

Supplemental Information for:

Separation and Characterization of Endogenous Nucleosomes by Native Capillary Zone Electrophoresis – Top-Down Mass Spectrometry (nCZE-TDMS)

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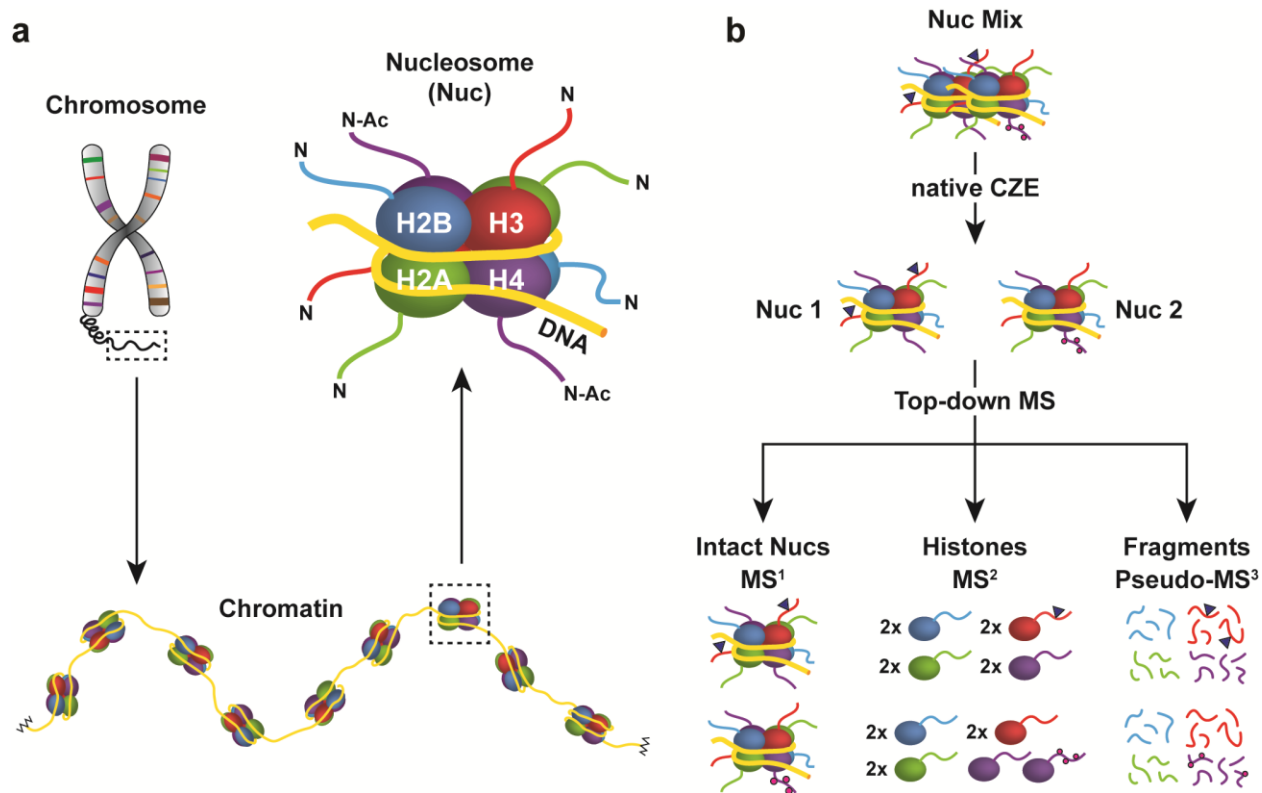


Figure S1: (a) Scheme of chromosome, chromatin, and nucleosome (Nuc). Chromosomes are macrostructures based on assemblages of chromatin, a chain repeating DNA : protein biopolymer. The Nuc, the smallest repeating unit, consists of ~147 bp DNA wrapped around two copies each of the core histones: H2A, H2B, H3, and H4. (b) Workflow of nCZE-TDMS system: Separation of different intact Nucs (e.g. proteoforms) in the nCZE dimension. TDMS analysis then follows in three steps: intact Nucs (MS¹), histone subunits (MS²), and peptide fragments (pseudo-MS³).

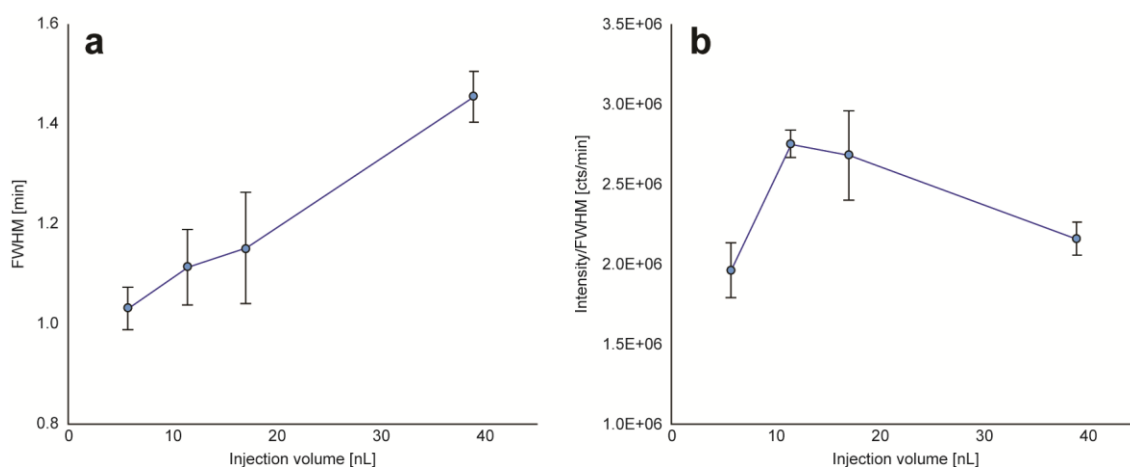


Figure S2. Optimization of injection volume. Different volumes of H3G34V ($c = 0.75 \mu\text{M}$, $n = 3$), ranging from ~ 5.7 to 38.0 nL (2.5 psi, 15 to 100 sec) were injected, which corresponds to roughly 0.9 to 6.0% of the total capillary volume. (a) Full-width half maximum (FWHM) was plotted against injection volume. (b) Ratio between intensity and FWHM was plotted against injection volume.

Optimization of injection volume: As would be expected, CZE peak widths increase proportional with the applied injection volume (**Figure S2a**). One appropriate measure to evaluate peak shape is to look at the ratio between peak intensity and full width half maximum (FWHM). The highest ratio was observed at 11.4 nL injection volume (**Figure S2b**). In addition, there was no significant improvement of absolute intensity observed for higher injection volumes. For this reason, 11.4 nL (2.5 psi, 30 sec) was selected for further experiments.

