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Site specific lysine acetylation of histones for nucleosome reconstitution using genetic code expansion in Escherichia coli.

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Corresponding Author:	Wenshe Liu UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	wsliu2007@tamu.edu;wliu@chem.tamu.edu
Order of Authors:	Chesley Marie Rowlett
	Wenshe Liu
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TITLE:

Site Specific Lysine Acetylation of Histones for Nucleosome Reconstitution Using Genetic Code Expansion in *Escherichia coli*

AUTHORS AND AFFILIATIONS:

Chesley Marie Rowlett[1], Wenshe Ray Liu[1]

[1]Department of Chemistry, Texas A&M University, College Station, Texas

Email Address of Co-Author:
Chesley Marie Rowlett (cmrowlett@tamu.edu)

Email Address of Corresponding Author:
Wenshe Ray Liu (wliu@chem.tamu.edu)

KEYWORDS:

nucleosome, acetylation, pyrrolysine, genetic code expansion, post translational modifications, protein modifications, histones

SUMMARY:

Here we present a method to express acetylated histone proteins using genetic code expansion and assemble reconstituted nucleosomes in vitro.

ABSTRACT:

Acetylated histone proteins can be easily expressed in *Escherichia coli* encoding a mutant, N^{ε}-acetyl-lysine (AcK)-specific *Methanosarcina mazi* pyrrolysine tRNA-synthetase (MmAcKRS1) and its cognate tRNA ($tRNA^{Pyl}$) to assemble reconstituted mononucleosomes with site specific acetylated histones. MmAcKRS1 and $tRNA^{Pyl}$ deliver AcK at an amber mutation site in the mRNA of choice during translation in *Escherichia coli*. This technique has been used extensively to incorporate AcK at H3 lysine sites. Pyrrolysyl-tRNA synthetase (PylRS) can also be easily evolved to incorporate other noncanonical amino acids (ncAAs) for site specific protein modification or functionalization. Here we detail a method to incorporate AcK using the MmAcKRS1 system into histone H3 and integrate acetylated H3 proteins into reconstituted mononucleosomes. Acetylated reconstituted mononucleosomes can be used in biochemical and binding assays, structure determination, and more. Obtaining modified mononucleosomes is crucial for designing experiments related to discovering new interactions and understanding epigenetics.

INTRODUCTION:

We have utilized PylRS and $tRNA^{Pyl}$ to synthesize and assemble reconstituted mononucleosomes with site specific acetylated histones. PylRS has proven invaluable as a genetic code expansion tool to produce proteins with post translational modifications (PTMs) and has been genetically evolved to incorporate about 200 different ncAAs. PylRS incorporates at an amber stop codon, removing competition from other amino acids during translation. PylRS was first discovered in

methanogenic archaea, and has since been utilized in chemical biology to incorporate novel reactive chemical groups into proteins[1,2].

MmAcKRS1 was evolved from *Methanosarcina mazei* PylRS and frequently used in our laboratory for the synthesis of acetylated proteins[3–6]. MmAcKRS1 delivers AcK at an amber mutation site in the mRNA of choice during translation in *Escherichia coli*. This technique has been previously used to incorporate AcK at K4, K9, K14, K18, K23, K27, K36, K56, K64, and K79 histone H3 lysine sites to study the activity of Sirtuin 1, 2, 6, and 7 on acetylated mononucleosomes[4–6]. Here we detail this method to express acetylated histones and reconstitute acetylated nucleosomes.

PROTOCOL:

1. Plasmid construction

1.1. Begin by deciding which histone protein will be acetylated and at which lysine site. Mutate the site to the amber stop codon (TAG) using site directed mutagenesis.

NOTE: There are four previously designed plasmids utilized for expression of histone proteins. All four histone proteins were cloned into the pETDuet-1 vector with an N-terminal histidine tag. Histone H4 also includes a SUMO tag, the origin of replication *colE1* with a copy number of approximately 40, ampicillin resistant, and a T7 promoter.

1.1.1. Design forward and reverse primers that contain a TAG mutation at the desired site (replacing an existing lysine codon with the amber stop codon) in one of the four histone protein plasmids.

1.1.2. Use the whole-plasmid PCR to amplify the TAG containing plasmid. Determine an appropriate annealing temperature for the primers. In general, the melting temperature (T_m) of the primers minus 5 ˚C will be sufficient. If difficulty is experienced obtaining a PCR product, a temperature gradient around T_m – 5 ˚C can be used to optimize the annealing temperature for amplification.

NOTE: In this experiment an in-house expressed PFU polymerase was used for 30 cycles with the following conditions: 94 ˚C for 30 s (denaturation), annealing temperature for 30 s, and 72 ˚C for 6 min (or 1 min per kilobase-pairs). For more details on this type of PCR method see Liu and Naismith[7].

1.1.3. Clean up the PCR product with a PCR clean up kit following manufacturer's recommendation. Analyze the PCR product by agarose gel electrophoresis and subsequent ethidium bromide staining.

1.1.4. Ligate the plasmid by incubating with T4 ligase overnight at 16 ˚C.

1.2. First transform TAG containing plasmid (Amp^R) into chemically competent *Escherichia*

coli. Pick a minimum of 4 colonies from the transformation and purify plasmid. Make cell stocks of each with glycerol using standard methods and store at -80 ˚C. Send for sequencing to confirm the desired TAG mutation.

