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Generation of recombinant nucleosomes containing site-specific DNA damage

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Summary

Eukaryotic DNA exists in chromatin, where the genomic DNA is packaged into a fundamental repeating unit known as the nucleosome. In this chromatin environment, our genomic DNA is constantly under attack by exogenous and endogenous stressors that can lead to DNA damage. Importantly, this DNA damage must be repaired to prevent the accumulation of mutations and ensure normal cellular function. To date, most in depth biochemical studies of DNA repair proteins have been performed in the context of free duplex DNA. However, chromatin can serve as a barrier that DNA repair enzymes must navigate in order find, access, and process DNA damage in the cell. To facilitate future studies of DNA repair in chromatin, we describe a protocol for generating nucleosome containing site-specific DNA damage that can be utilized for a variety of *in vitro* applications. This protocol describes several key steps including how to generate damaged DNA oligonucleotides, the expression and purification of recombinant histones, the refolding of histone complexes, and the reconstitution of nucleosomes containing site-specific DNA damage. These methods will enable researchers to generate nucleosomes containing site-specific DNA damage for extensive biochemical and structural studies of DNA repair in the nucleosome.

Keywords

DNA damage; base excision repair; nucleosomes; chromatin

1. Introduction

DNA is constantly exposed to endogenous and exogenous stressors that lead to the accumulation of DNA damage. Oxidative stress is a primary source of DNA damage that contributes approximately 10^4 lesions per cell, per day [1]. Well over 100 different forms of oxidative DNA base lesions exist including the prevalent examples of 8-oxo-7,8-dihydro-2’-deoxyguanosine (8oxoG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), 8-oxox-7,8-dihydro-2’-deoxyadenosine (8oxoA), thymine glycol, and 5-hydroxy-2’-deoxycytidine (OH5C). In general, oxidative damage results in changes to the structure of the DNA base. These alterations increase the likelihood that the damaged base will form non-canonical base pairing that can result in the accumulation of

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genomic transversions and/or transitions [2,3]. Ultimately, these oxidative DNA lesions must be repaired to prevent the accumulation of mutations that can lead to genomic instability and several different disease states [4,5].

The base excision repair (BER) pathway is responsible for the repair of most oxidative DNA base damage, requiring efficient recognition of oxidative DNA damage [6,7]. We direct readers to other excellent reviews of BER for more details about the pathway [8–10]. Briefly, BER is initiated by one of several different DNA glycosylases, which recognize damaged DNA bases [7,11]. The DNA glycosylase then excises the damaged DNA base resulting in the formation of a baseless sugar moiety known as an apurinic/apyrimidinic site (AP-site). The resulting AP-site is then processed by AP Endonuclease 1 (APE1), resulting in a 5'-nick in the DNA backbone. The 5'-nicked substrate is then processed by two distinct enzymatic activities of Polymerase (Pol) β. The lyase activity of DNA Pol β removes the 5'-nick generating a one nucleotide gapped DNA substrate. Pol β then utilizes its DNA synthesis activity to fill the gap with a non-damaged nucleotide, generating a 3'-nick. After incorporation of the non-damaged nucleotide, the XRCC1-DNA ligase III complex seals the 3'-nick, ultimately restoring the coding potential of the DNA.

Mechanistic insight into the function of BER enzymes has largely come from *in vitro* studies using free duplex DNA, see reference [12] and associated references. However, cellular DNA often exists as chromatin, where genomic DNA is packaged into a fundamental repeating unit known as the nucleosome [13,14]. The nucleosome is composed of an octameric core of histone proteins: H2A, H2B, H3 and H4, that wraps ~147bp of DNA [15]. The robust interaction between histone proteins and the nucleosomal DNA can act as a significant barrier to BER proteins, which must be overcome to effectively complete repair of the damaged DNA. We direct the readers to several excellent reviews for further information on how nucleosome structure regulates BER enzymes [16–22]. Over the past twenty years, initial progress has been made to define how BER proteins repair DNA damage in the context of the nucleosome [23–66]. However, rapid progress has been hampered by the lack of comprehensive step-by-step protocols for generating nucleosomes containing site-specific DNA damage.

