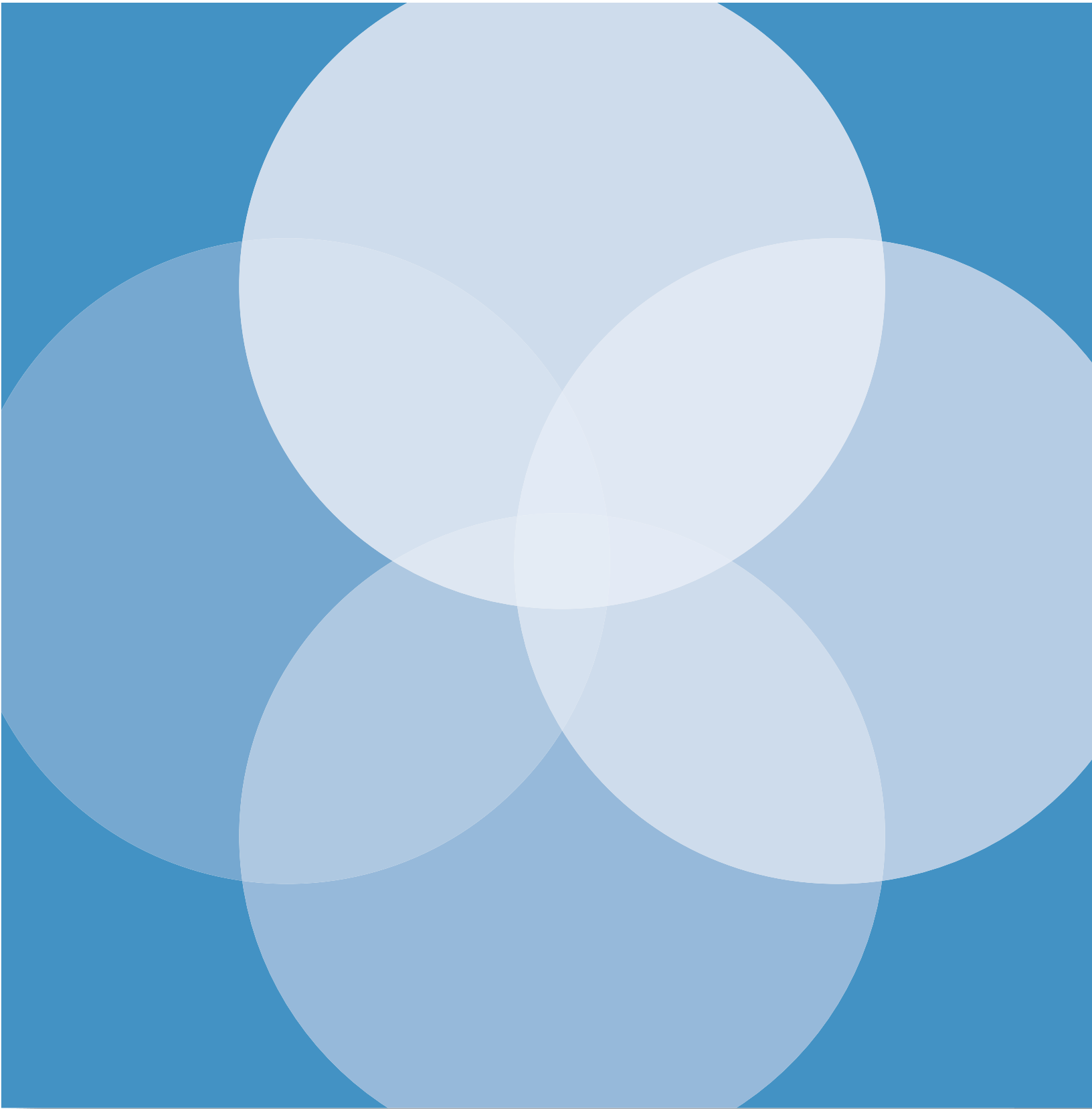
EpiCypher

Anatomy of a Recombinant Nucleosome

An EpiCypher White Paper



Anatomy of a Recombinant Nucleosome

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Nucleosome substrates are emerging tools for the *in vitro* study of chromatin and epigenetic regulation, with applications from basic research to high-throughput drug screening. The advent of recombinant designer nucleosomes (dNucs) carrying fully defined covalent modifications of the histone proteins (e.g. lysine acylation) or wrapping DNA (e.g. 5' methylcytosine) enables novel or dramatically improved approaches (e.g. reader binding, enzymatic assays, antibody profiling) for next-generation chromatin research. This document proposes the minimal thresholds for the assembly and quality validation of these reagents; essential if they are to yield reproducible, biologically-relevant data.