1.3. Once the sequence is confirmed, co-transform the TAG-containing plasmid (Amp^R) with pEVOL-AckRS (Chl^R) into chemically competent CobB (the only histone lysine deacetylase known in *E. coli*.) deletion BL21 cells using the heat shock method. pEVOL is a plasmid with a mid-copy-number to low-copy-number *p15A* origin.

NOTE: The pEVOL series of plasmids were constructed based on previous studies that showed an increased expression of the orthogonal aaRS/$tRNA^{Tyr}$ pair was effective in decreasing protein truncation and in increasing overall yields of mutant proteins[8]. If CobB deletion cells are not accessible, proceed with chemically competent BL21 cells. CobB is a Sirtuin-like histone lysine deacetylase and Nicotinamide can be added at a 5 mM final concentration during histone protein expression to inhibit CobB as an alternative approach[9].

1.4. Make a cell stock and store at -80 ˚C.

2. Acetylated histone protein expression

2.1. Prepare 1 L of autoclaved 2YT media (or volume of choice) in a culture flask.

2.2. Inoculate 20 mL of 2YT media containing the appropriate antibiotics with the co-transformed cell stock that has the TAG-containing plasmid (Amp^R) and pEVOL-AckRS (Chl^R) and grow at 37 ˚C to OD = 0.6 (4-6 h).

2.3. Add the appropriate antibiotics to the 1 L of autoclaved 2YT media and inoculate with the 20 mL starter culture. Grow at 37 ˚C to OD = 0.6-0.8 (2-3 h).

2.4. Add inducing agents and acetyllysine (AcK) to the final concentrations of IPTG 1 mM, 0.2% arabinose, and AcK 5 mM.

2.5. Grow the culture at 37 ˚C for 6-8 h.

2.6. Pellet cells at 2,700 x *g* for 15 min.

2.7. Store the cell pellet at -80 ˚C overnight.

2.8. From this step onwards keep the sample on ice all times. Dissolve the cell pellet in 50 mL of histone lysis buffer per 1 L of culture.

2.9. Sonicate according to the following cycle: 1 s on, 1 s off, total on time: 3 min at 60% amplitude.

2.10. Pellet inclusion bodies at 41,600 x *g* for 45 min.

2.11. Discard the supernatant and resuspend inclusion bodies in 30 mL of histone lysis buffer. Pellet inclusion bodes at 41,600 x *g* for 30 min.

2.12. Discard the supernatant and resuspend inclusion bodies in 30 mL of pellet wash buffer. Pellet inclusion bodies at 41,600 x *g* for 30 min.

2.13. Discard the supernatant and dissolve inclusion bodies in 25 mL of 6 M Guanidine Hydrochloride (GuHCl) buffer. Incubate with agitation at 37 ˚C for 1 h.

NOTE: If needed, inclusion bodies can be incubated with agitation overnight at 4 ˚C.

2.14. Pellet inclusion bodes at 41,600 x *g* for 45 min. Incubate the supernatant with 1 mL of Ni-NTA resin equilibrated with 6 M GuHCl buffer for 2 h.

2.15. Proceed with Ni-NTA purification under denatured conditions. Wash the column with 3 column volumes of column wash buffer. Elute acetylated histone protein with 10 mL of elution buffer.

NOTE: Attempting to concentrate the protein here is an option but acetylated histones are very unpredictable and can easily precipitate. It is not recommended to concentrate past 2 mL in total volume.

2.16. Extensively dialyze the acetylated histone protein against 5% acetic acid buffer to remove salts. Dialyze for 3 h at 4 ˚C, exchanging for fresh buffer a minimum of 6 times.

2.17. Aliquot and lyophilize protein, and store indefinitely at -80 ˚C. Wild type histone proteins generally yield 10-50 mg/L whereas acetylated histone proteins yield less than 10 mg/L depending on the specific lysine site. For example, H3K79Ac averages 5 mg/L.

3. Wild-type histone protein expression

3.1. To assemble full nucleosomes, express all 4 histone proteins. When expressing and purifying wild type histones the protocol is the same as the acetylated histones except the following:

3.1.1. Do not perform co-transformation. Do not use pEVOL-AcKRS for wild type histone expression. Adjust antibiotics accordingly.

3.1.2. Do not use a CobB deletion cell line or add Nicotinamide, AcK, or arabinose during induction of cellular expression.

4. Preparation of 601 DNA

NOTE: A previously designed optimized DNA sequence to direct nucleosome positioning is assembled with histone octamers to produce mononucleosome with high efficiency. This sequence is referred to as 601 DNA or the Widom sequence. This sequence has become the standard DNA sequence for in vitro studies of nucleosomes from chromatin remodeling assays to single molecule measurements[10].

4.1. To prepare significant quantities of 601 DNA for nucleosome assembly, transform pGEM-3z/601 into Top 10 cells and grow 10 mL of culture for plasmid amplification and extraction. Use a plasmid extraction kit and follow manufacturer's recommendation.

4.2. Use an in-house expressed PFU polymerase (more efficient) for this amplification protocol. Set up a PCR reaction: For 250 µL reaction, use 207.5 µL autoclaved MQ H_2O, 25 µL 10x PFU Buffer, 5 µL Forward Primer: ctggagaatcccggtgccg, 5 µL Reverse Primer: acaggatgtatatatctgacacg, 5 µL dNTP mix, 2.5 µL PFU enzyme.