To overcome this challenge, we present a step-by-step protocol for the reconstitution of recombinant NCPs containing site-specific DNA damage. This protocol was adapted from the landmark method from Dyer and Luger et al. described over 15 years ago, as well as the many labs who have made significant progress on methods for generating damaged DNA substrates for the *in vitro* reconstitution of nucleosomes [67,65,68,64,39,69,43,70]. Initially, we describe a ligation-based method for generating a 147bp Widom 601 strong positioning DNA substrate containing site-specific DNA damage [71]. We then describe the expression and purification of human histone proteins from *E. coli* and the salt-dialysis method for refolding recombinant histone proteins into histone H2A/H2B dimers and histone H3/H4 tetramers. Finally, we describe a salt-dialysis method for reconstituting NCPs using the Widom 601 strong positioning DNA containing site-specific DNA damage, and how to purify these NCP substrates for downstream *in vitro* biochemical or structural biology experiments. Importantly, the utility of this step-by-step protocol is highlighted by recent

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work from our lab using this method to generate NCPs containing THF, enabling us to determine the structural basis for APE1 processing DNA damage in the NCP [72].

2. Materials

2.1 Instruments and equipment

1. PCR Thermal cycler
2. Thermo-Fisher Owl A-series horizontal gel system or equivalent
3. Bioreactor or Shaking incubator
4. QSonica Q500 Sonic dismembrator or equivalent
5. Stir plate
6. Gravity flow column with filter unit
7. 3,000 Da MWCO Dialysis Membrane
8. 10,000 Da MWCO Centrifugal Filter Unit
9. AKTA Pure protein purification system or equivalent
10. Superdex 200 Increase 10/300 GL or equivalent
11. Biocomp Gradient Master 108 or equivalent
12. Beckman Coulter 38.5 mL Open-Top Ultra Centrifuge tube or equivalent
13. SW 32 Ti Swinging-Bucket Rotor or equivalent
14. Optima XE-100 Ultracentrifuge or equivalent.

2.2 Reagents

1. Project specific oligonucleotides (design described in section 3.1.1)
2. Agarose
3. New England Biolabs T4 DNA ligase or equivalent
4. New England Biolabs 10X T4 DNA ligase buffer or equivalent
5. New England Biolabs 6x gel loading dye or equivalent
6. Ethidium bromide or equivalent DNA visualizing reagent
7. pET3a-histone H2A plasmid
8. pET3a-histone H2B plasmid
9. pET3a-histone H3 plasmid
10. pET3a-histone H4 plasmid
11. New England Biolabs BL21 (DE3) pLysS competent cells or equivalent
12. Novagen Rosetta™ 2(DE3) pLysS competent cells or equivalent

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13. Luria broth (LB) - Casein Digest Peptone 10g/L, Sodium Chloride 10g/L, Yeast Extract 5g/L
14. LB agar plates containing 100 μg/ml ampicillin
15. LB agar plates containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol
16. Centrum multivitamin or equivalent, 1 tablet dissolved in 50 mL ddH_2O
17. Triton X-100
18. Dimethyl sulfoxide (DMSO)
19. Cytiva Q-sepharose Fast Flow anion exchange chromatography resin or equivalent
20. Cytiva SP-sepharose Fast Flow cation exchange chromatography resin or equivalent

2.3 Buffers, Solutions, and Media

1. 1x TS: 10mM Tris (pH-7.5) and 10mM NaCl
2. 1x TBE: 90mM Tris-base, 90mM Boric Acid, and 2mM EDTA (pH-8.3)
3. 10% denaturing Urea-PAGE gel solution: 10% 29:1 acrylamide:bis-acrylamide, 8M Urea, and 1X TBE
4. 1x TE: 10 mM Tris (pH-7.5) and 1 mM EDTA
5. Gel extraction buffer: 200mM NaCl, 1mM EDTA
6. 10x M9 Minimal media pH (7.2): 335 mM Na_2HPO_4•$7H_2O$, 220 mM KH_2PO_4, 10 mM NaCl, 20mM NH_4Cl, supplemented with 0.5% glucose, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, and 1.0% vitamin solution. (See Note 1).
7. 20% Glucose solution (w/v)
8. 1 M $MgSO_4$
9. 1 M $CaCl_2$
10. 100 mg/mL Ampicillin
11. 25 mg/mL Chloramphenicol
12. 1M Isopropyl β- d-1-thiogalactopyranoside (IPTG)
13. Histone lysis buffer: 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM benzamidine, 5 mM beta mercaptoethanol, 1 mM EDTA
14. Guanidinium buffer: 20 mM Tris pH 7.5, 6 M Guanidinium hydrochloride, 10 mM DTT
15. 8 M urea
16. 8 M urea, 100 mM NaCl
17. 8 M urea, 200 mM NaCl

