: For most accurate measurement, perform triplicate readings. If sample is too viscous, readings will not be consistent. Try higher dilutions (e.g. 1:100) or more vigorous vortexing / sonication.

4. Use molar extinction coefficient for DNA to calculate concentration:

$$DNA\ Concentration = OD_{260} \times Dilution\ Factor \times 50\ ng/\mu L$$

Crosslinked ChIP

1. After chromatin sonication, follow steps in the user-specific protocol to digest proteins and reverse crosslinking. In general:
 - a. Add elution buffer
 - b. Incubate samples at 65°C while shaking 4 hours – overnight
 - c. Add Proteinase K
 - d. Incubate samples at 60°C while shaking for 1 hour
2. Purify DNA (e.g. PCR Purification Kit, phenol:chloroform extraction, Serapure/AMPure, or equivalent method)
3. Remove a small aliquot of purified DNA. Dilute 1:100 using TE buffer.
4. Use a spectrophotometer to determine nucleic acid content (OD₂₆₀)
5. Use molar extinction coefficient for DNA to calculate concentration:

$$DNA\ Concentration = OD_{260} \times Dilution\ Factor \times 50\ ng/\mu L$$

Appendix 2: Frequently Asked Questions

- **Why do I need SNAP-ChIP?**

Think of SNAP-ChIP as a spike-in control: both positive (nucleosomes with the PTM of interest) and negative (unmodified nucleosome) controls are incorporated into a single spike-in. The standard method of performing qPCR for positive and negative genomic locus controls to evaluate performance of a ChIP experiment serves as an assumed proxy of on-target recovery, but does not directly assess recovery of the intended PTM. SNAP-ChIP provides the first ever direct and quantitative assessment of on-target recovery in every ChIP experiment. Additionally, the inclusion of related PTMs in one convenient spike-in panel gives information about antibody specificity, reducing concerns about the potential for undetected cross-reactivity to lead to incorrect assignment of biological function to a specific PTM².

- **Will the barcodes overlap with genomic DNA sequences from my samples?**

SNAP-ChIP barcodes are designed to be compatible with multiple species (e.g. human, mouse, fly, and yeast) such that their genomic DNA can be readily distinguished without issue.

- **What results can I expect with the standard spike-in amount?**

At a spike-in volume of 2 μ L per 10 μ g of chromatin, qPCR cycle threshold (Ct values) for the Input samples is expected to be in the low ~20's. In a standard ChIP-sequencing experiment (30 million reads per sample), Input read counts for the individual SNAP-ChIP barcoded nucleosomes is expected to range from ~200-500. Based on these general expectations, users can make an educated decision about how much to scale the spike-in volume up or down in conditions where different amounts of sample chromatin are used.

- **Why would I use a native ChIP-protocol with micrococcal nuclease (MNase) digestion vs. crosslinking/sonication?**

SNAP-ChIP is directly compatible with both native and crosslinked approaches though the former is recommended. Crosslinking can impact antibody specificity and enrichment because crosslinked chromatin becomes stickier and more susceptible to epitope masking.

In our experience, signal-to-noise ratios are often decreased in crosslinked samples compared to native ChIP.

In contrast, a native nuclei preparation that is micrococcal nuclease digested to yield >95% pure mononucleosomes will generate samples that more closely resemble the SNAP-ChIP spike-ins (i.e. unfixed mononucleosomes). As a result, data obtained from the SNAP-ChIP controls will be most representative of the experimental samples.

- **I've never done native ChIP. Can you recommend a protocol?**

See Brand et al. (2008)⁴ for detailed native ChIP methods.

- **Are there any guidelines for how to run the sequencing?**

Paired end sequencing is recommended for several reasons:

1. Because the reverse barcodes (unique to each dNuc within a panel) are towards the 3' end of the NPS (**Figure 3**), they will not be reachable from the top strand in many NGS configurations (e.g. single end 50, 75 or 100 bp sequencing). Thus, half of the data associated with the SNAP-ChIP spike-ins (those from the top strand) cannot be confidently aligned to a specific nucleosome in the panel and will be discarded. The read depth associated with the SNAP-ChIP spike-ins will be concomitantly reduced.
2. Paired end sequencing allows read filtering to eliminate data associated with dinucleosomes (immunoprecipitated more efficiently than mononucleosomes¹ and thus overrepresented in the sequencing data). This bias can be mitigated by excluding fragments sized > 220 bp from analysis.

- **Will the spike-ins affect the required sequencing depth?**

The SNAP-ChIP spike-ins represent << 1% of total nucleosomes in the sample, so sequencing depth is unaffected.

- **Can you recommend specific histone PTM antibodies?**

EpiCypher has performed extensive antibody testing for various histone PTMs. For more information, inquire at info@epicypher.com.

Appendix 3: Considerations for Antibody Specificity Testing

NOTE: Below are practical guidelines for conducting a limited qPCR test using primers specific to a small subset of barcoded nucleosomes in the K-AcylStat Panel. This way, users can monitor the most likely sources of antibody cross-reactivity before proceeding to NGS, where specificity against the full K-AcylStat Panel is captured in the sequencing data. An antibody is never considered "SNAP-ChIP Certified" until it can be tested against the full panel, since antibodies can exhibit unpredicted cross-reactivity to various PTMs².

- Sensitivity to combinatorial PTMs: The K-AcylStat Panel contains lysine acetylation alone as well as in combination with other PTMs (e.g. H3K27ac + S28ph, tetra-Ac H3, H4, and H2A). Antibody enrichment (post-IP recovery of the target PTM) can be influenced by the presence of neighboring modifications. For example, antibody enrichment can be either enhanced (**Figure 7**) or inhibited by combinatorial PTMs.