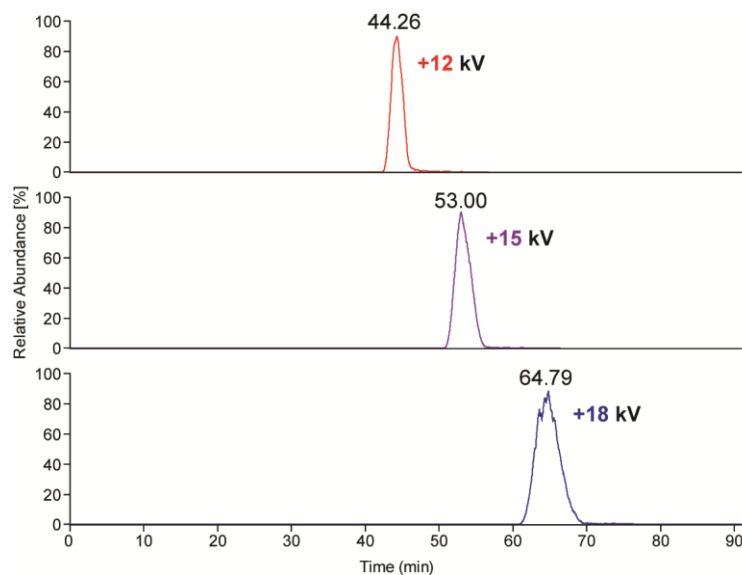


Figure S3. Evaluation of influence of separation voltage on nucleosome migration. H2BK120ub ($c = 500$ nM) was analyzed at three different voltage settings: +12, +15, and +18 kV. The other method parameter settings were kept constant, including 40 mM AmAc ($\text{pH} \approx 6.8$) as BGE and a supplemental pressure of 2.5 psi. The systematic increase in migration time by changing the HV from +12 to +18 kV, while maintaining other parameters, is a strong indication that net charge of the nucleosome complexes is negative under these conditions.

native CZE	
N	S G R G K Q G G K A R A K A K T R A S R A G L Q F 25
26	P V G R V H R L L R K G N Y S E R V G A G A P V Y 50
51	L A A V L E Y L T A E I L E L A G N A A R D N K K 75
76	T R I I P R H L Q L A I R N D E E L N K L L G K V 100
101	T I A Q G G V L P N I Q A V L L P K K T E S H H K 125
126	A K G K C

native DI	
N	S G R G K Q G G K A R A K A K T R A S R A G L Q F 25
26	P V G R V H R L L R K G N Y S E R V G A G A P V Y 50
51	L A A V L E Y L T A E I L E L A G N A A R D N K K 75
76	T R I I P R H L Q L A I R N D E E L N K L L G K V 100
101	T I A Q G G V L P N I Q A V L L P K K T E S H H K 125
126	A K G K C

Figure S4. Comparison of fragmentation performance between native CZE and native direct infusion (DI) for the analysis of histone H2A after ejection from H3K27me3 nucleosomes by pseudo MS³ TD experiments. The same number of mass spectra were averaged for each technique for comparison. Fragmentation profiles and coverage were determined to be similar between these two approaches and most fragments were picked up by both techniques. This indicates no significant loss of fragmentation quality due to the front end CZE separation.

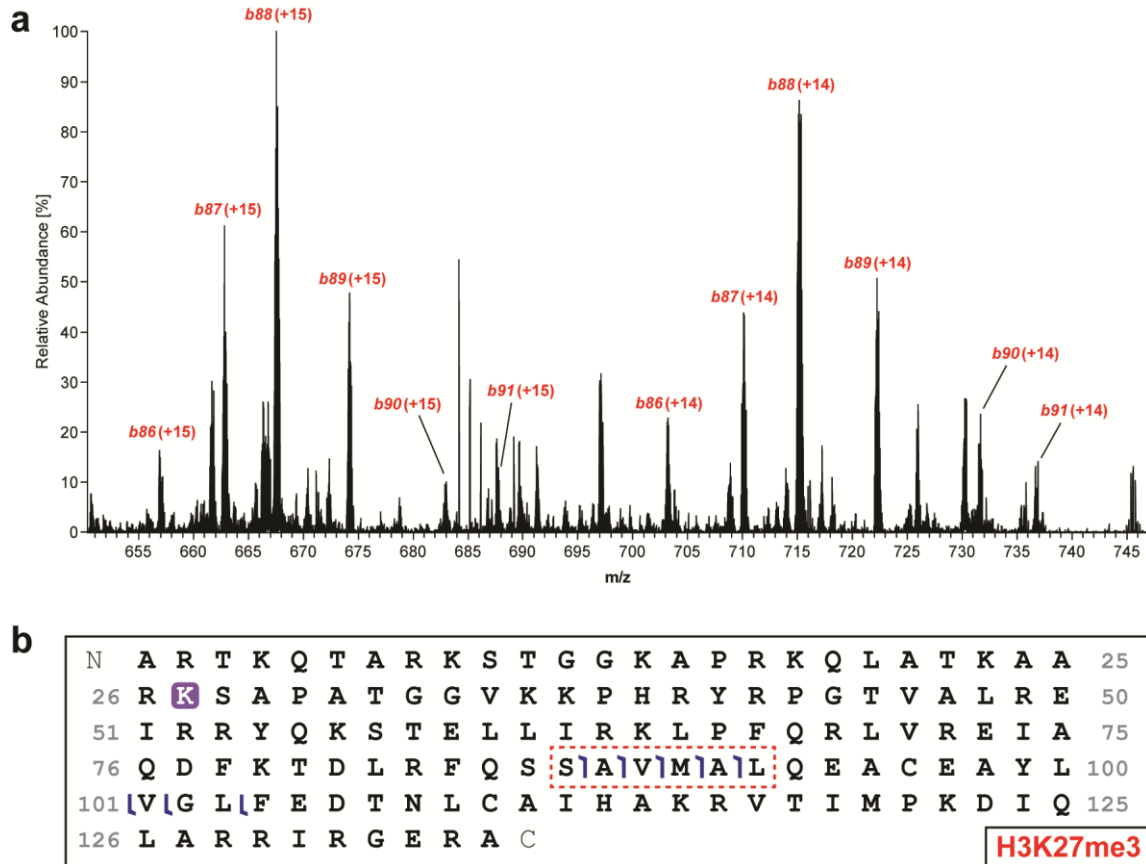


Figure S5. (a) Highlighted section of pseudo-MS³ mass spectrum showing diagnostic trimethylated *b*-ions after ejection and fragmentation (HCD) of histone H3K27me₃ from intact Nuc (*c* = 1 μM). Predominantly, +14 and +15 charge states were observed for these fragment ions. (b) Graphical fragment maps of peptides observed from H3K27me₃. Detected *b*-ions exhibiting a mass shift matching a tri-methylation are highlighted (red, dashed box).