4.3. Use an annealing temperature of 52 ˚C for 30 s and an extension time of 30 s if using PFU polymerase. Otherwise follow the manufacturers recommended conditions.

4.4. Once PCR is done, use a PCR clean up kit of choice to purify the PCR product.

5. Assembly of histone octamer

5.1. Dissolve aliquots of histone protein pellets H2A, H2B, H3, and H4 so that there is a separate stock of each histone protein in GuHCl Buffer with a total volume of 100 µL.

5.2. Calculate the concentration of each histone protein by measuring the A_{280} absorbance.

5.3. If absorbance is greater than 1 for any protein, dilute with GuHCl buffer to get a more accurate concentration.

5.4. Combine H2A and H2B proteins in a 1:1 molar ratio and dilute to a total protein concentration of 4 µg/µL. Repeat for H3 and H4.

5.5. Dialyze sequentially at 4 ˚C against 2 M TE buffer overnight, 1 M TE buffer for 2 h and 0.5 M TE buffer for 5 h.

NOTE: The second and third steps of dialysis causes unstable conformations of protein to precipitate out. It is expected to see heavy precipitation at these steps.

5.6. Remove precipitates by centrifugation at 16,800 x *g* at 4 ˚C.

5.7. Again, calculate the concentration of histone dimers (H2A/H2B mixture) and tetramers (H3/H4 mixture) measuring the A_{280} absorbance.

5.8. Mix dimers and tetramers in a 1:1 molar ratio and adjust NaCl to 2 M by the addition of solid NaCl.

5.9. Histone octamer can be stored at 4 ˚C and is more stable than dimers and tetramers. Never freeze histone octamer as this can cause disassembly.

5.10. If assembling with acetylated histones, simply replace the wild type protein with the acetylated protein in the procedure.

6. Nucleosome assembly

6.1. Use 100x TE buffer and solid NaCl to adjust 601 DNA to 2M TE buffer. Add the 601 DNA in 2M TE buffer to histone octamer in a molar ratio of 0.85:1 to 0.90:1. A lower ratio of DNA may be added if free DNA is present in the gel shift analysis.

6.2. Transfer the DNA-histone mixture a dialysis bag and place in about 200 mL of 2M TE buffer (or more if assembling multiple samples) and very gently stir at 4 ˚C.

6.3. Set a peristaltic pump to purge to slowly drip in no salt TE buffer. When the volume has roughly doubled, pour out half the volume. Do this at least 4 times in total. With a pump this can take 4-8 h depending on the starting volume. Nucleosomes form when the salt concentration is reduced to 150 mM (measured by salinity meter).

6.4. After the salt concentration is reduced to 150 mM, dialyze against a 20 mM TE buffer overnight.

6.5. Remove precipitates by centrifugation and measure the concentration of the nucleosomes by A_{260} reading using a plate reader.

6.6. Add His-TEV protease (TEV: nucleosome 1:30, w:w) and incubate for overnight at 4 ˚C to remove all histidine tags. Remove the histidine tag impurities from the nucleosome solution by Ni-NTA resin pull down.

6.7. To position the nucleosome and homogenize the sample, incubate at 60 ˚C for 1 h.

NOTE: For acetylated sites that are particularly unstable, this step may not be advisable.

6.8. Store nucleosomes for short term (a few weeks) at 4 ˚C.

6.9. For long-term storage, dialyze nucleosomes against storage buffer and store at -80 ˚C.

REPRESENTATIVE RESULTS:

Dimers, tetramers, and octamers can be assessed by running a 12% SDS PAGE gel (**Figure 1** and **Figure 2**). Here you can see that some of the acetylated tetramers have a lower yield than others

(**Figure 1**). In fact, the closer to the core region, the lower the yield observed. This is most likely due to the acetylation interfering with the assembling of the tetramer the closer you get to the core regions. A similar affect is observed after assembling octamers (**Figure 2**). After assembling octamers with 601 DNA sequence, the nucleosome can be assessed using a 5% 1x TBE Native PAGE gel followed with staining with ethidium bromide (**Figure 3**). Before TEV digestion the nucleosome band is observed near the 10k base pair mark. After TEV digestions the nucleosome band shifts down relative to the ladder. Before thermal positioning, the observed nucleosome bands will be very broad, and possibly streak (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Histone dimers and tetramers. Representative 12% SDS PAGE gel of wild type histone dimer (lane 2) and H3 acetylated tetramers (lanes 3-9).

Figure 2: **Histone octamers.** Representative 12% SDS PAGE gel of H3 acetylated histone octamers (lanes 2-11).

Figure 3: Assembled his tagged nucleosome complex. Representative 1x TBE Native PAGE gel of wild type nucleosome complex (lane 2) compared to free 601 DNA (Lane 4) visualized by ethidium bromide.

Figure 4: Assembled TEV digested nucleosome complex. Representative 1x TBE Native PAGE gel of wild type TEV digested nucleosome complex, and free 601 DNA. A comparison of before and after nucleosome thermal positioning.