Conclusion

We define key quality metrics for each step towards recombinant nucleosome assembly, including modified histone synthesis and purification, octamer assembly and purification, and nucleosome assembly. We consider the criteria described herein to represent the minimal requirement to validate these reagents for use in chromatin research and drug discovery applications.

Recombinant nucleosomes as physiological substrates for drug discovery and development

EpiCypher has pioneered the commercial development of recombinant nucleosomes for next generation epigenetic assays and tool development (see **Table 1** for a list of our nucleosome based products). Mounting evidence demonstrates that nucleosomes are the optimal substrates to characterize many chromatin regulators [1-4]. For example, NSD2 methyltransferase (associated with oncogenic reprogramming in multiple myeloma [5, 6]) requires nucleosomal substrates for in vitro activity [7]. Similarly, recent work with SETD8 methyltransferase demonstrated a 10,000-fold decrease in Km of SAM cofactor and IC50 of SAH or sinefungin inhibitors when recombinant nucleosomes (from EpiCypher) were used in place of peptide substrates (**Box 1**) [8].

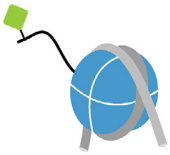
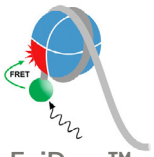
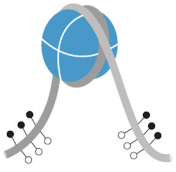
	Characteristics	Advantages
 dNucs™	**Designer Nucleosomes** • Fully recombinant human histones • Contain physiological histone PTMs • 601 nucleosome positioning sequence (biotinylated)	• Stably positioned nucleosome • Suitable for enzyme assays and high-through put screening (modification addition or removal) • Suitable for protein-protein interaction studies involving the modification of interest
 rNucs™	**Recombinant Nucleosomes** • Fully recombinant human histones • 601 nucleosome positioning sequence (available in biotinylated and non-biotinylated forms)	• Devoid of post-translational modifications • Stably positioned nucleosome • Suitable for enzyme assays, inhibitor testing and high-throughput screening (modification addition)
 oncoNucs™	**Oncogenic Nucleosomes** • Fully recombinant human histones • Contains specific K-to-M mutations associated with cancer • 601 nucleosome positioning sequence (biotinylated)	• Study effects of mutations on enzyme activity • Suitable for high-throughput screening and inhibitor testing • Can be used for structural studies
 vNucs™	**Histone Variant Nucleosomes** • Fully recombinant human histones • Includes one of several histone variants • 601 nucleosome positioning sequence (biotinylated)	• Study effects of mutations on enzyme activity • Suitable for high-throughput screening and inhibitor testing • Can be used for structural studies
 EpiDyne™	**Chromatin Remodeling Assay Substrate** • Fully recombinant human histones • Nucleosome positioning sequence with an added nucleosome acceptor sequence • Functionalized DNA or histones to enable HTS assay development	• Suitable for high-throughput screening and inhibitor testing • Stably positioned nucleosome • Can be used for structural studies
 Methyl DNA	**Methyl DNA Designer Nucleosomes** • Fully recombinant human histones • Contain physiological DNA modifications • 601 nucleosome positioning sequence (biotinylated)	• Stably positioned nucleosome • Suitable for enzyme assays and high-throughput screening (modification addition or removal) • Suitable for protein-DNA crosstalk interaction

TABLE 1

List of recombinant nucleosome products available from EpiCypher.

Nucleosomes carrying defined covalent modifications (termed designer nucleosomes or 'dNucs') also provide excellent substrates for a range of reader and eraser assays. For instance, binding of the BRD4 tandem bromodomain is significantly increased when lysine residues on both histone H3 and H4 are acetylated [4], while LSD1 demethylase and the SIRTUIN family of histone deacetylases require acetylated nucleosomes to mimic *in vivo* target selectivity [9-13].