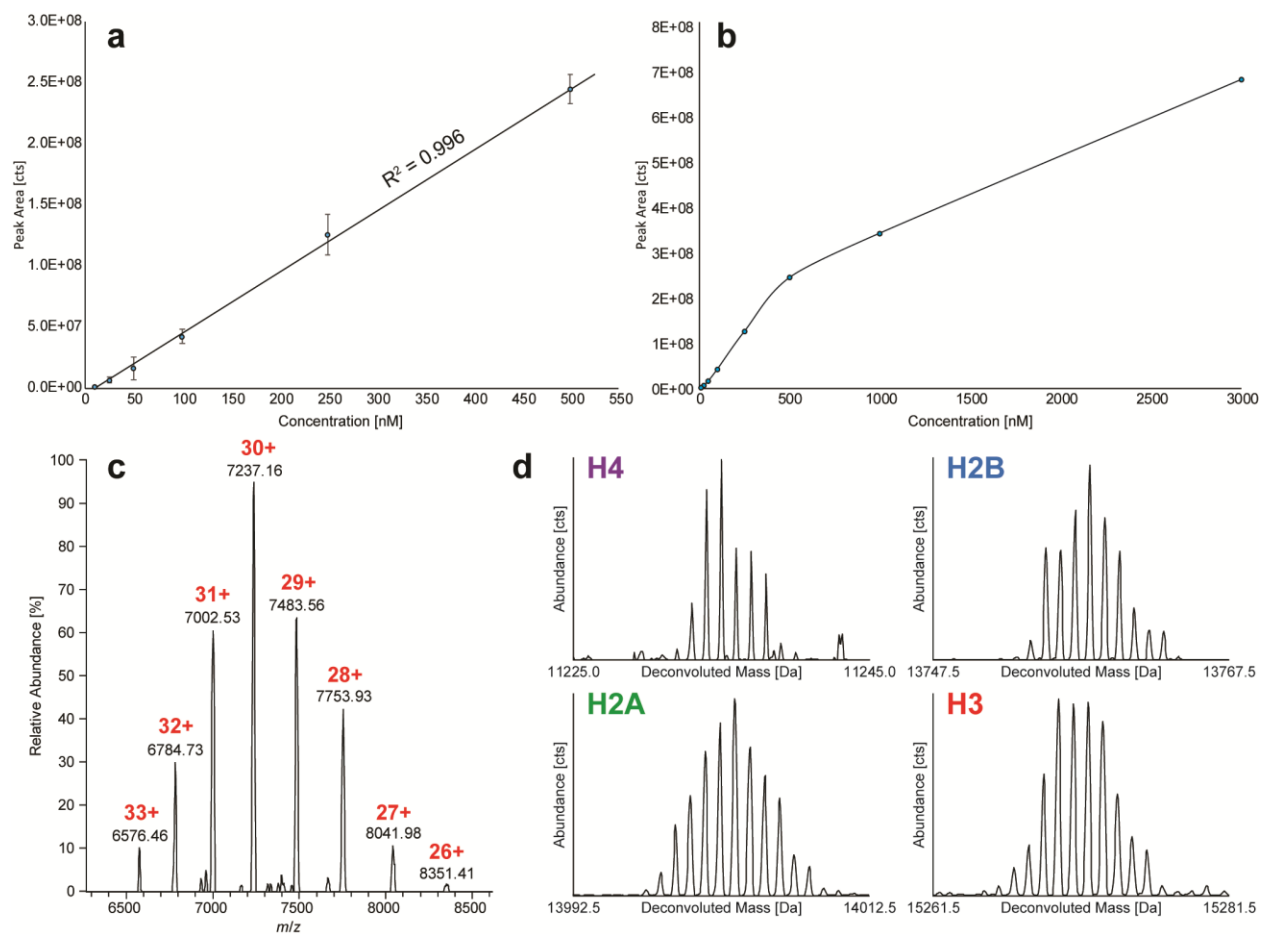


Figure S6: Regression and determination of LOD: **(a)** Linear range was determined as 10 to 500 nM (110 amol to 5.7 fmol) with $R^2 = 0.996$ and **(b)** dynamic range was observed between 10 to 3000 nM. **(c)** Mass spectrum of intact nucleosome at lowest analyzed concentration (10 nM) close to LOD with an average S/N of 12.3 ± 1.0 . **(d)** Deconvoluted isotopic distributions for histones ejected from unmodified recombinant Nucs measured at 62.5 nM.

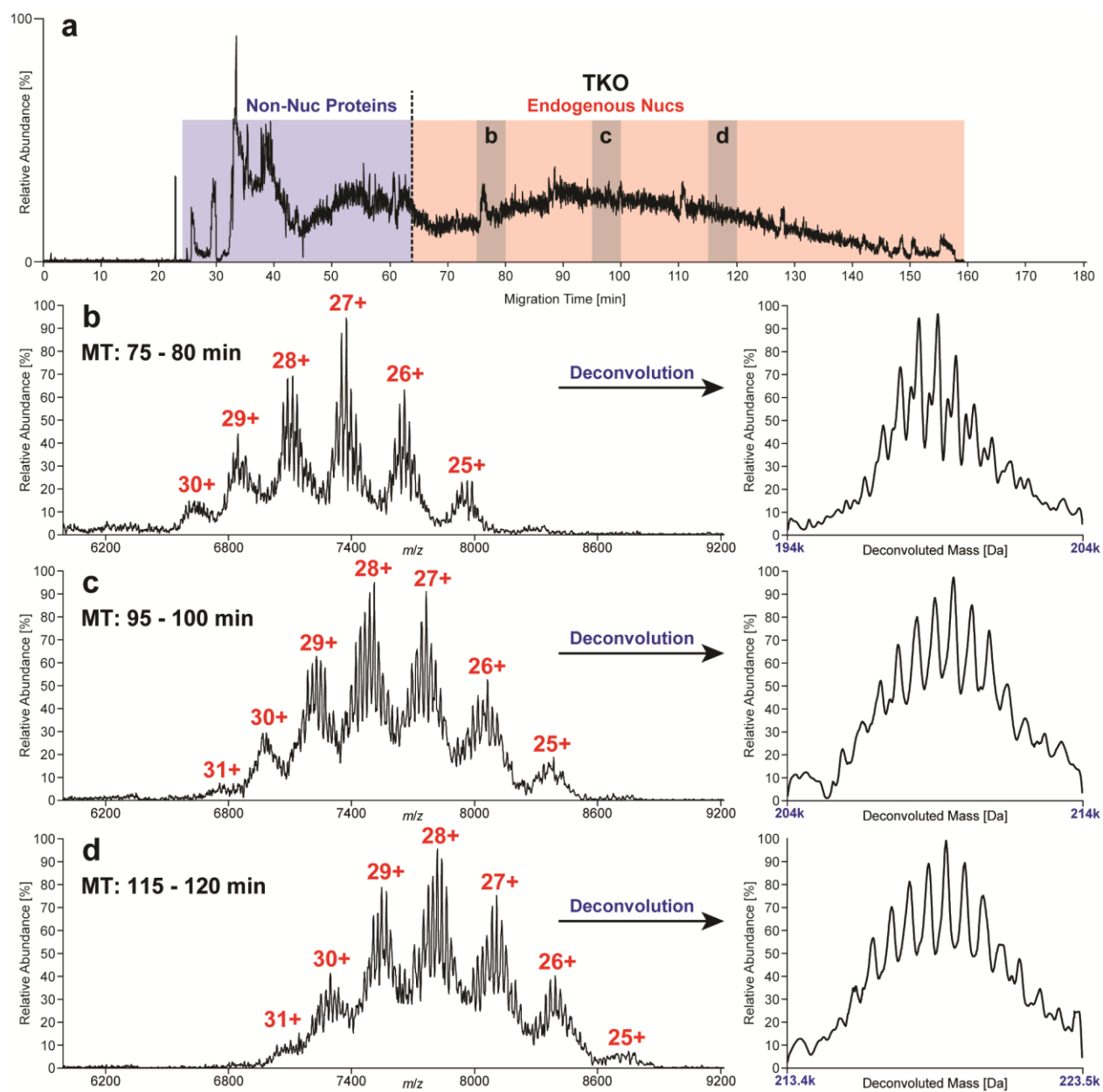


Figure S7. Separation of endogenous nucleosomes (endoNucs; ~10 mg/mL) derived from TKO cells. Total Ion Electropherograms (a) divided into two major regions: (i) non-Nuc proteins and (ii) endoNucs. Raw and deconvoluted mass spectra (b-d) of three exemplary sections (75 – 80 min, 95 – 100 min, 115 -120 min). Several “nucleoforms” were observed in each section. In general, the intact mass of nucleoforms increased with migration time and an average mass shift of 620.2 ± 18.5 Da was observed between neighboring peaks, which corresponds to a single base pair difference in length of the associated DNA ($\Delta m_{GC} = 618.4$ Da, $\Delta m_{AT} = 617.4$ Da).

