SNAP-ChIP®

OncoStat™ Panel

Panel Version 1.0

EpiCypher™


EpiCypher™

SNAP-ChIP OncoStat Panel

Panel Version 1.0

Catalog No. 19-2001 (10 ChIP Reactions)
Catalog No. 19-2100 (100 ChIP Reactions)

Store at -20°C Upon Receipt

User Manual Version 1.0

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Description

SNAP-ChIP (**S**ample **N**ormalization and **A**ntibody **P**rofiling **Ch**romatin **I**mmuno**P**recipitation) uses DNA-barcoded designer nucleosomes (dNucs) bearing distinct post-translational modifications (PTMs) or histone mutations as next-generation spike-in ChIP controls. EpiCypher's SNAP-ChIP panels are directly compatible with your current ChIP workflow, with semi-synthetic nucleosomes bearing the PTM or mutation of interest immunoprecipitated and processed alongside sample chromatin (**Figure 1**). Recovery of the associated DNA barcodes can then be deciphered by quantitative PCR (ChIP-qPCR) or Next-Generation Sequencing (ChIP-seq). For the first time, users can directly monitor antibody capability (specificity and enrichment) in a ChIP experiment by measuring the recovery of on- and off-target nucleosomes. The SNAP spike-ins can be further used as a defined standard to normalize your ChIP data, thereby controlling for technical variability across experiments. These uses set SNAP-ChIP apart from any other spike-in controls currently available.

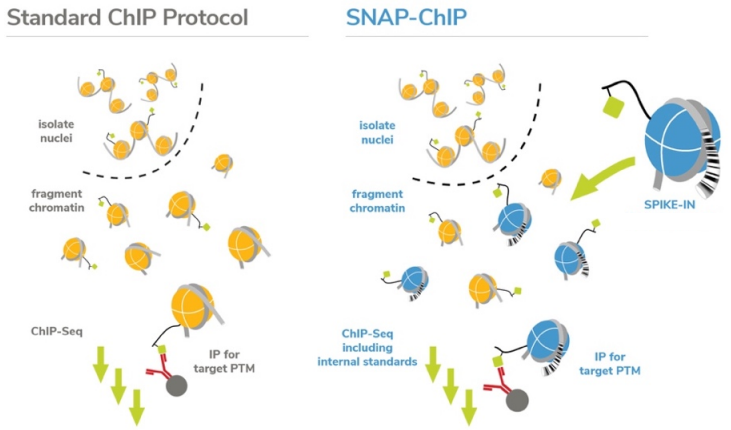

Figure 1. Overview of SNAP-ChIP approach (adapted from ICeChIP technology[1]). A pool of recombinant nucleosomes with defined PTMs or mutations identified by unique DNA barcodes is added to sample chromatin prior to immunoprecipitation (IP). Capture of the barcoded nucleosomes (on- and off-target) allows the user to assess antibody specificity / enrichment, monitor technical variability, and normalize experiments. Of note, the qualitative recovery of barcoded nucleosomes (monitored by qPCR) provides a useful STOP / GO capability prior to Next-Generation Sequencing (NGS).

EpiCypher's oncogenic mutation status panel, or "OncoStat" Panel, is the second product in our SNAP-ChIP line. The panel consists of a pool of nucleosomes harboring seven well-studied histone H3.3 mutations that have been implicated in cancer (histone H3.3K4M, H3.3K9M, H3.3K27M, H3.3G34R, H3.3G34V, H3.3G34W and H3.3K36M) plus a wild-type H3.3 control (**Figure 2**). Further, each of these eight nucleosomes is wrapped by two independent DNA barcodes (16 total barcodes in the panel), allowing for an internal technical replicate in each ChIP reaction. 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, supporting the generation of high quality ChIP data.