In addition to assays that examine the addition, recognition or removal of histone modifications, recombinant nucleosomes can also be made compatible with chromatin remodeling assays (see EpiDyne™ product family in **Table 1**). Of note, the SWI/SNF family of chromatin remodeling complexes are emerging as high value therapeutic targets, with research indicating the need for nucleosome substrates to accurately interrogate the ATPase-dependent function of these enzymes.

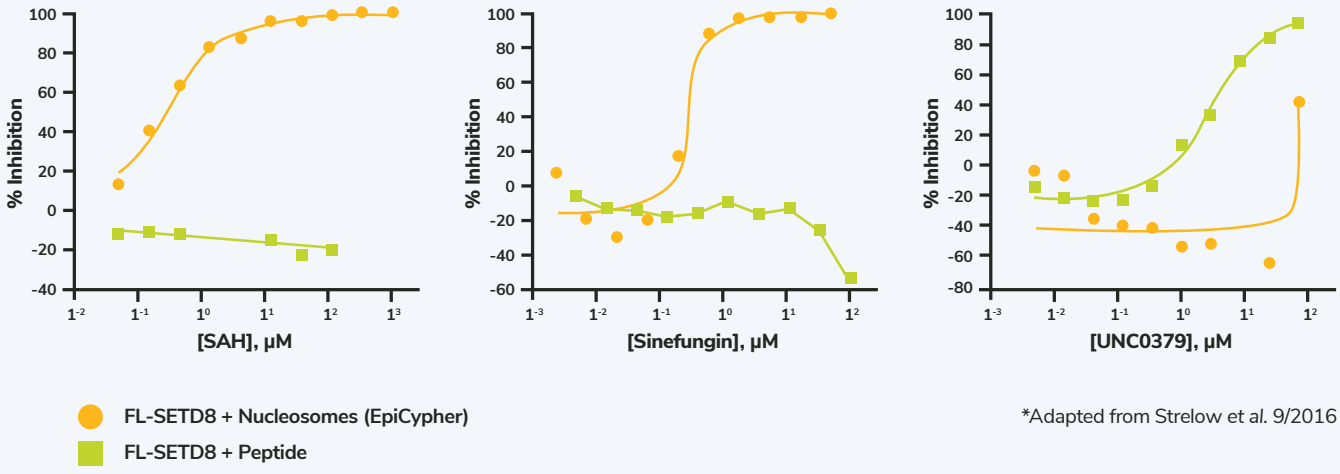
SETD8 is a histone targeting methyltransferase strongly associated with various cancers

- SETD8 is the sole histone monomethyltransferase that targets histone H4 at lysine 20 (H4K20).
- SETD8 is overexpressed in many cancer tissues, including bladder, lung, leukemia and pancreatic.

The use of nucleosome substrates improves binding of SAM analogs to SETD8 [8]

- Km of the SAM cofactor is 10,000-fold lower when SETD8 targets nucleosomes vs. synthetic peptides or recombinant free histones.
- SETD8 inhibitors developed using histone peptide substrates fail to target the enzyme in contact with nucleosomes (see UNC0379 below), demonstrating the criticality of physiological substrates for drug discovery and development.

Inhibition profile of three inhibitors of SETD8 (full-length) using either a peptide substrate or recombinant nucleosomes



*Adapted from Strelow *et al.* 9/2016

BOX 1

UNC0379 fails to inhibit SETD8 on nucleosomes. Profiling three inhibitors of full-length SETD8 using peptide or recombinant nucleosome substrates (adapted from Strelow et al. 2016).

A Biochemical View

As the commercial development of recombinant nucleosomes becomes standardized, it is critical to establish key quality metrics to define these reagents. A nucleosome is comprised of two distinct elements: the histone octamer (two copies of each histone protein; H2A, H2B, H3 and H4) and associated DNA (≥147bp). Variation or heterogeneity (in identity or quality) can be introduced by the individual components (e.g. distinct designer or variant histones) or the (in)efficiency of octamer / nucleosome assembly. **Table 2** lists the key quality criteria we have established for histone preparation through octamer and nucleosome assembly. **Figure 1** shows the essential QC elements to ensure the delivery of a fully fit-for-purpose designer nucleosome.