DISCUSSION:

It is essential to follow this protocol in every detail during an experiment. Nucleosomes are not very stable and much trial and error has gone into determining this protocol. It is key to remove precipitates at every step (or whenever observed) because particulates can easily interfere with the assembling processes. Always keep histone samples on ice. If nucleosomes are stored at 4 ˚C for too long, they can spontaneously disassemble. Be sure to check any samples by Native PAGE if stored at 4 ˚C for more than 2 weeks before use in any experiment. Avoid vortexing octamers and nucleosomes as this can also cause them to disassemble. If issues are encountered assembling dimers, tetramers, or octamers this is usually indicative of poor protein quality. Check the protein quality by 15% SDS-PAGE. One option if protein purity is low is to dialyze the protein against pure water. This will cause heavy precipitation and greatly reduce protein yield, but will produce a purer sample. The incorporation and nucleosomal stability of each histone acetylation site varies widely. In general, the closer the site is to the N-terminal domain, the lower the yield during histone protein expression. For nucleosome stability, the closer the acetylation site is to the nucleosome core or DNA binding sites, the less stable the assembled nucleosome. If stability issues are encountered, it is crucial to always keep the nucleosome sample on ice. It may be necessary to omit the nucleosome positioning step at 60 ˚C as this may cause disassembly or precipitation. A major limitation of acetylated nucleosomes is their instability. It may be impossible to preform assays at higher temperatures such as 37 ˚C without causing nucleosomal precipitation. If experimental procedures allow, perform them at 4 ˚C to prevent precipitation of

the acetylated nucleosome.

This method of producing nucleosomes is particularly useful for producing modified nucleosomes for binding experiments and structure determinations. This method produces nucleosomes with higher purity and a known nucleosomal DNA sequence, which eliminates confounding variables resulting in better controlled experiments and higher resolution images. It is crucial for structure determinations that the nucleosome sample is homogenous with a known DNA sequence, otherwise it will be all but impossible to obtain high resolution images.

There is an abundance of potential applications of this method. Specifically, in the field of epigenetics. It can be incredibly difficult to obtain modified proteins. Obtaining modified proteins is crucial for probing the structure and function of the writers, readers, and erasers in the many epigenetic pathways that are poorly understood. We have previously utilized this technique to probe the accessibility of nucleosomal DNA to Pst1 digestion at the H3 acetylation sites K18, K36, and K56 by assembling each mutant octamer with a modified 601 DNA sequence that contains a Pst1 digestion site. This technique can be further modified to incorporate NCAAs other than AcK. PylRS can be engineered to incorporate a host of other NCAAs. We have also incorporated N^{ϵ}-(7-azidoheptanoyl)-L-lysine (AzHeK) by amber codon in *Escherichia coli* for recombinant expression of several AzHeK-containing histone H3 proteins to investigate the histone deacylation activity of SIRT7 at several acetylation sites. This approach revealed that SIRT7 has high activity towards deacylation of H3K36 and is also catalytically active to deacylate H3K37. H3K36 deacylation was further showed to be nucleosome dependent and can be significantly enhanced by adding a short double-stranded DNA to the acyl-nucleosome substrate that mimics the bridging DNA between nucleosomes in native chromatin[6].

This method can also be modified to produce wild type nucleosomes with no modifications which can be useful in a variety of scenarios. Our group recently published a structure in collaboration with Dr. Pingwei Li's group showing the tight binding of cyclic GMP-AMP synthase (cGAS) to a negatively charged acidic patch formed by histone H2A and H2B via its second DNA binding site[11]. cGAS is a dsDNA sensor that catalyzes the synthesis of a cyclic dinucleotide cGAMP, which mediates the induction of type I interferons through the STING-TBK1-IRF3 signaling axis[12–16].

The benefit of using modified nucleosomes is its similarity to the native state of DNA and histone proteins which can be used as a significantly better model to probe the activities or binding abilities of any number of epigenetic-related proteins. There are numerous examples of limited or entirely absent enzyme activity towards naked DNA or histone peptide substrates that when probed with nucleosome substrates drastically changes results. As with the example mentioned above, a novel deacylation site was discovered for SIRT7 by using a nucleosome substrate that would have otherwise been unknown. It is crucial to use native substrates when probing these types of systems. This technique can be used to probe the reading, writing, and erasing activity or binding capability of any modification that can be incorporated by the pyrrolysine genetic code expansion technique. The pyrrolysyl-tRNA synthetase is already natively promiscuous and has been engineered for a host of other substrates already opening the door for using this technique in any number of systems making the primary limiting factor the creativity of the researcher.

ACKNOWLEDGMENTS:

We would like to thank Dr. Wesley Wang for laying the groundwork for this protocol and his valuable mentorship.

DISCLOSURES:

There are no competing financial interests to declare by any of the authors.