Of note, the OncoStat panel is designed to be fully compatible with EpiCypher's SNAP-ChIP K-MetStat™ Panel (Catalog #19-1001) in the same ChIP experiment. The K-MetStat Panel consists of a pool of nucleosomes carrying fifteen well-studied, disease relevant lysine methyl marks on histones H3 and H4 (mono-, di- and tri-methylated H3K4, H3K9, H3K27, H3K36, and H4K20) plus an unmodified control. Compatibility of the OncoStat and K-MetStat panels allows maximum user flexibility with experimental design.

Additional modification-specific dNuc panels (e.g. lysine acylation, arginine methylation, lysine ubiquitylation) are currently in development.

Figure 2. Schematic depicting the 7 oncogenic mutations plus wild type control included in the OncoStat Panel. Each nucleosome is wrapped by two unique DNA barcodes, providing an internal technical replicate for each mutation of interest.

SNAP-ChIP Advantages

- Homogenous, fully defined standards that faithfully represent target mononucleosomes in the experimental sample
- EpiCypher nucleosomes 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 in ChIP:
    - Panels contain a pool of related nucleosomes (PTMs or histone mutants) that allow users to determine antibody specificity (recovery of on- vs. off-target nucleosomes in the panel)
    - Determination of antibody enrichment (amount of target nucleosome immunoprecipitated relative to Input)
- Ability to monitor technical variability between samples
- Defined standards enable universal normalization across experiments
- 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 clone[2]; **Figure 3**). Embedded within the NPS are two 22 bp DNA "barcode" regions. The 5' Forward Barcode is the same for every nucleosome in both the OncoStat and K-MetStat Panels. However, the 3' Reverse Barcode is unique to each nucleosome in the two panels. Further, each recombinant nucleosome represented in the panels are 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 sequencing[1]. The full sequence of each barcoded DNA is available from the SNAP-ChIP product page (www.epicypher.com).

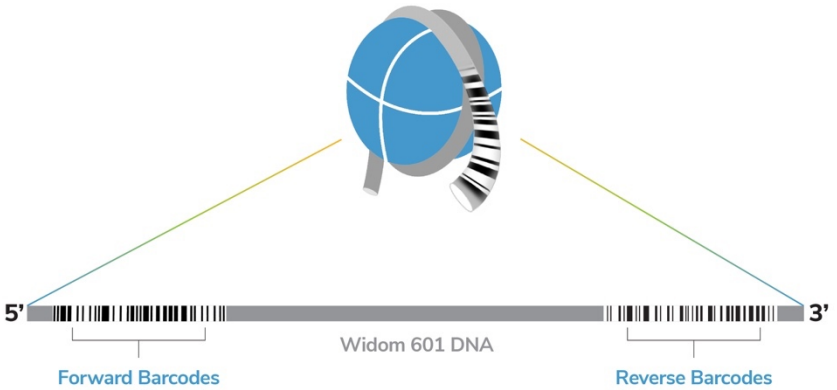

Figure 3. Schematic depicting the DNA barcoding scheme in each SNAP-ChIP panel. Two 22 bp barcode regions are embedded within the 147 bp nucleosome positioning sequence (gray). The Forward Barcode is shared by all nucleosomes in the K-MetStat and OncoStat panels, while the Reverse Barcode is unique to each nucleosome in the panels. Further, each nucleosome is wrapped by two distinct reverse barcodes, providing an internal technical replicate for each PTM or histone mutation of interest.

Materials Required but not Supplied

SNAP-ChIP OncoStat Panel contains enough reagent to perform 10 (Catalog #19-2001) or 100 (Catalog #19-2100) ChIP reactions (each from ~10^6 cell equivalents) in the user-preferred workflow.

ChIP-seq

- Standard ChIP and library preparation 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 user can adapt the protocol to alternate platforms.