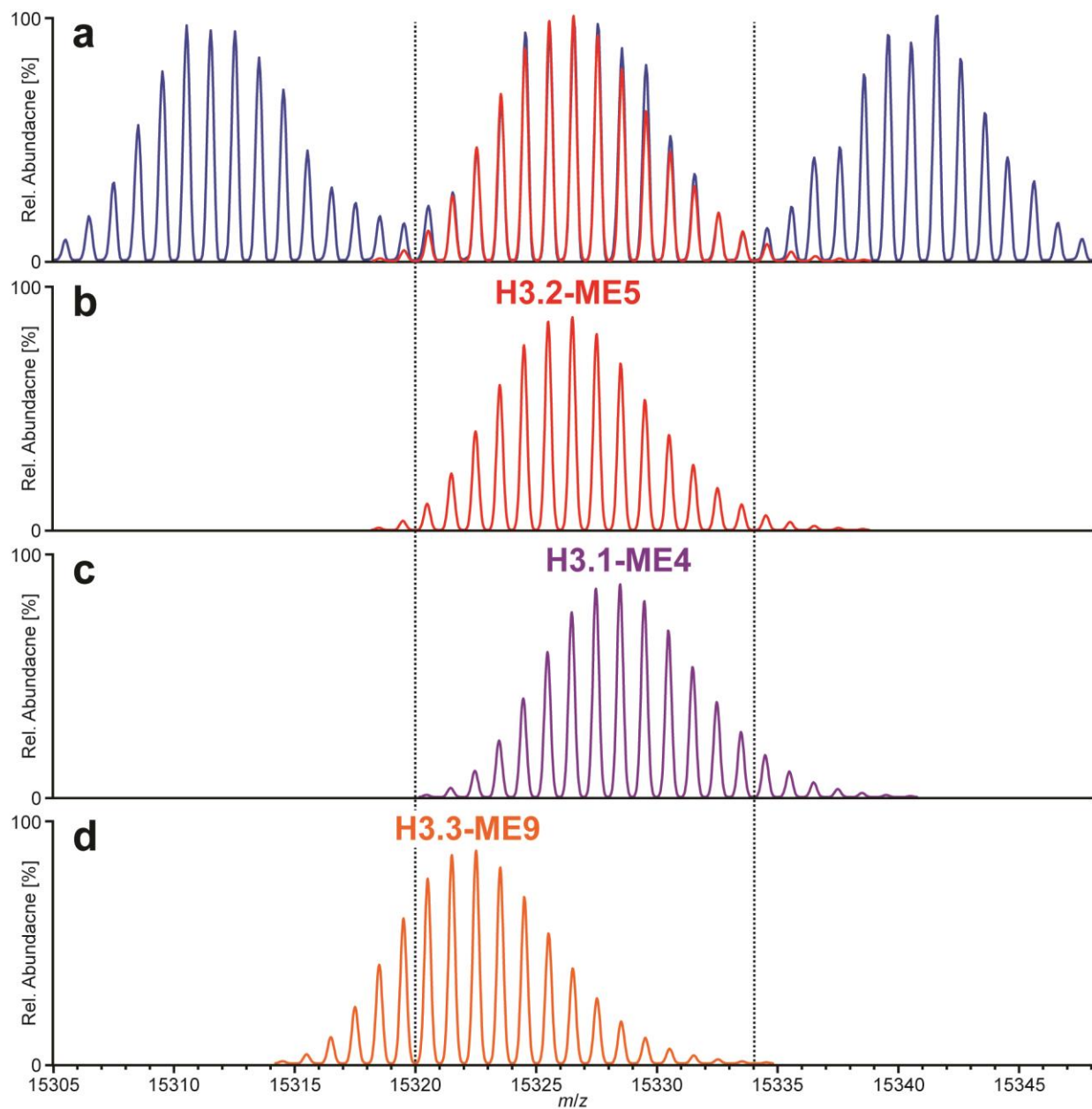


Figure S8. Comparison of observed and theoretical isotopic distributions of H3 proteoforms. **(a)** Deconvoluted mass spectrum (blue) of NTKO in the range from 15,305 to 15,350 Da showing the three major H3 proteoforms. The theoretical isotopic distribution of the H3.2 proteoform exhibiting five methyl equivalents (ME) is overlaid (red), indicating a high match with the observed data. Calculated theoretical distributions of proteoforms **(b)** H3.2-ME5, **(c)** H3.1-ME4, and **(d)** H3.3-ME9.

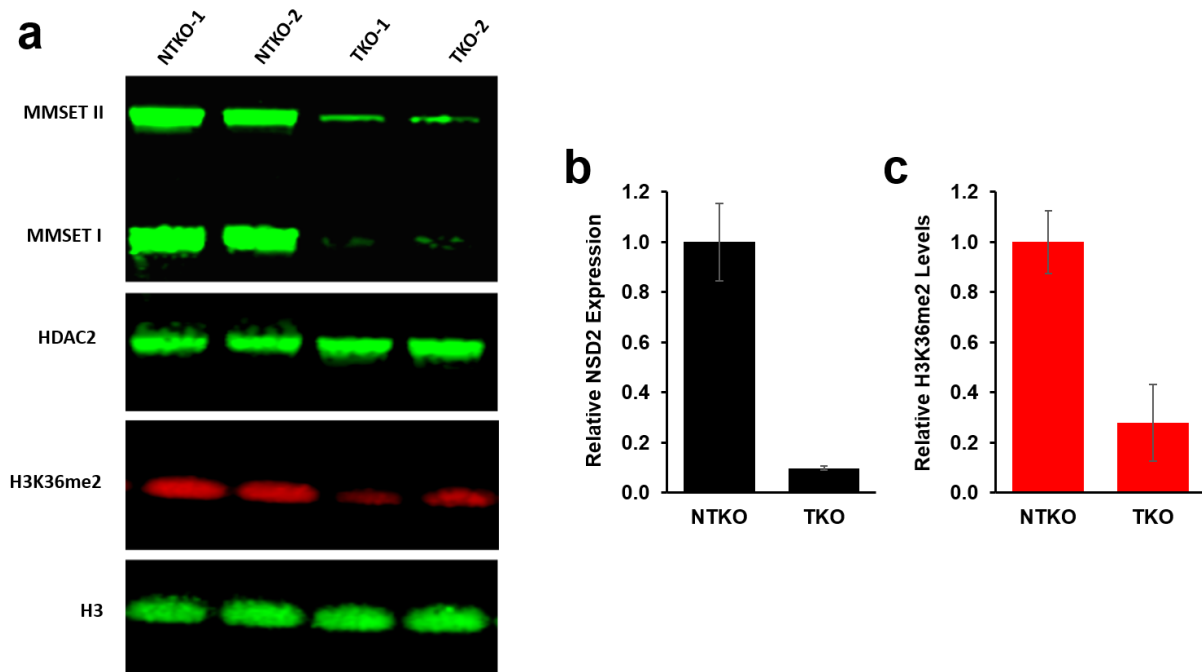


Figure S9. Immunoblot analysis showing NSD2 (MMSET) and H3K36me2 levels in NSD2-high (NTKO) and NSD2-low (TKO) isogenic cells derived from the human multiple myeloma cell line KMS11. (A) Reduction of both full-length (MMSET II) and the short isoform (MMSET I) of NSD2 in TKO compared to NTKO cells. Decrease in NSD2 levels is associated with a drop in global H3K36me2 levels. HDAC2 was used as a loading control for NSD2 blots. Total H3 was used as a loading control for H3K36me2 blots. (B, C) Relative quantification of NSD2 levels (normalized to HDAC2) and H3K36me2 (normalized to total H3) in NTKO and TKO cells. Data represent the mean \pm SD (n =2).