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Figure 1

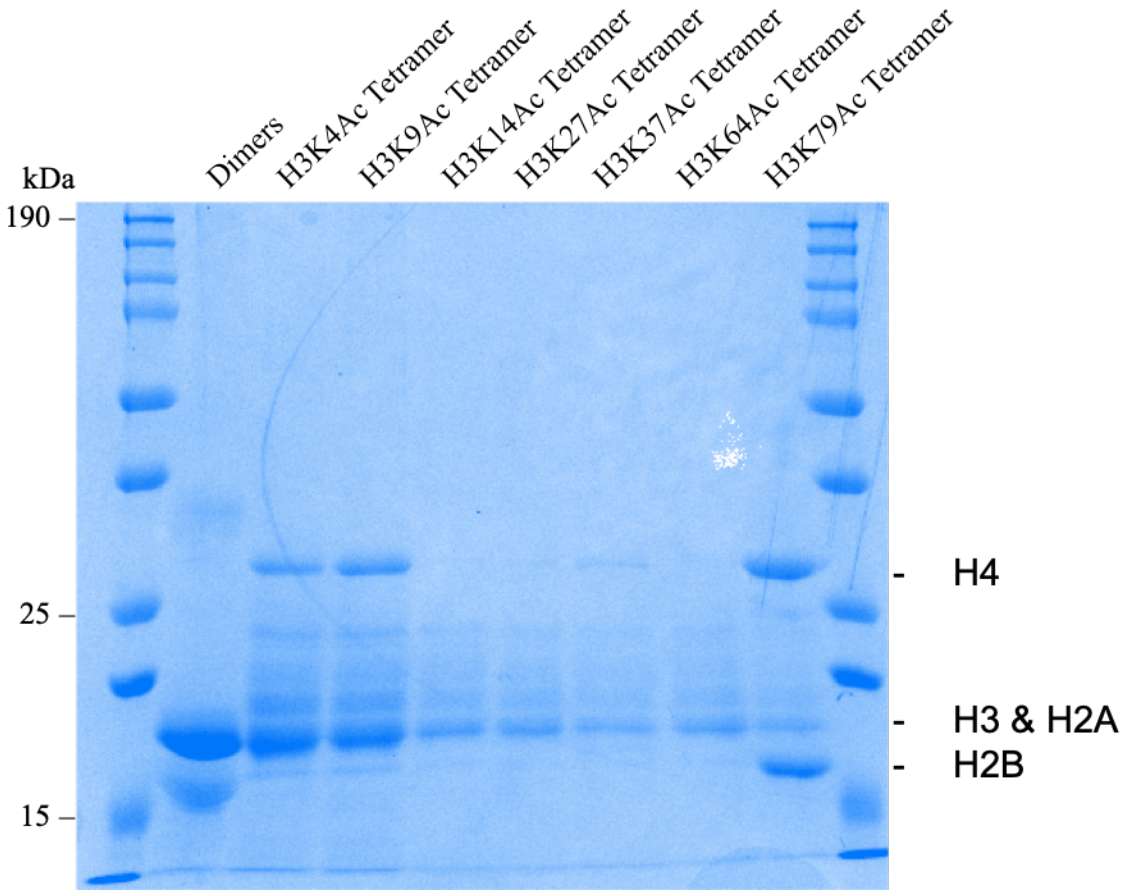


Figure 2

Click here to access/download;Figure;Figure 2.pdf