- Standard ChIP reagents (user specific)
- qPCR Master Mix: e.g. Bio-Rad iTaq™ Universal Probes Supermix
- qPCR Plates: e.g. Bio-Rad HSP9601
- qPCR Plate Seals: e.g. Bio-Rad MSB1001
- qPCR Machine: e.g. Bio-Rad CFX Connect™
- qPCR Forward Primer (same for all nucleosomes in the OncoStat and K-MetStat Panels): e.g. IDT Technologies, Eurofins Scientific

  5' - CGT ATC GCG CGC ATA ATA - 3'
- qPCR Reverse Primers (unique to each nucleosome in the OncoStat and K-MetStat Panels): Primer sequences corresponding to each nucleosome can be downloaded directly from the product page at www.epicypher.com. Primers can be purchased from any third-party vendor (e.g. IDT Technologies, Eurofins Scientific).
- qPCR Probe (for TaqMan applications, common to all nucleosomes in all EpiCypher SNAP-ChIP Panels): order as a PrimeTime® qPCR Probe from IDT Technologies

  5' - 6-FAM™/TCT AGC ACC GCT TAA ACG CAC GTA/3' - Iowa Black® FQ

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**, Step 1, p.8).
2. After immunoprecipitation and DNA isolation, use qPCR to assess whether ChIP has successfully (and specifically) enriched the target of interest. Use this information to decide whether to invest in NGS (STOP/GO capability). For example, does your anti-H3.3K27M sufficiently distinguish mutant from wild-type nucleosomes?
3. NGS data will give further information regarding antibody specificity for all nucleosomes in the panel, identifying additional potential cross-reactivities. If all is as expected, NGS data can be corrected for technical variability by using recovery of the SNAP-ChIP target as a normalization standard.

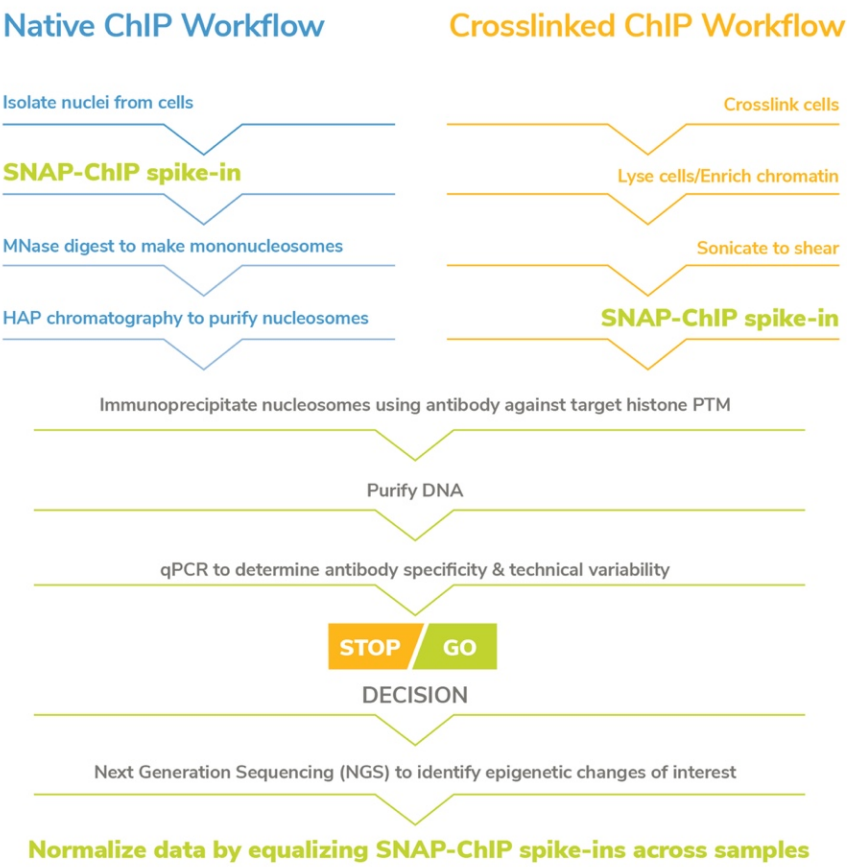

Figure 4. SNAP-ChIP is compatible with both native and crosslinked ChIP protocols, where a simple spike-in of the panel enables the assessment of antibody specificity and technical variability prior to investment in NGS (STOP/GO decision). SNAP-ChIP can also be used to normalize experimental data, improving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

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, users should evaluate compatibility with SNAP-ChIP.