Immunoblot Analysis: Nuclear extracts from NTKO and TKO cells were prepared using the Nuclear Extract Kit (Active Motif) following the manufacturer's protocol. Nuclear extracts (50 μ g proteins for NSD2/HDAC2 and 20 μ g for H3K36me2/H3) were separated by SDS gel electrophoresis on a 4-12 % NuPAGE Bis-Tris gel (ThermoFisher Scientific) and transferred using an iBlot system and iBlot Gel Nitrocellulose Transfer Stacks (ThermoFisher Scientific). Blots were blocked in Pierce Protein-Free Blocking Buffer (ThermoFisher Scientific) for 1 hour. Blots were then incubated with primary antibodies-NSD2 (PMID: 18156491), HDAC2 (Millipore # 05-814),

H3K36me2 (Cell Signaling Technology #2901S), H3 (Cell Signaling Technology # 14269S) - diluted in blocking buffer (1:1000), overnight at 4°C. Membranes were washed 3 times with PBS-Tween and incubated with the secondary antibody solutions - goat anti-mouse, Alexa Fluor Plus 800 (ThermoFisher Scientific # A32730) or goat anti-rabbit, Alexa Fluor Plus 680 (ThermoFisher Scientific # A32734) - diluted in PBS-tween (1:10,000) for 1 hour at room temperature. Membranes were then washed 3 times in PBS-Tween and scanned on an Odyssey CLx instrument (LICOR).

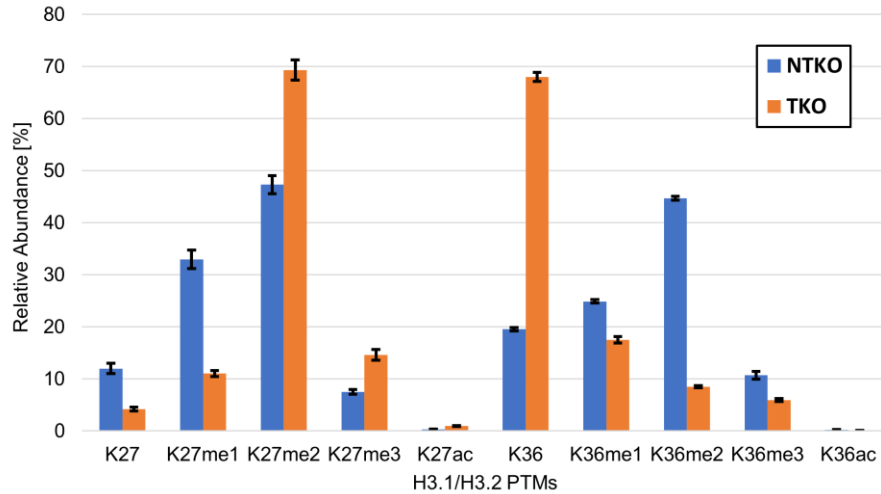


Figure S10. Relative quantification of H3.1/H3.2 proteoforms associated with K27 and K36 from NTKO (blue, n =3) and TKO (orange, n = 3) cells by bottom-up proteomics (LC-MS/MS). As expected, a decrease of K36me2 in TKO cells could be observed. Concurrently, an increase in K27me2, K27me3 and K27ac was detected. Samples were prepared and analyzed based on the protocol of Zheng *et al.* (Reference 51). Error bars represent standard deviations.

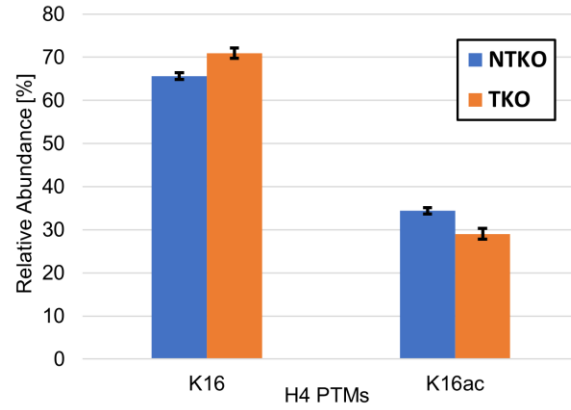


Figure S11. Relative quantification of H4K16 acetylation from NTKO (blue, n =3) and TKO (orange, n = 3) cells by bottom-up proteomics (LC-MS/MS). Samples were prepared and analyzed based on the protocol of Zheng *et al.* (Reference 51). Error bars represent standard deviations.

Table S1: Overview of steps of the three developed native CE methods. Rinsing steps were performed at 100 psi.

Step	Capillary	Standard	High-resolution	High-throughput
Rinse: 0.1 M HCl	SL	5 min	5 min	3 min
Rinse: BGE	CL	3 min	3 min	2 min
Rinse: BGE	SL	5 min	5 min	3 min
Injection	SL	2.5 psi, 30 sec	2.5 psi, 30 sec	2.5 psi, 30 sec
Ramp Up	SL	5 psi, 1 min	2.2 psi, 1 min	15 psi, 1 min
Separation	SL	+18 kV, 5 psi, 30 min	+18 kV Synthetic Nucs: 2.2 psi, 115 min EndoNucs: 3.0 psi, 180 min	+18 kV, 15 psi, 6 min
Ramp Down	SL	5 psi, 5 min	5 psi, 5 min	15 psi, 4 min
Total Run Time	-	~50 min	~135 min	~20 min

SL = separation line, CL = conductive line

Table S2: Parameters of MS methods applied for Nuc characterization using CZE-TDMS.

OT Instrument	QE-EMR		UHMR
Mode	MS ¹	MS ²	Pseudo-MS ³
Scan Range [<i>m/z</i>]	500 – 10,000	500 – 10,000	500 – 12,000
Isolation Range [<i>m/z</i>]	2,000 – 10,000	6,000 – 9,000	1002.5 ± 15 (H2A)
Resolution	7,500; 15,000	120,000	70,000
In-Source CID [eV]	none	25 - 50	-
In-Source Trapping	-	-	off
Trapping Voltage	-	-	60
Microscans	20	10	10
Max Injection [μ s]	30	500	1000
AGC target	3e6	3e6	1e6
Extended Trapping [eV]	120	120	-
HCD Fragmentation	-10	-10	35
Pressure Regulator	4 (~1.2e-9 mbar)	1 (~6e-10 mbar)	-

Table S3: Determination of intra- and inter-day reproducibility for nCZE-TDMS method for nucleosome characterization. Designer nuc H3K27me3 ($c = 1 \mu\text{M}$) was analyzed five consecutive times on two different days. Relative standard deviation (RSD) values were calculated for migration time (MT), peak area and peak intensity. Peak area and intensity were based on the extracted ion electropherograms (EIEs) of the five most intense charge states.

Day 1 (intra-day)				Day 2 (intra-day)			
Run	MT [min]	Area [cts]	Intensity [cts]	Run	MT [min]	Area [cts]	Intensity [cts]
1	20.44	$2.60 \cdot 10^8$	$3.80 \cdot 10^6$	6	20.23	$2.92 \cdot 10^8$	$4.16 \cdot 10^6$
2	20.59	$2.74 \cdot 10^8$	$4.18 \cdot 10^6$	7	20.35	$2.59 \cdot 10^8$	$3.74 \cdot 10^6$
3	20.59	$2.83 \cdot 10^8$	$4.29 \cdot 10^6$	8	20.20	$2.53 \cdot 10^8$	$3.82 \cdot 10^6$
4	20.42	$2.63 \cdot 10^8$	$3.78 \cdot 10^6$	9	20.49	$2.57 \cdot 10^8$	$3.93 \cdot 10^6$
5	20.61	$2.13 \cdot 10^8$	$3.73 \cdot 10^6$	10	20.51	$2.62 \cdot 10^8$	$3.91 \cdot 10^6$
Mean	20.53	$2.58 \cdot 10^8$	$3.96 \cdot 10^6$	Mean	20.36	$2.62 \cdot 10^8$	$3.91 \cdot 10^6$
Stdev	0.09	$2.70 \cdot 10^7$	$2.58 \cdot 10^5$	Stdev	0.14	$1.71 \cdot 10^7$	$1.58 \cdot 10^5$
RSD	0.45%	10.46%	6.53%	RSD	0.70%	6.51%	4.04%
				Day 1 & 2 (inter-day)			
				Mean	20.44	$2.60 \cdot 10^8$	$3.94 \cdot 10^6$
				Stdev	0.15	$2.14 \cdot 10^7$	$2.03 \cdot 10^5$
				RSD	0.71%	8.22%	5.16%

Table S4: Relative quantification of NTKO/TKO histone proteoforms using nCZE-TDMS. Comparison between histone proteoforms from NTKO (IGH-NSD2; H3K36me2^{hi}) and TKO (NSD2^{+/-}; H3K36me2^{lo}) cells using spectra averaged over the entire endoNuc migration range (n = 3). Statistically significant increases (blue) and decreases (red) of histone proteoforms in TKO samples are highlighted.