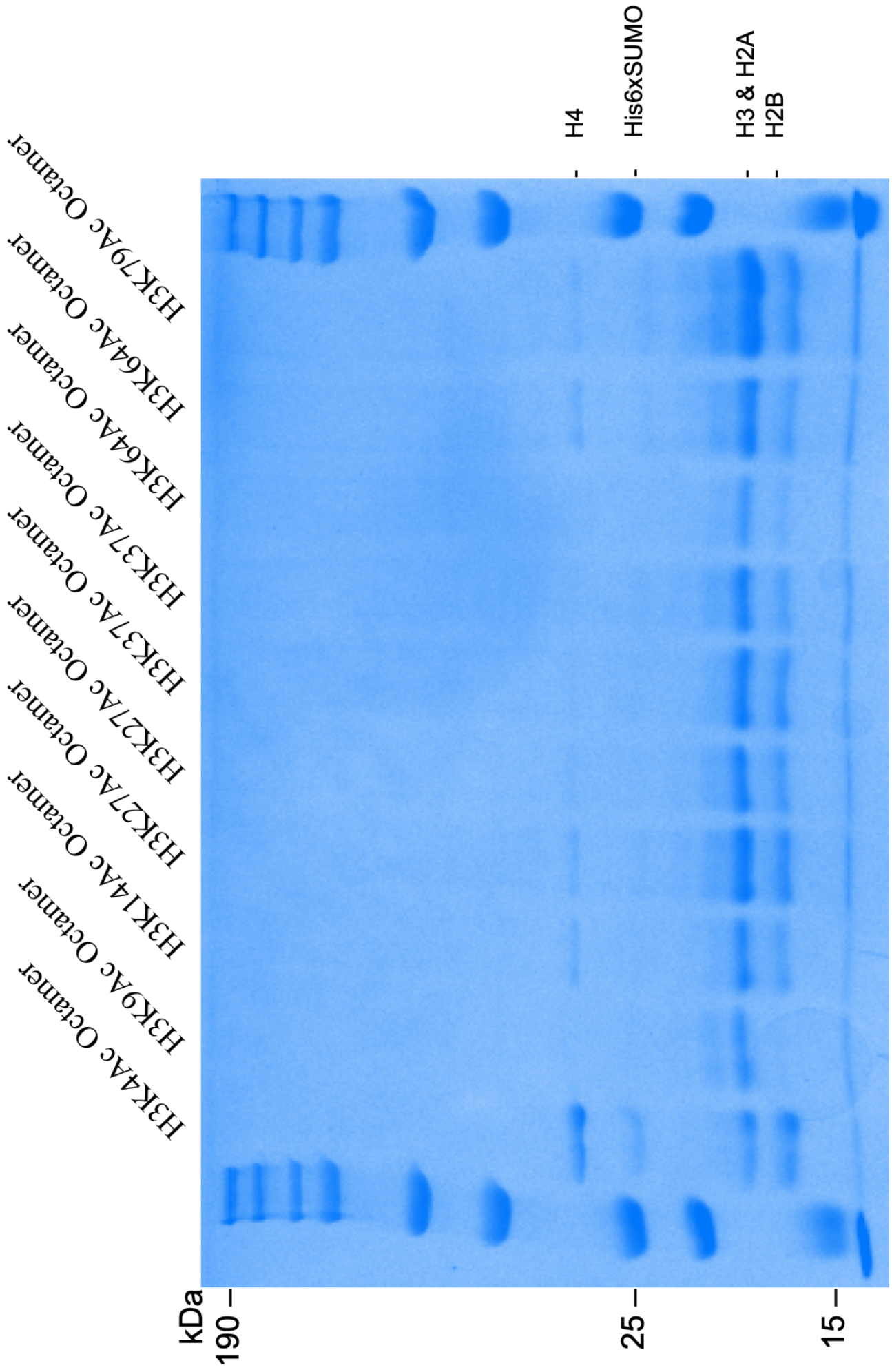


Figure 3

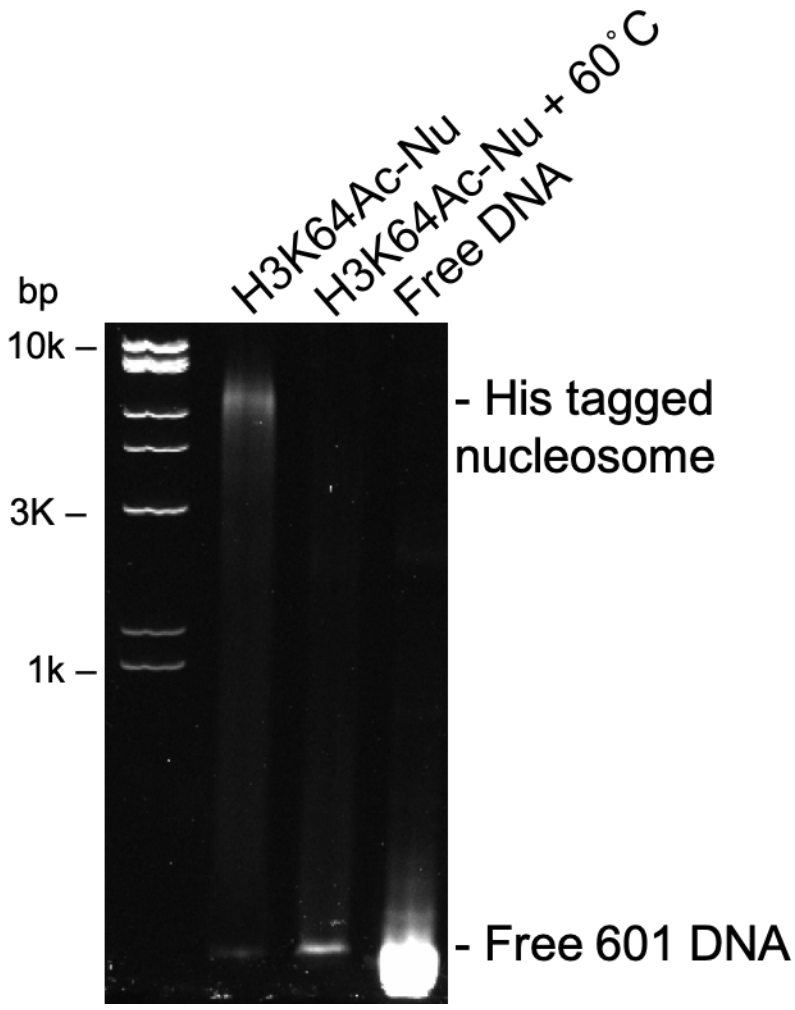
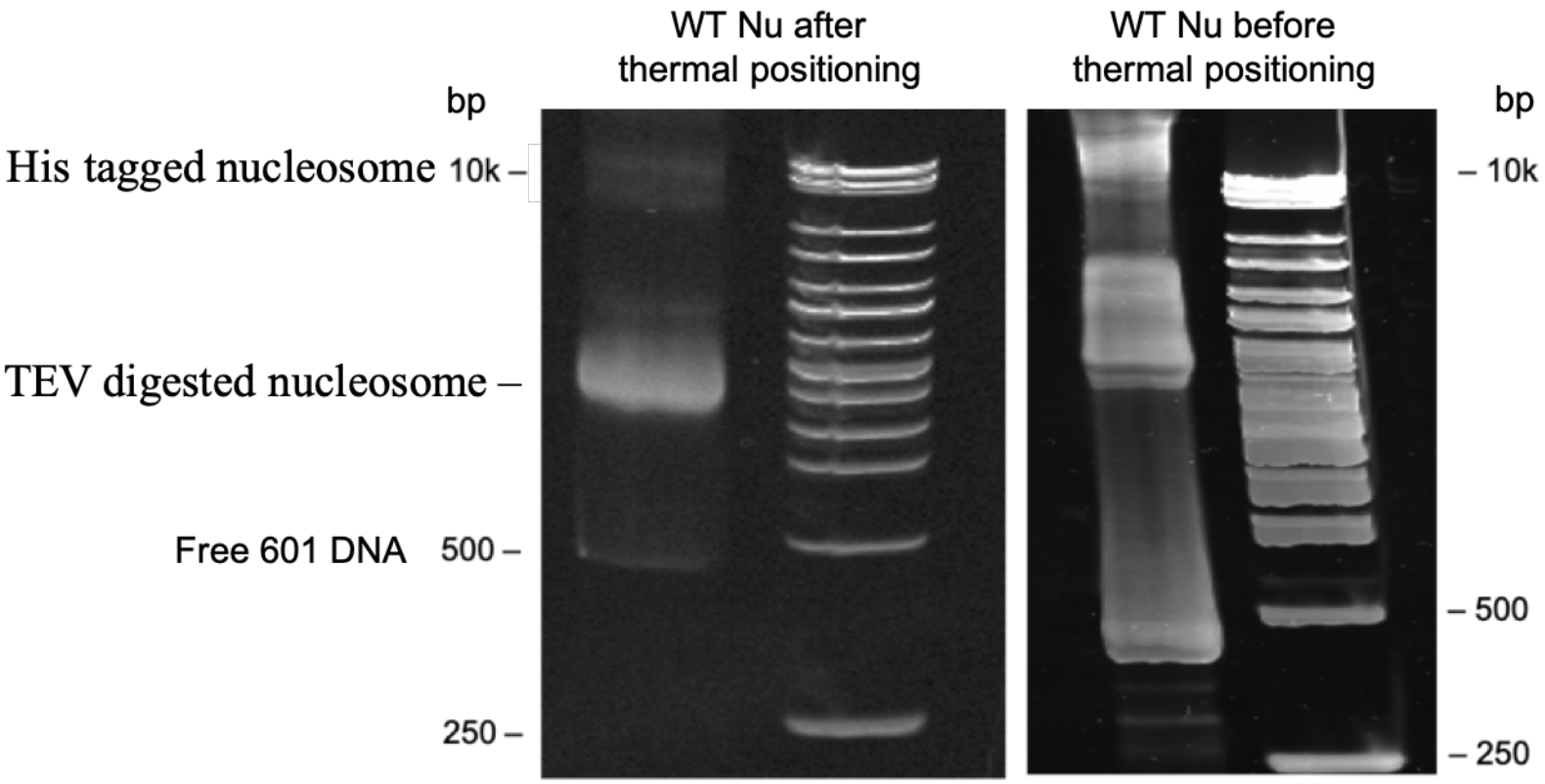


Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.5 M TE Buffer	NA	NA	0.5 M NaCl, 20 mM Tris, 1 mM EDTA, pH 7.8
1 M TE Buffer	NA	NA	1 M NaCl, 20 mM Tris, 1 mM EDTA, pH 7.8
100x TE Buffer	NA	NA	
2 M TE Buffer	NA	NA	2 M NaCl, 20 mM Tris, 1 mM EDTA, pH 7.8
20 mM TE Buffer	NA	NA	20 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 7.8
6 M GuHCl			6M guanidinium chloride, 20 mM Tris, 500 mM NaCl, pH 8.0
Acetyllysine			
Column Wash Buffer	NA	NA	6 M urea, 500 mM NaCl, 20 mM Tris, 20 mM imidazole pH 7.8
Elution Buffer	NA	NA	
Fisherbrand Variable-Flow Chemical Transfer Pump	Fischer Scientific	15-077-67	
His-TEV protease			
Histone Lysis Buffer	NA	NA	60 mM Tris, 100 mM NaCl, 0.5% Triton-X100 (v/v), 1 mM PMSF pH 8.0

Ni-NTA Resin			6 M urea, 500 mM NaCl, 20 mM Tris, 250 mM imidazole, pH 7.8
PCR Clean-Up Kit	Epoch Life Sicences	2360050	
Pellet Wash Buffer	NA	NA	60 mM Tris, 100 mM NaCl, pH 8.0
petDUET-His-SUMO-TEV-H4			
petDUET-His-TEV-H2A			
petDUET-His-TEV-H2B			
petDUET-His-TEV-H3			
pEVOL-AckRS	Addgene	137976	
pGEM-3z/601	Addgene	26656	
Storage Buffer	NA	NA	20 mM NaCl, 20 mM Tris, 20 mM NaCl, 1 mM EDTA, 20% glycerol, pH 7.8
Thermocycler			

Dear Editors and Reviewers,

We thank you very much for the insightful feedback and have addressed each point below. We believe that after revision we have greatly improved this manuscript and appreciate the consideration for publication. Our responses are below each concern outlined below.

Editor's comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have taken the time to proofread the manuscript to remove spelling errors and standardize abbreviations.

2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

We believe we have corrected this throughout the manuscript.

3. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added more detail to specific sections such as both PCR protocols and added references for more information on PCR. We have also added more details to the nucleosome assembly section to clarify the use of the peristaltic pump.

4. Section 1: Besides the general instructions, please include some specific example that can be included in the video and that will help the readers/viewers see the implementation of your protocol. For details that will not be included in the video, as stated above, please cite references.