2. Spike-in 2 µL SNAP-ChIP per 10 µg sample chromatin (~10^6 cell equivalents per ChIP). If more or less chromatin is used, scale the spike-in volume accordingly.

   NOTE: see Appendix 1 "Quantification of Sample Chromatin"

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

   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, 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 ΔΔCt 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.
- 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 context[3].
- 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**).

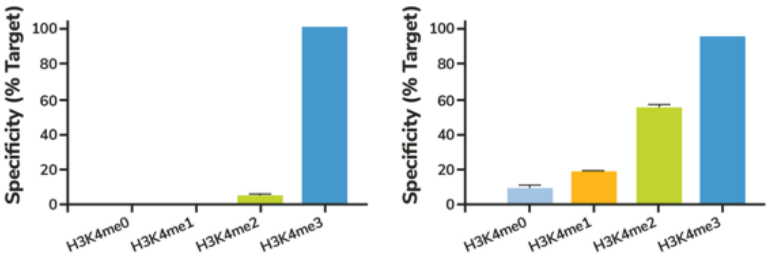

Figure 5. The importance of determining platform-specific antibody capability in ChIP is illustrated using EpiCypher's SNAP-ChIP K-MetStat Panel (Catalog #19-1001). A SNAP-ChIP experiment was conducted using two commercially available ChIP grade antibodies to trimethylation at histone H3, lysine 4 (H3K4me3). qPCR for barcodes corresponding to SNAP-ChIP nucleosomes bearing alternate H3K4 methyl states shows that **Antibody #1** (left) is highly specific for H3K4me3, exhibiting <3% cross-reactivity with H3K4me2. In contrast, **Antibody #2** (right) shows ~60% cross-reactivity with H3K4me2, 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 nucleosome compared to off-target nucleosomes in the SNAP-ChIP Panel.

   NOTE: It is recommended at minimum to check the ability of the antibody to immunoprecipitate the oncogenic mutation of interest without cross-reacting with wild-type nucleosomes.

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.

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

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

4. If proceeding to ChIP-seq, all barcodes will be captured in the NGS data. Align the read counts to the SNAP-ChIP barcodes (see product page at www.epicypher.com) to determine the enrichment (IP/Input) for each individual nucleosome in the panel.

Ct (Cycle Threshold) Mean	ΔCt	ΔΔCt	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 nucleosome immunoprecipitated by the ChIP antibody and "off-target" refers to any other nucleosome 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.

NOTE: The directions below describe normalization of SNAP-ChIP-qPCR experiments. For normalization of NGS data, see published bioinformatic analysis methods[1]. For more information, inquire at info@epicypher.com.

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) 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 nucleosomes contributes sample data which cannot be accounted in the normalization. As an example: the biological perturbation under study differentially impacts the antibody on- (H3.3G34R) and off-target (H3.3 WT) 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 H3.3G34R ChIP experiment, use the barcodes corresponding to the H3.3G34R nucleosome in the SNAP-ChIP panel).

   NOTE: See the % Enrichment calculation (p.10) to calculate the % Input for ChIP-qPCR.