Histone Proteoform	NTKO		TKO		t-test	
	Mean	StdDev	Mean	StdDev	p-value	α -value
H4-K20met1	3.54%	0.02%	3.26%	0.08%	3.91E-03	
H4-K20met2	3.50%	0.06%	3.42%	0.10%	0.328	
H4-NtAc	4.22%	0.01%	5.55%	0.17%	5.50E-03	
H4-K20met1-NtAc	8.47%	0.16%	8.36%	0.21%	0.523	
H4-K20met2-NtAc	50.62%	0.63%	54.15%	0.90%	5.19E-03	6.25E-03
H4-K20me3-NtAc / H4-K16Ac-NtAc	4.23%	0.16%	4.38%	0.12%	0.273	
H4-R3met1-K20met3-NtAc / H4-K20met1-K16Ac-NtAc	3.61%	0.15%	2.93%	0.10%	2.60E-03	
H4-K20met2-K16Ac-NtAc	21.82%	0.18%	17.95%	0.83%	1.42E-03	
H2B PF1	2.13%	0.03%	1.98%	0.18%	0.296	
H2B PF2	2.73%	0.06%	2.52%	0.18%	0.123	
H2B PF3	5.07%	0.15%	4.79%	0.27%	0.185	
H2B.1-K	18.30%	0.13%	20.15%	0.12%	5.55E-05	6.25E-03
H2B.1-C/E/F/G/I/O	29.95%	0.12%	31.71%	0.44%	2.61E-03	
H2B.2-E/F	34.46%	0.26%	32.58%	0.66%	0.0102	
H2B.1-D	6.50%	0.15%	5.44%	0.34%	7.92E-03	
H2B.1-M	0.85%	0.04%	0.83%	0.07%	0.670	
H2A.Z	1.89%	0.06%	2.24%	0.31%	0.181	
H2A.1-H-NtAc	1.99%	0.04%	1.53%	0.03%	6.02E-05	
H2A PF3	1.35%	0.03%	1.20%	0.08%	0.0325	
H2A.2-C-NtAc	10.57%	0.02%	10.72%	0.21%	0.350	
H2A.2-B-NtAc	9.08%	0.04%	9.87%	0.33%	0.0528	
H2A.J-NtAc	0.80%	0.02%	0.80%	0.03%	0.783	
H2A. PF7	1.64%	0.04%	1.57%	0.03%	0.0806	
H2A.1	2.99%	0.05%	2.82%	0.07%	0.0293	3.57E-03
H2A.1C / H2A.1-Q104met1	1.38%	0.02%	1.27%	0.04%	0.0118	
H2A.1-C-Q104met1	7.14%	0.28%	6.57%	0.21%	0.0469	
H2A.1-B/E	50.14%	0.55%	52.62%	1.32%	0.0394	
H2A.1-C-NtAc	6.96%	0.22%	5.94%	0.11%	1.91E-03	
H2A.1-B/E-Q104met1	2.21%	0.07%	1.61%	0.09%	7.23E-04	
H2A.1-B/E-NtAc	1.84%	0.01%	1.24%	0.06%	2.66E-03	
H3.3-ME3	1.64%	0.13%	2.13%	0.10%	6.61E-03	
H3.3-ME4	3.11%	0.19%	3.88%	0.07%	2.66E-03	
H3.3-ME5	4.33%	0.10%	5.29%	0.05%	1.26E-04	
H3.2-ME2	5.89%	0.18%	7.75%	0.10%	8.74E-05	
H3.2-ME3	11.22%	0.25%	15.21%	0.39%	1.20E-04	
H3.2-ME4	20.17%	0.14%	28.56%	0.35%	2.64E-06	5.00E-03
H3.2-ME5	21.94%	0.12%	21.22%	0.26%	1.25E-02	
H3.2-ME6	19.71%	0.17%	9.92%	0.34%	1.52E-06	
H3.2-ME7	9.30%	0.20%	4.47%	0.19%	7.18E-06	
H3.2-ME8	2.69%	0.28%	1.57%	0.08%	2.55E-03	

PF = proteoform, met = methylation, Ac = acetylation, NtAc = N-terminal acetylation, ME = methyl equivalent

Table S5: Relative quantification of NTKO/TKO histones using nCZE-TDMS. Comparison between histones from NTKO and TKO cells using spectra averaged over the entire endoNuc migration range (n = 3).

Histone	NTKO	TKO
H4	6.04 \pm 0.07%	5.81 \pm 0.24%
H2B	47.24 \pm 0.74%	47.02 \pm 2.42%
H2A	36.20 \pm 0.60%	36.54 \pm 2.00%
H3	10.52 \pm 0.24%	10.63 \pm 0.75%

Table S6: Comparison between front, middle and end section of the endoNuc migration time window for NTKO/TKO endoNuc samples analyzed by nCZE-TDMS. Electropherograms were divided into three equally long sections and deconvoluted.

Histone	NTKO			TKO		
	Front	Middle	End	Front	Middle	End
H4	5.97 ±0.07%	5.78 ±0.08%	7.38 ±0.21%	5.93 ±0.31%	5.52 ±0.16%	6.48 ±0.44%
H2B	38.16 ±0.95%	51.68 ±0.53%	58.66 ±0.85%	38.07 ±1.67%	52.06 ±1.29%	59.01 ±3.83%
H2A	42.42 ±0.67%	33.49 ±0.51%	27.25 ±0.81%	42.42 ±1.97%	33.31 ±0.97%	28.66 ±3.04%
H3	13.45 ±0.29%	9.05 ±0.16%	6.70 ±0.22%	13.58 ±0.62%	9.10 ±0.47%	5.85 ±1.00%

Table S7: Comparison between front, middle and end section of the endoNuc migration time window for **NTKO** (IGH-NSD2; H3K36me2^{hi}) endoNuc samples analyzed by nCZE-TDMS. t-tests were performed between front and end sections. Statistically significant increases (blue) and decreases (red) of histone proteoforms over the course of the nCZE separation are highlighted.