ASSEMBLY PROCESS	VALIDATION METHOD	METRIC	NOTES
Histone purification	Coomassie	> 95% pure	Purification tags can affect downstream applications
Modified histone (dNuc)	Analytical HPLC	> 95% pure	Unnatural PTM linkages can affect downstream applications
	HRMS	Single peak; +/- 1 Dalton expected mass	Essential secondary validation to ensure PTM was incorporated properly
Octamer assembly	Coomassie	Equal stoichiometric ratio	Changes in ratio indicate that assembly reaction was suboptimal
Nucleosome assembly	Native PAGE	No free DNA; minimal higher order species	Free DNA can alter enzyme activity / specificity; Higher order species indicate heterogeneous positioning of octamer on DNA template
	Immunoblot	PTM specific signal	Demonstrates that PTM is incorporated in the correct location on the histone

TABLE 2

Quality metrics for recombinant (and designer) nucleosome assembly. HRMS = high-resolution ass spectrometry; PTM = Post-translational modification; dNuc = designer nucleosome.

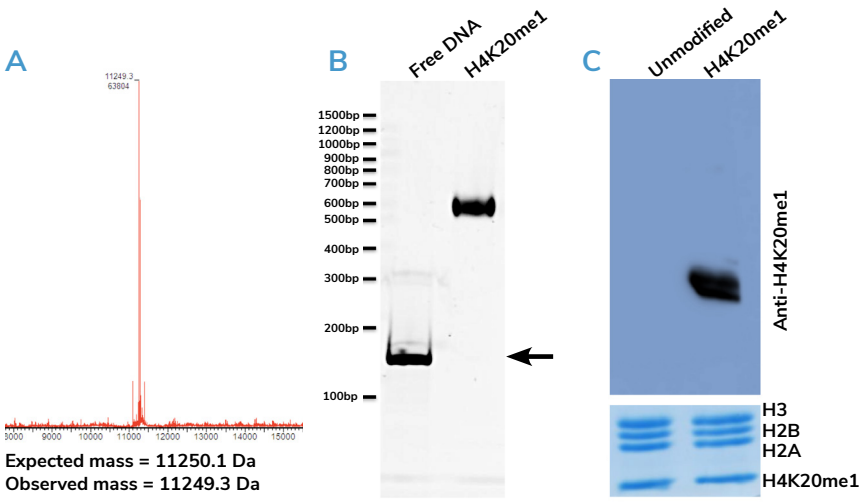


FIGURE 1

Representative QC data for designer nucleosome manufacturing (e.g. H4K20me1) (A) High resolution mass spectometry of H4K20me1 free histone. Expected mass 11,250.1 Da / Determined 11,249.3 Da. (B) Free DNA (147bp 601 Widom Nucleosome Positioning Sequence; Catalog No.: 18-0005) and H4K20me1 dNucs (Catalog No.: 16-0331) were resolved by native PAGE (note absence of free DNA in the latter indicating efficient assembly). Arrow indicates lack of free DNA in final nucleosome preparation. (C) Recombinant nucleosome (unmodified; Catalog No.: 16-0006) and H4K20me1 dNuc were denatured, resolved by SDS-PAGE and immunoblotted with anti-H4K20me1 to demonstrate the appropriate reactivity (Top); or Coomassie stained to monitor histone stoichiometry (Bottom).

Recombinant nucleosome assembly: 1. Histone Preparation

Histone proteins can be readily expressed and purified from *E. coli* as recombinants. Since bacteria lack most post-translational modification capabilities, the resulting purified histones are free from preexisting background PTMs, providing a homogenous and highly defined product. Recombinant histones should be >95% pure by Coomassie staining (*i.e.* no contaminating proteins detected) prior to octamer assembly. For maximum flexibility in downstream assays, histones should also be free from any affinity tags for purification (terminal extensions have been shown to disrupt the activity of some chromatin-targeting enzymes [14]).

We have further developed and validated processes to produce highly pure (>95% by analytical HPLC) designer histone proteins carrying unique PTMs, which are used for dNuc assembly. It is important to ensure that the desired PTMs are incorporated using scar-less methodologies that recapitulate the native histone primary structure. Unnatural PTM analogs (such as MLAs [15]) are undesirable, since these have been shown to disrupt interactions with chromatin regulating proteins and PTM-specific antibodies (see Cowles et al., EpiCypher White Paper, 2016; [16-18]).