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18. 8 M urea, 300 mM NaCl
19. 8 M urea, 400 mM NaCl
20. 8 M urea, 500 mM NaCl
21. 8 M urea, 600 mM NaCl
22. 8 M urea, 700 mM NaCl
23. High-salt reconstitution buffer: 20 mM Tris-HCL pH 7.5, 2 M NaCl, 1 mM EDTA, 0.5 mM benzamidine, 1mM DTT (added fresh).
24. No-salt reconstitution buffer: 20 mM Tris-HCL pH 7.5, 1 mM EDTA, 0.5 mM benzamidine, 1mM DTT (added fresh).
25. 10% sucrose in 1X TE
26. 40% sucrose in 1X TE
27. 5% Native PAGE gel: 5% 59:1 acrylamide:bis-acrylamide and 0.2X TBE

3. Methods

3.1 Generation and purification of damaged DNA substrates

Several methods exist for incorporating site-specific DNA damage into the 601 strong positioning DNA sequence. These methods include oligonucleotide synthesis strategies [57,24,64,43], PCR amplification [65,37], nickase-based methods [39,40,73], and ligation-based methods [74,75,70,66]. We point the readers to an excellent and comprehensive review on the different methods for generating site-specific DNA damage for nucleosome reconstitution [76]. Here, we describe a T4-DNA ligase-based method for site-specific incorporation of the apurinic/apyrimidinic site analogue tetrahydrofuran (THF) into the Widom 601 strong positioning DNA sequence containing a 6-Carboxyfluorescein (6-FAM) label [71]. This modular methodology can be performed at large scales in a cost-effective manner, ultimately providing an optimal way of generating damaged DNA for nucleosome reconstitutions (for additional benefits of the T4 DNA ligase-based system see section 3.1.1).

3.1.1 Design of damaged DNA substrates—The T4 DNA ligase-based method described here allows for the site-specific incorporation of DNA damage within a 147 bp Widom 601 strong positioning DNA sequence. The first step is to design oligonucleotide (oligo) fragments that correspond to each strand of the 601 DNA sequence. A diagram of the oligo design can be found in Fig. 1a. For simplicity, we refer to these two DNA strands as the J-strand (non-damaged strand) and the I-strand (damaged strand, see note 2), which is modeled after the nomenclature used from the first crystal structure of a nucleosome containing the Widom 601 sequence [77]. The J-strand is split into two oligonucleotides that consist of 74 bp (J-strand oligo 1) and 73 bp (J-strand oligo 2) of DNA. The I-strand is then split into three oligonucleotides that consist of 50 bp (I-strand oligo 1), 50 bp (I-strand oligo 2), and 47 bp (I-strand oligo 3) of DNA. The length of these component oligos are kept

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under 100 bps to enable easy access to commonly used oligo synthesis companies, such as Integrated DNA Technologies. The sequences for each of the 5 oligos are as follows:

- J-strand oligo 1 –

  5′ ATCGGATGTATATATCTGACACGTGCCTGGAGACTAGGGAGTAATCCC CTTGGCGGTTAAAACGCGGGGGACAG**3′**

- J-strand oligo 2 –

  5′/Phos/ CGCGTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAG CGGCCTCGGCACCGGGATTCTCGAT**3′**

- I-strand oligo 1 –

  5′ **[FAM]**ATCGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAG CTC**3′**

- I-strand oligo 2 –

  5′/Phos/ TAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCC A**3′**

- I-strand oligo 3 –

  5′/Phos/ AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATAT**/THF/** CATCCGAT**3′**

The oligo design is such that each oligo has at least 25 bp of complimentary DNA between every J- and I-strand DNA oligo. Importantly, J-strand oligo 2, I-strand oligo 2, and I-strand oligo 3 also all contain 5′-phosphate groups at the end of the DNA that is required for ligation (Fig. 1a). When mixed at equimolar ratios and annealed, the resulting 147bp DNA sequence contains three ligation spots with a 5′-phosphate juxtaposed to a 3′-OH. The three nicks can then be sealed by T4 DNA ligase generating the full-length 147 bp DNA sequence containing DNA damage. In the example shown in Fig. 1A, the THF is the 9th position from the end of the DNA on the I-strand oligo 3. However, this ligation-based method is built to enable movement of the THF to any location on the I-strand or J-strand of the DNA, with only minor exceptions (see note 3).

The benefits of using the T4-DNA ligase-based approach are numerous. First, the relatively small oligo size means purchasing numerous oligos containing DNA damage is cost-effective, which is not the case with full-length 147 bp DNA oligos containing DNA damage. Next, the ligation reactions and purification scheme can be performed in large-scale batches enabling the generation of large quantities of damaged DNA. Next, the modularity of the system means that moving the DNA damage to different locations in the 601 DNA sequence requires simply ordering a single additional oligo. Finally, the modularity also allows for the incorporation of various fluorescent tags, protein-attachment modifications,

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and additional DNA damage types. This ultimately means that nucleosome substrates containing DNA damage can be generated for a variety of BER substrates and different *in vitro* experimental techniques.