Histone Proteoform	Front Section		End Section		t-test	
	Mean	StdDev	Mean	StdDev	p-value	α -value
H4-K20met1	4.29%	0.16%	2.63%	0.08%	8.43E-05	
H4-K20met2	3.90%	0.22%	2.86%	0.12%	2.07E-03	
H4-NtAc	4.25%	0.32%	4.20%	0.08%	0.817	
H4-K20met1-NtAc	8.93%	0.24%	7.26%	0.26%	1.21E-03	
H4-K20met2-NtAc	49.63%	0.65%	56.40%	1.15%	8.77E-04	6.25E-03
H4-K20me3-NtAc / H4-K16Ac-NtAc	4.22%	0.33%	3.66%	0.36%	0.0797	
H4-R3met1-K20met3-NtAc / H4-K20met1-K16Ac-NtAc	4.32%	0.23%	2.50%	0.06%	3.67E-04	
H4-K20met2-K16Ac-NtAc	20.64%	0.39%	20.48%	0.28%	0.596	
H2B PF1	2.08%	0.05%	1.76%	0.11%	0.0110	
H2B PF2	2.66%	0.09%	2.30%	0.11%	0.0126	
H2B PF3	5.38%	0.19%	4.08%	0.22%	3.06E-03	
H2B.1-K	18.48%	0.13%	17.74%	0.11%	9.04E-04	6.25E-03
H2B.1-C/E/F/G/I/O	29.98%	0.14%	31.12%	0.27%	2.95E-03	
H2B.2-E/F	34.01%	0.30%	36.86%	0.53%	1.28E-03	
H2B.1-D	6.33%	0.19%	5.71%	0.27%	0.0313	
H2B.1-M	1.17%	0.06%	0.42%	0.02%	2.70E-05	
H2A.Z	1.66%	0.08%	1.36%	0.09%	0.0110	
H2A.1-H-NtAc	1.96%	0.04%	1.77%	0.06%	7.85E-03	
H2A PF3	1.34%	0.05%	1.19%	0.04%	0.0142	
H2A.2-C-NtAc	10.14%	0.04%	10.85%	0.13%	7.55E-04	
H2A.2-B-NtAc	8.87%	0.07%	8.99%	0.17%	0.319	
H2A.J-NtAc	0.80%	0.03%	0.66%	0.03%	1.40E-03	
H2A. PF7	1.76%	0.06%	1.19%	0.04%	1.92E-04	
H2A.1	3.28%	0.13%	2.18%	0.08%	2.54E-04	3.57E-03
H2A.1C / H2A.1-Q104met1	1.49%	0.04%	1.06%	0.04%	1.43E-04	
H2A.1-C-Q104met1	7.65%	0.29%	5.38%	0.22%	4.11E-04	
H2A.1-B/E	49.09%	0.66%	56.73%	1.07%	4.57E-04	
H2A.1-C-NtAc	7.12%	0.25%	5.93%	0.33%	7.41E-03	
H2A.1-B/E-Q104met1	2.35%	0.09%	1.62%	0.11%	8.27E-04	
H2A.1-B/E-NtAc	2.42%	0.02%	1.09%	0.04%	8.16E-07	
H3.3-ME3	1.73%	0.10%	1.73%	0.21%	0.978	
H3.3-ME4	3.25%	0.14%	3.11%	0.14%	0.284	
H3.3-ME5	4.36%	0.04%	4.20%	0.36%	0.531	
H3.2-ME2	5.90%	0.10%	5.31%	0.14%	3.83E-03	
H3.2-ME3	11.17%	0.20%	11.65%	0.46%	0.177	
H3.2-ME4	20.17%	0.04%	19.50%	0.20%	0.0264	5.00E-03
H3.2-ME5	21.49%	0.10%	22.73%	0.31%	2.61E-03	
H3.2-ME6	19.76%	0.19%	20.65%	0.27%	9.37E-03	
H3.2-ME7	9.40%	0.33%	8.96%	0.26%	0.145	
H3.2-ME8	2.76%	0.25%	2.17%	0.35%	0.0734	

PF = proteoform, met = methylation, Ac = acetylation, NtAc = N-terminal acetylation, ME = methyl equivalent

Table S8: Comparison between front, middle and end section of the endoNuc migration time window for **TKO** (NSD2^{+/-}; H3K36me2^{lo}) endoNuc samples analyzed by nCZE-TDMS. t-tests were performed between front and end sections. Statistically significant increases (blue) and decreases (red) of histone proteoforms over the course of the nCZE separation are highlighted.

Histone Proteoform	Front Section		End Section		t-test	
	Mean	StdDev	Mean	StdDev	p-value	α -value
H4-K20met1	3.63%	0.11%	2.48%	0.07%	1.05E-04	6.25E-03
H4-K20met2	3.72%	0.05%	2.86%	0.14%	5.05E-04	
H4-NtAc	5.35%	0.06%	5.57%	0.34%	0.380	
H4-K20met1-NtAc	8.65%	0.40%	7.57%	0.24%	0.0164	
H4-K20met2-NtAc	52.00%	2.04%	60.95%	2.68%	0.0100	
H4-K20me3-NtAc / H4-K16Ac-NtAc	4.79%	0.23%	3.19%	0.57%	0.0109	
H4-R3met1-K20met3-NtAc / H4-K20met1-K16Ac-NtAc	3.64%	0.58%	1.93%	0.23%	8.91E-03	
H4-K20met2-K16Ac-NtAc	18.22%	1.39%	15.45%	1.89%	0.109	
H2B PF1	1.96%	0.20%	1.69%	0.11%	0.112	
H2B PF2	2.48%	0.22%	2.06%	0.20%	0.0710	
H2B PF3	4.87%	0.30%	3.80%	0.40%	0.0211	
H2B.1-K	20.10%	0.26%	19.82%	0.22%	0.231	6.25E-03
H2B.1-C/E/F/G/I/O	31.69%	0.55%	33.12%	0.40%	0.0217	
H2B.2-E/F	32.35%	0.86%	34.72%	0.76%	0.0231	
H2B.1-D	5.46%	0.40%	4.30%	0.42%	0.0255	
H2B.1-M	1.10%	0.11%	0.48%	0.07%	1.19E-03	
H2A.Z	2.14%	0.33%	1.67%	0.17%	0.0944	3.57E-03
H2A.1-H-NtAc	1.50%	0.06%	1.37%	0.11%	0.143	
H2A PF3	1.31%	0.09%	1.00%	0.05%	0.0220	
H2A.2-C-NtAc	10.55%	0.11%	10.21%	0.75%	0.519	
H2A.2-B-NtAc	9.71%	0.36%	9.47%	0.51%	0.552	
H2A.J-NtAc	0.84%	0.06%	0.67%	0.04%	0.0132	
H2A. PF7	1.71%	0.01%	1.35%	0.16%	0.0571	
H2A.1	3.06%	0.12%	2.31%	0.17%	3.13E-03	
H2A.1C / H2A.1-Q104met1	1.36%	0.04%	1.00%	0.03%	3.45E-04	
H2A.1-C-Q104met1	7.00%	0.22%	4.96%	0.13%	1.58E-04	
H2A.1-B/E	51.52%	1.58%	59.37%	1.40%	2.98E-03	
H2A.1-C-NtAc	6.23%	0.17%	4.37%	0.27%	5.66E-04	
H2A.1-B/E-Q104met1	1.71%	0.16%	1.09%	0.20%	0.0131	
H2A.1-B/E-NtAc	1.46%	0.11%	1.15%	0.58%	0.459	
H3.3-ME3	2.17%	0.12%	2.30%	0.27%	0.493	5.00E-03
H3.3-ME4	3.79%	0.18%	4.09%	0.30%	0.209	
H3.3-ME5	5.33%	0.10%	5.29%	0.88%	0.947	
H3.2-ME2	7.82%	0.12%	7.55%	0.29%	0.203	
H3.2-ME3	15.24%	0.58%	15.45%	0.56%	0.686	
H3.2-ME4	28.20%	0.39%	28.71%	0.70%	0.325	
H3.2-ME5	21.18%	0.31%	20.99%	0.20%	0.441	
H3.2-ME6	10.16%	0.27%	9.58%	0.19%	0.0359	
H3.2-ME7	4.54%	0.21%	4.29%	0.27%	0.261	
H3.2-ME8	1.56%	0.16%	1.74%	0.34%	0.449	