Rigorous quality control of modified histones (*i.e.* the starting material) is the key first step in the assembly of fully-defined and homogenous dNucs. All EpiCypher modified histones are validated by analytical HPLC and high resolution mass spectrometry (HRMS). HPLC traces should show a single eluting species, indicating > 95% histone purity (e.g. **Figure 2A**). Parallel HRMS should exhibit a single peak within 1 dalton of expected mass, with no additional mass to charge (m/z) signals of any significance (e.g. **Figure 2B**). **Figure 2C** shows a modified histone that failed QC: this sample exhibited high HPLC purity but poor HRMS peak purity, indicating product contamination and demonstrating the importance of secondary validation. Undesirable species (e.g. methionine oxidation as in **Figure 2C**) could induce structural alterations, and impact electrostatic or hydrophobic interactions, thus compromising the efficiency of nucleosome assembly.

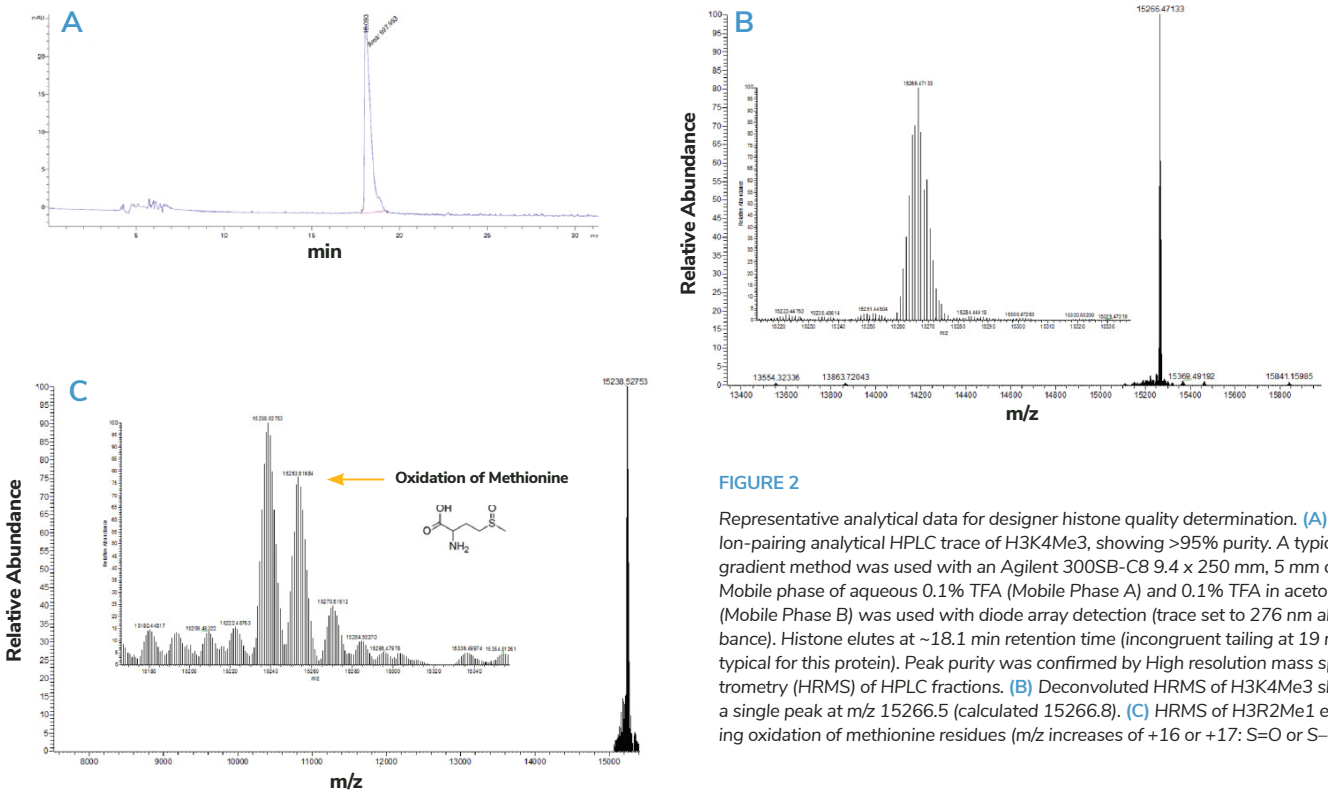


FIGURE 2

Representative analytical data for designer histone quality determination. (A) *Ion-pairing analytical HPLC trace of H3K4Me3, showing >95% purity. A typical gradient method was used with an Agilent 300SB-C8 9.4 x 250 mm, 5 mm column: Mobile phase of aqueous 0.1% TFA (Mobile Phase A) and 0.1% TFA in acetonitrile (Mobile Phase B) was used with diode array detection (trace set to 276 nm absorbance). Histone elutes at ~18.1 min retention time (incongruent tailing at 19 min is typical for this protein). Peak purity was confirmed by High resolution mass spectrometry (HRMS) of HPLC fractions.* (B) *Deconvoluted HRMS of H3K4Me3 showing a single peak at m/z 15266.5 (calculated 15266.8).* (C) *HRMS of H3R2Me1 exhibiting oxidation of methionine residues (m/z increases of +16 or +17: S=O or S–OH).*

2. Octamer Assembly

QC validated histones are assembled into octamers, which are further purified by FPLC and analyzed by SDS-PAGE / Coomassie staining to ensure subunit integrity and stoichiometry (**Figure 3**; lanes 1-2). Contamination with additional protein species or deviation from a 1:1:1:1 ratio could indicate histone degradation or poor assembly, so it is imperative to adequately resolve each histone. Sample overloading (**Figure 3**; lane 3) is a common issue that undermines data analysis, and is thus to be avoided. Of note, changes to histone composition (e.g. point mutations or PTM addition) can impact protein mobility in SDS-PAGE and must be determined empirically and accommodated. As an example the disease-associated lysine to methionine mutations [19] in our oncoNuc product line (**Table 1**) induce a mobility shift sufficient to compress H3K4M into H2B (**Figure 3**; Lane 4).