5. Apply the formula:

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

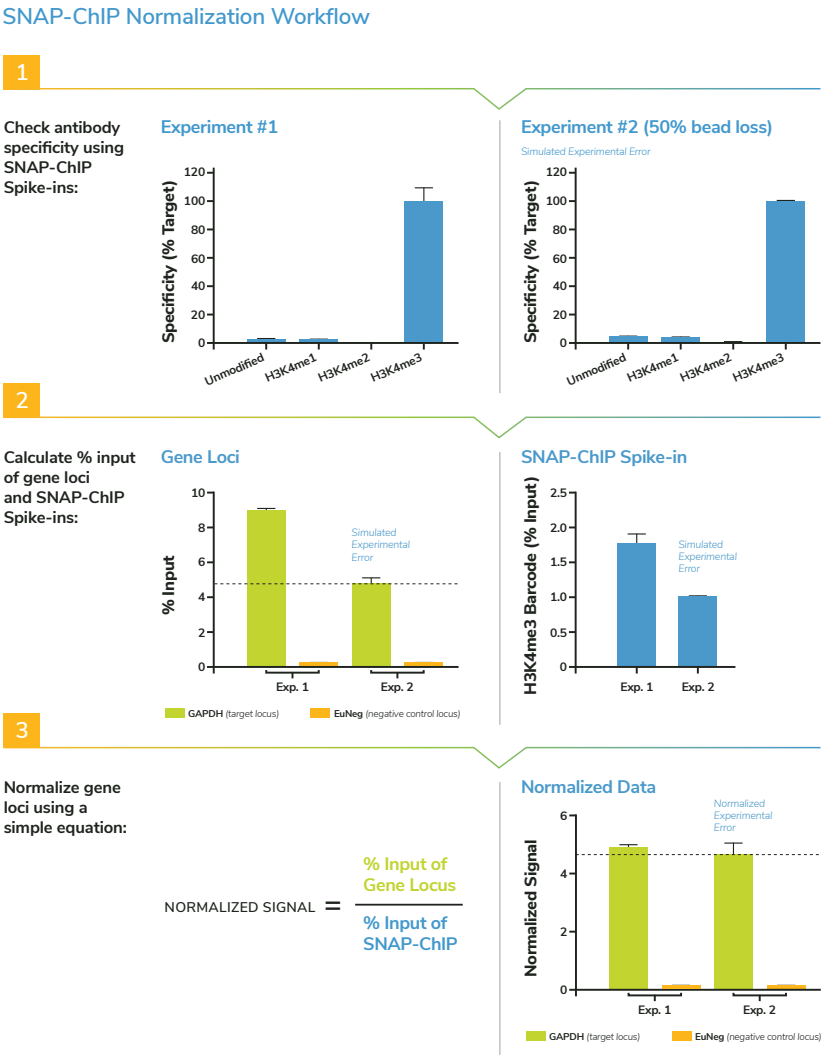

Figure 6. Overview of data normalization using the SNAP-ChIP approach. Technical variability was simulated by the loss of bead-antibody conjugates (Experiment #2). (**1**) A check of antibody specificity using the SNAP-ChIP K-MetStat spike-ins (critical before proceeding to normalization) confirmed that the antibody enriched for the intended target (H3K4me3) but not related species (H3K4me0, H3K4me1, or H3K4me2). (**2**) Examination of genomic loci (GAPDH and euchromatin negative control region, EuNeg: left panel) revealed a difference between two experiments that could indicate biological perturbation. However, the SNAP-ChIP spike-in (right panel) also shows this discrepancy, indicating that the observed difference is due to technical variability in the ChIP procedure. (**3**) A simple equation can be applied to resolve this issue and elucidate true biological change(s).

Appendix 1: Quantification of sample chromatin

NOTE: The guidelines below are for UV absorbance measurement of DNA. When working with small numbers of cells (<10^6) 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 (OD_{260}).

   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:

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

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, 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 (OD_{260})
5. Use molar extinction coefficient for DNA to calculate concentration:

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

Appendix 2: Frequently Asked Questions

- **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.

- **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 more sticky and 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 yield 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 nucleosome 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 mononucleosomes[1] 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 or oncogenic mutation antibodies?**

  EpiCypher and our collaborators have performed extensive antibody specificity studies for lysine methylation, lysine acetylation, and oncogenic mutation status, with additional studies ongoing. For more information, inquire at info@epicypher.com.

References

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