PF = proteoforms, met = methylation, Ac = acetylation, NtAc = N-terminal acetylation, ME = methylation equivalent

Method S1: Preparation of TKO and NTKO mononucleosomes

Cells were collected and resuspended in 2.5 pelleted cell volumes (PCV) of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 340 mM sucrose, 0.5 mM PMSF, 0.5 mM benzamidine) supplemented with 5 mM β -mercaptoethanol and 1 \times cOmplete EDTA-free Protease Inhibitor Cocktail (Roche). A similarly supplemented volume of buffer A (2.5 PCV) containing 0.2% (vol/vol) Triton X-100 detergent was also prepared simultaneously. The resuspended cell pellet was completely homogenized by pipetting and light vortexing, and the detergent-containing buffer A was added to the cell suspension, mixed by inversion, and allowed to incubate on ice for 10 min with occasional gentle mixing to thoroughly lyse the cells. The resultant nuclei were pelleted by centrifugation (1,300g, 4 °C, 5 min), and the supernatant was aspirated and discarded. Next, the nuclei pellet was resuspended in 6 PCV of supplemented buffer A and centrifuged through a 35-mL sucrose cushion (10 mM HEPES, pH 7.9, 30% (wt/vol) sucrose) in a 50-ml conical tube supplemented with 5 mM β -mercaptoethanol and 1 \times cOmplete EDTA-free Protease Inhibitor Cocktail (1,300g, 4 °C, 10 min). The sucrose cushion purification of the nuclei was repeated until the cushion remained free of cell debris after centrifugation. The pelleted nuclei were then gently resuspended in 2 PCV buffer A (supplemented as above). The DNA concentration in the nuclei resuspension was calculated (A_{260}) by hypertonic lysing of the nuclei by mixing 5 μ L of the nuclei resuspension with 495 μ L of 2 M NaCl. The suspension was diluted to a DNA concentration of 1.5 μ g/ μ L, and CaCl₂ was added to the nuclei to a final concentration of 1 mM. The suspension was aliquoted into 2-mL microcentrifuge tubes and equilibrated in a 37 °C water bath for 5 min. Digestion of the nuclei to nucleosomal species was initiated by adding 1 U MNase (Worthington, prepared in supplemented buffer A) for every 70 μ g DNA, and the samples were incubated at 37 °C for 15 min. The digestion was then quenched by adding 0.5 M EGTA to a final concentration

of 10 mM, mixing by inversion and placing the samples on ice. The final volume of the digested material was noted. Approximately 10 μ g DNA was purified (Qiagen DNA Cleanup kit) and resolved on a 2% agarose gel in 0.5 \times TBE to confirm the extent of digestion by fragment size distribution in the population. NaCl (2 M) was added dropwise to the digested chromatin while mixing on a magnetic stir plate at 4 $^{\circ}$ C to a final concentration of 650 mM. The resulting material was cleared by centrifugation at 12,000g and 4 $^{\circ}$ C to pellet any insoluble material before size-exclusion chromatography. The material was fractionated with a HiPrep 26/60 Sephacryl S-300 HR column equilibrated with supplemented buffer A, as above, containing 650 mM NaCl using an ÄKTA Prime Plus FPLC (GE Life Sciences). Samples (10 μ g) of the individual fractions containing nucleosomes were purified (Qiagen DNA Cleanup kit) and resolved on a 2% agarose gel in 0.5 \times TBE. Only those fractions that contained mononucleosomal-associated DNA fragments (~150 bp) were pooled for further analysis.

Discussion S1: Optimization of “higher-resolution” CZE method

Though partial separation was achieved using the “standard” CZE method, there is still room for significant improvement. Therefore, several different method conditions were evaluated, including ammonium acetate concentration (20 – 40 mM), supplemental pressure (2.2 – 5.0 psi), and pH (6.0 – 8.0). The concentration of AmAc did not affect the overall separation performance significantly (e.g. $R_{20\text{mM}} = 0.365$ vs. $R_{40\text{mM}} = 0.366$). A change in the pH of the BGE did not lead to better separation performance. However, if the pH is too low (≤ 6.0), no intact nucleosome signal was observed, possibly due to issues with complex stability. Moreover, appreciable metal adduct formation was observed at pH 8.0, decreasing overall signal quality. Given that the Nucs are counter-migrating in the system due to their negative net charge, balancing the supplemental pressure and mobility of the Nucs represents a key factor to improve separation performance. Although migration times and peak widths increase noticeably when decreasing the supplemental pressure from 5.0 psi to 2.2 psi, electrophoretic resolution is significantly improved from $R = 0.37$ to 1.09 (see **Figure 3**).

Discussion S2: Optimization of “high-throughput” CZE method

With potential for widespread deployment of the nCZE-TDMS platform, we developed a “high-throughput” CZE method tailored for the quality control environment during semi-synthetic Nuc manufacturing. For this purpose, we adapted the “standard” CZE method to decrease overall run time but still resolve Nuc-related peaks from salt/matrix compounds (**Figure S12**).

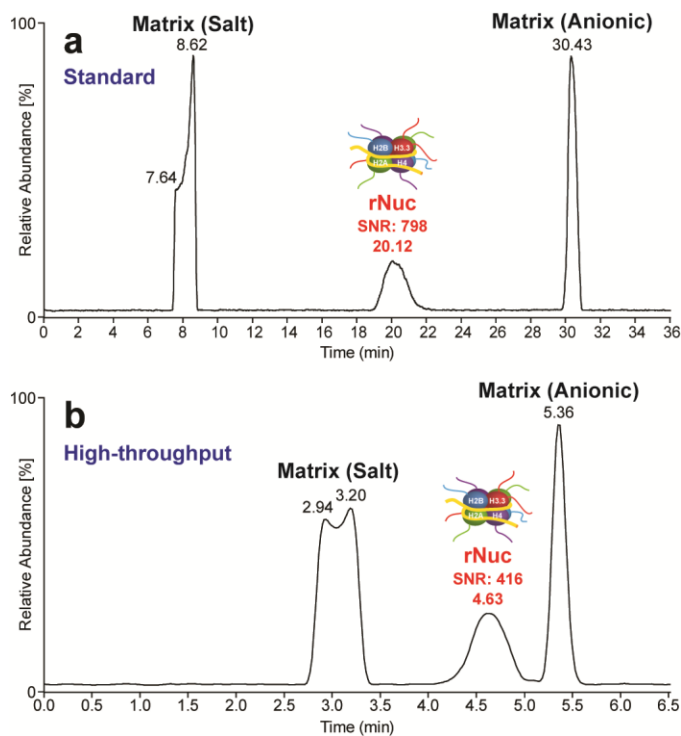


Figure S12. Comparison of Total Ion Electropherogram of “standard” (run time: 50 min) and “high-throughput” (run time: 20 min) CZE method analyzing unmodified recombinant Nuc ($c = 1 \mu\text{M}$). SNR values were determined at FWHM. While the higher-throughput method can baseline-resolve Nuc from matrix, there is a two-fold drop in sensitivity compared to the standard CZE method.

The influence of supplemental pressure was tested in a range between 5.0 and 30 psi and either maintained constant over the course of analysis or varied in different sections of the method. A constant pressure above 15 psi lead to issues regarding resolution of Nuc and matrix compounds.

In addition, varying the pressure during analysis resulted in electrospray process instabilities and thus, potential negative effects on reproducibility. We determined that a constant supplemental pressure of 15 psi, in combination with some changes to the preconditioning steps, yielded stable and reproducible signal, and reduced total run time from 50 to < 20 min per analysis (**Figure S12**). The Nuc peak could be detected in < 5 min using the “high-throughput” CZE, which represents a major improvement versus ~20 min using the “standard” CZE method.