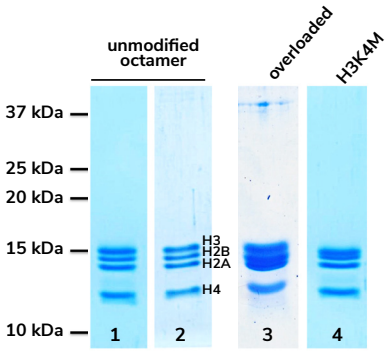


FIGURE 3

Examination of histone proteins in a purified octamer by SDS-PAGE. Histone octamers (2 µg of indicated sample per lane) were resolved by 15% SDS-PAGE (30% acrylamide : 0.8% bis acrylamide (w/v)) and visualized with Coomassie Brilliant Blue G-250. Lanes 1 and 2 illustrate properly resolved histone octamers in which all species can be assigned. Lane 3 illustrates overloaded octamers, with compressed, unresolved histone species. Certain histone mutations (e.g. K-to-M) can induce mobility shift: e.g. the compression of H3K4M into H2B in Lane 4.

3. Nucleosome Assembly

Recombinant nucleosomes can be assembled using natural or artificial nucleosome positioning sequences (NPS) that favor assembly without the assistance of histone chaperones or ATPases. Such sequences could include Xenopus 5S, MMTV, human NCP-TA2 or the synthetically derived Widom 601 [20]. The DNA template (nucleotide length, chemical modification, etc.) can be varied to accommodate various downstream applications. At EpiCypher, most nucleosomes are assembled using 147 bp Widom 601 DNA [+/- biotin], though longer sequences can support specific applications, such as chromatin remodeling assays (EpiDyne™). Additionally, altering the length of linker DNA can dramatically alter the binding and / or enzymatic activity of some chromatin regulators [21].

The quality validation of nucleosomes following DNA assembly is essential. Contaminating free DNA can induce an aberrant activity in chromatin modifying enzymes (e.g. NSD2) and is thus to be avoided. Moreover, suboptimal assembly can result in a heterogeneous mix including mispositioned nucleosome species). One approach to monitor such events is native PAGE, where an efficient assembly using ~150bp DNA should yield only a single species (given the limited positioning options available) with reduced mobility relative to unassembled free DNA (**Figure 4A**). Assembling octamers onto longer DNA templates gives some inherent freedom for nucleosome positioning, even if using a strong NPS located centrally or terminally [22].