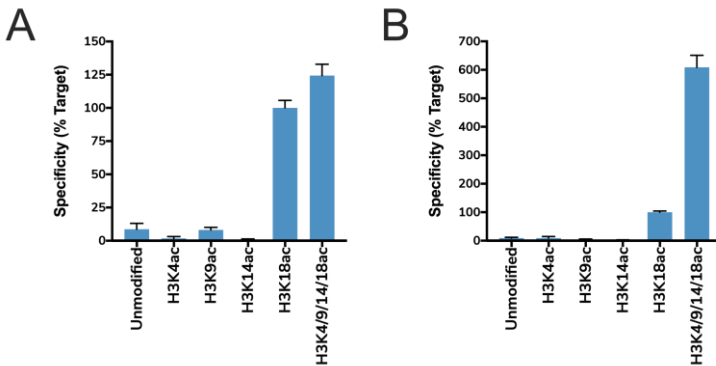


Figure 7. Antibodies can be affected by the presence of neighboring PTMs. (A) An antibody to H3K18ac exhibits similar recovery of the target PTM when present alone or in combination with nearby acetylations (tetraAc-H3: H3K4/9/14/18ac). (B) A different H3K18ac antibody is greatly influenced by the presence of nearby PTMs, as it exhibits >6-fold improved recovery of the target PTM for the tetraAc-H3 SNAP-ChIP nucleosome compared to H3K18ac alone.

- Extended acylation marks: Lysine acylation states have a high degree of structural similarity, and thus represent especially challenging antibody targets. The extended acylation states butyryl (bu) and crotonyl (cr) are included in the K-AcylStat panel at H3K9, H3K18 and H3K27. When designing a targeted panel to confirm antibody

specificity on qPCR before NGS, these marks should be taken into consideration (**Figure 8**).

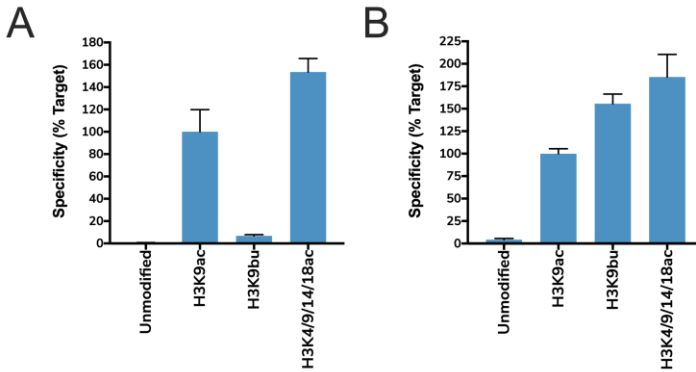


Figure 8. A SNAP-ChIP experiment using two commercially available H3K9ac antibodies. One of the antibodies has specific recovery of the target when alone as well as in combination with nearby acetylations (H3K4/9/14/18ac) (A). It does not cross-react with H3K9bu. In contrast, the other H3K9ac antibody prefers the butyryl mark over single acetylation at H3K9 (B).

- **Sequence Similarities:** Certain histone tail regions, such as H4K5 / H2AK5, and H3K9 / H3K27 (**Figure 9**), share similar amino acid flanking sequences, making these likely sources of antibody cross-reactivity that should be checked before proceeding to NGS.

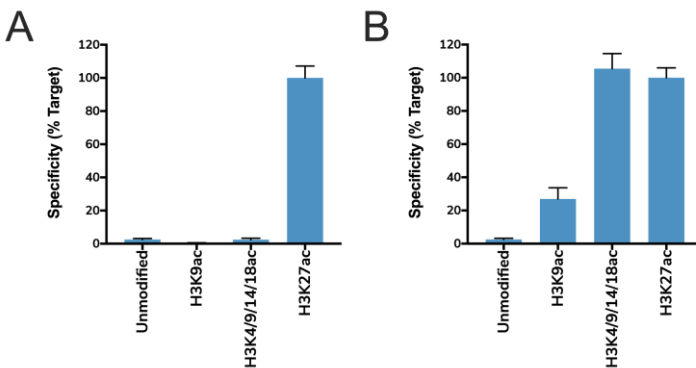


Figure 9. A SNAP-ChIP experiment using two commercially available H3K27ac antibodies. One antibody does not cross-react with H3K9ac despite the shared sequence similarity of the flanking amino acids (A). In contrast, the other H3K27ac antibody cross-reacts with H3K9ac, and its cross-reactivity is further amplified by the presence of nearby acetylations (H3K4/9/14/18ac) (B).

References

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- 2 Shah, R. N. et al. Examining the Roles of H3K4 Methylation States with Systematically Characterized Antibodies. *Molecular cell*, doi:10.1016/j.molcel.2018.08.015 (2018).
- 3 Lowary, P. T. & Widom, J. New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning. *Journal of molecular biology* 276, 19-42, doi:10.1006/jmbi.1997.1494 (1998).
- 4 Brand, M., Rampalli, S., Chaturvedi, C. P. & Dilworth, F. J. Analysis of epigenetic modifications of chromatin at specific gene loci by native chromatin immunoprecipitation of nucleosomes isolated using hydroxyapatite chromatography. *Nature protocols* 3, 398-409, doi:10.1038/nprot.2008.8 (2008).