5. Section 4: what are the PCR conditions?

As noted above, we have added details on procedure, conditions, and a few references for both sections involving PCR.

6. Section 5: lines 131-132: what do you mean by "dissolve and combine like aliquots of histone protein pellets …"?

This was meant to emphasize that all aliquots of H4 were combined, all aliquots of H3 were combined etc. We have rewritten this sentence to be more clear.

7. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

We have included the space and highlighted sections for the video.

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

In our first paragraph, we have added some details on the critical steps within the protocol including some modifications and troubleshooting of our technique as well as the major limitations. The next several paragraphs then discuss the significance with respect to existing methods and the extensive future applications of the technique. We have used some of our previous work as examples.

Reviewers' comments:

Reviewer #1:

Major Concerns:

None

Minor Concerns:

1. Line 55, describe key features of the plasmid used to express histone, such as origin, copy number and promoter.
2. Line 68, briefly describe the acetyllysine incorporation system. Origin and copy number of the plasmid? Which AckRS and tRNA mutants are used? How is the expression of AckRS and tRNA controlled, i.e. promoters? A reference is also needed here.
3. Line 106, Can the authors comment on the yield of the modified protein? How many mg of protein is obtained from 1 L culture?

This reviewer has brought up an excellent point brought up and we have added this information for all the plasmids. We have added several sentences describing each of the plasmids used as well as their origin, copy number and promoter of each. We have also added estimates of protein yield of wild type versus acetylated proteins.

Reviewer #2:

Major Concerns:

None

Minor Concerns:

For the most part, the protocol is easy to follow. I have just a few suggestions:

1. I know that there will be a table of abbreviations, but I urge the authors to define non-common abbreviations in the protocol. And, do so for relatively naïve readers. For example, pEVOL-AckRS, GuHCl and 601 DNA.

This issue was brought up by the editors and our first reviewer as well. We have gone through and defined and standardized all abbreviations as well as included a short introduction to 601 DNA and its origins.

2. Be a little more explicit. Don't make your readers assume or infer your meaning. In plasmid construction 1.1, the authors mention 4 histone protein plasmids, but these plasmids were never introduced. Also, the authors never explain how to do whole-plasmid PCR (1.2).

See our responses above. We believe we have successfully addressed these issues.

3. When the authors introduce CobB, they should mention that it is the only KDAC in E. coli. Since CobB is a sirtuin, then it makes sense that the sirtuin inhibitor nicotinamide can be used instead of a cobB deletion mutant.

This is excellent information that should be included. We have pulled a some references and included the background information on CobB and the rationale behind using nicotinamide as an alternative.

4. Please refrain from jargon. I know that "knock-out" is in common usage, but the formal term is "deletion," which is more explicit.

This terminology has been updated.

5. Under plasmid construction 3, I think the authors have made a typo - shouldn't it be 3.1 and not 1.1?

These typos have been fixed.

6. The authors mention the use of nicotinamide to inhibit the sirtuin CobB, but they never say when to add the nicotinamide nor the appropriate concentration.

We have added the appropriate concentration and note to add it with inducers.

7. On line 206, please define "extended periods."

In this case, we have added that it means more than 2 weeks.

Reviewer #3:

Major Concerns:

1. Line 61: Is it PFU DNA polymerase , PFU turbo DNA polymerase? The authors should clarify with the vendor name.

We express an in-house construct of PFU polymerase that we do not have complete sequence information for. Really any polymerase is acceptable for PCR. We have added that information to our manuscript, but is secondary to the procedure.

2. Line 160: liquid transfer pump should be changed to peristaltic pump. And it is also better to put the speed (and/or length) of no salt TE buffer.

We have added more details on how this is done. Our pump does not have a speed setting beyond the vague options of slow, fast, and purge, but we have included our specific method in the protocol with this pump.

3. Figure 1, the authors should label the lanes. I cannot see the bands of tetramers in H3K14Ac, H3K27Ac, H3K37Ac, and H3K64Ac.

We have added markers for each histone protein which should make this more clear. Some of the bands are very faint because we work at low protein concentrations once we are at this point in the protocol.

4. Figure 2, what are upper bands (~30 kDa and ~25kDa) in the H3K4Ac?

We have added protein markers. We believe the ~25kDa band is left over His6x-SUMO but it is difficult to be sure.

5. Figure 3, the authors should remove the gel well part. And it is also good to load at least one of acetylated nucleosomes as well.

This is an excellent point and we have replaced Figure 3 with an example of H3K64Ac assembled nucleosome.

6. Figure 4, there are three panels, the authors labelled after nucleosome thermal positioning in the center. What are left and right panel? The authors should describe in the legends. And it is also nicer with at least one of acetylated nucleosomes. Should we apply thermal positioning even in the destabilized acetylated nucleosome?

We have updated this figure to be more clear and removed one of the panels since it's not explicitly necessary. It's certainly possible that thermal positioning could further destabilize the nucleosome and have included an example of this in figure 3. It may not always be advised especially for acetyl lysine sites that are known to be more unstable. We have updated our protocol to include this.

We deeply appreciate the constructive feedback and opportunity to submit this manuscript for your reconsideration.

Best,

Dr. Wenshe Ray Liu & Chesley Marie Rowlett

Texas A&M University
Room 213, Chemistry Building
580 Ross St, College Station, TX 77844