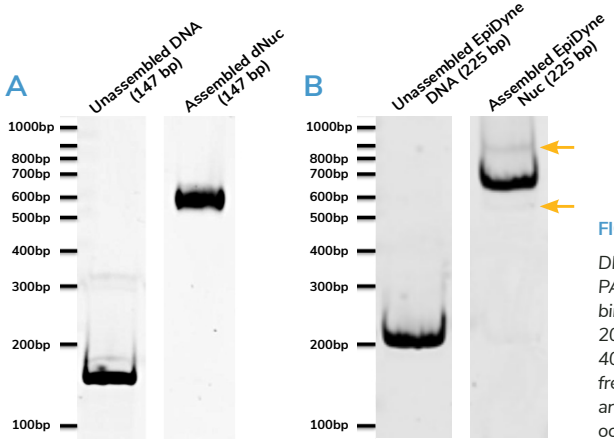


FIGURE 4

DNA shift from Free DNA to fully assembled Nucleosomes. (A-B) 6% Native PAGE was used to examine the shift from free DNA to fully assembled recombinant dNuc or EpiDyne nucleosome. The first lane in A and B was loaded with 200ng of DNA that was used in a subsequent nucleosome assembly. In lane 2, 400ng of the resulting nucleosomes were loaded onto the gel. No contaminating free DNA is observed, indicative of a quality nucleosome assembly. Yellow arrows denote additional nucleosome species, likely caused by heterogeneous octamer assembly on the nucleosome positioning sequence DNA.

3. Nucleosome Assembly *continued*

As an example, EpiDyne-FRET™ (catalog no. 16-4201) represents a terminally positioned nucleosome on a > 200 base pair DNA sequence, and several assembly subspecies are thus possible (**Figure 4B**). Products with a terminally positioned nucleosome will migrate more quickly than those centrally positioned: the mobility difference is thought to be due to the relative amounts of contiguous free-DNA. Multiple higher order species indicate a heterogeneous assembly, which could lead to unpredictable (and thus unacceptable) variability between production lots. This is a particular concern for EpiDyne-FRET, where an appropriately positioned mononucleosome completes a FRET pair, leading to emission of light at 647nm when Cy3 is stimulated (**Figure 5**). Free DNA or heterogeneous nucleosome positioning leads to assay interference and is thus avoided in all production lots (**Figure 5**). Alternate remodeling readouts (such as FP, AlphaLISA™and TR-FRET) also benefit from a homogenous nucleosome pool. In each case, controlling the nucleosome positioning minimizes potential batch-to-batch variation which maximizes production of lots for large-scale screening.

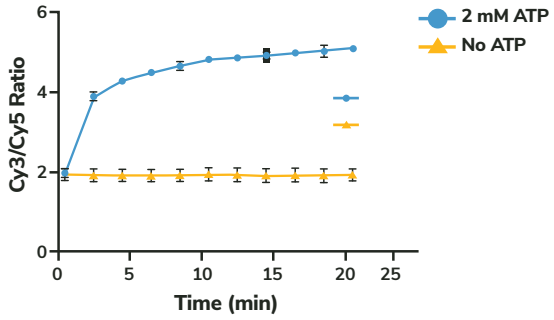


FIGURE 5

RSC/ATP-dependent nucleosome remodeling reaction. EpiDyne-FRET nucleosomes (20 nM) were incubated with RSC chromatin remodeler (10 nM) in the presence or absence of 2 mM ATP (conditions previously determined as optimal for this enzyme: data not shown). Upon ATP addition, reactions were read in an Envision Multi-label plate reader at times indicated. Data is presented as the mean±SD of the Cy3/Cy5 ratio (N=6) [see Note (iv)].

Conclusion

Recombinant designer nucleosomes are an emerging technology in the field of epigenetic study and targeted drug discovery. They provide access to novel therapeutic targets resistant to DNA or histone peptide-based approaches. As these higher reagents become more readily available, standardized validation criteria are critical if results are to be accurate and reproducible. Toward this goal, we have developed a series of quality metrics for each step towards recombinant nucleosome assembly, including modified histone synthesis, histone purification, octamer assembly and purification, and nucleosome assembly. We consider these criteria to represent the minimal requirement to validate these reagents for use in chromatin research and drug discovery applications.

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