3.1.2 Ligation of damaged DNA substrates

1. Dissolve oligos with 1x TS to a final concentration of 1 nmol/μL.
2. Mix the oligos in a 1:1 stoichiometry at a concentration of 1 nmol/μL (see note 4).
3. Anneal the oligos using a thermal cycler by heating to 95 °C before cooling to 4 °C at a rate of 0.1 °C/sec.
4. Dilute the annealed oligos to a final volume of 190 μL with H_2O and 10X T4 DNA ligase buffer (NEB).
5. Ligate the annealed DNA oligos by the addition of 10 μL of T4 DNA ligase (NEB) for a final reaction volume of 200 uL.
6. Incubate the ligation reactions at 25 °C for 24–48 hours.
7. Run a 10% denaturing Urea-PAGE gel (10% 29:1 acrylamide:bis acrylamide, 8M Urea, and 1x TBE) to monitor the progress of the ligation reaction. (Fig. 1b, see note 5).

3.1.3 Purification of damaged DNA substrates

1. Pour a 10% denaturing Urea-PAGE gel (10% 29:1 acrylamide:bis-acrylamide, 8M Urea, and 1x TBE).
2. Load the denaturing Urea-PAGE gel with equal volume of ligated DNA mixed with 6x gel loading dye.
3. Run the denaturing Urea-PAGE gel at 125 V for 1 hour.
4. Visualize the ligated DNA via the fluorescent tag, or by staining with ethidium bromide. An example gel of the ligation reaction before purification can be found in Fig. 1b.
5. Excise the ligated 147bp DNA from denaturing Urea-PAGE gel and cut the excised fragments into small pieces.
6. Extract the ligated 147bp DNA from the gel fragments by soaking in 20 mL of DNA extraction buffer for 24 hours. Repeat the extraction at least twice to ensure collection of all the damaged DNA.
7. Buffer exchange the extracted DNA with 1X TE at least 5 times using a centrifugal filter unit (10,000 Da MWCO).
8. Concentrate the extracted DNA in the centrifugal filter unit (10,000 Da MWCO) to a final concentration of 10 μM (see note 6)

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3.1.4 Annealing and storage of damaged DNA substrates

1. Anneal the purified 147bp oligos using a thermal cycler by heating to 95 °C before cooling to 4 °C at a rate of 0.1 °C/sec.
2. Determine the concentration of resuspended 147 bp DNA spectroscopically using the theoretical extinction coefficient for the respective 147 bp DNA sequence. Typical yields are ~10–15% of starting material.
3. Aliquot the 147 bp damaged DNA in 5 nmol aliquots and store frozen at −20 °C indefinitely (see note 7 and 8). An example of a purified 147bp damaged DNA can be found in Fig. 1c.

3.2 Histone expression and purification

Generating recombinant nucleosomes requires milligrams quantities of histone H2A, H2B, H3 and H4 proteins. To generate these quantities, recombinant histones are expressed in *E. coli* and purified extensively using a protocol modified from Dyer and Luger et, al. [67]. The expression, purification, and long-term storage process for each individual histone is similar, with only minor differences highlighted below.

3.2.1 Histone Plasmid Generation—The inserts corresponding to the coding region of human histone H2A (UniProt identifier: P0C0S8), H2B (UniProt identifier: P62807), H3 C110A (UniProt identifier Q71DI3), and H4 (Uniprot identifier: P62805) genes were cloned into a tagless pet3a expression vector.

3.2.2 Histone Expression

1. Perform a fresh transformation using NEB BL21 (DE3) pLysS competent cells for histone H2A, H3, and H4 growths. For histone H2B, perform a fresh transformation using Novagen Rosetta™ 2 (DE3) pLysS competent cells. Perform the transformation following the manufacturers protocol for the respective cells.
2. Plate the transformation on an LB agar plate with antibiotic specific for that histone.
    - H2A – 100 μg/ml ampicillin
    - H2B – 100 μg/ml ampicillin 25 μg/ml chloramphenicol
    - H3 – 100 μg/ml ampicillin
    - H4 – 100 μg/ml ampicillin
3. Take a streak of colonies from the transformation and inoculate 35mL of LB Broth containing antibiotics (50 ug/mL ampicillin and/or 12.5 ug/mL chloramphenicol).
4. Grow the inoculated culture in a shaking incubator at 37°C until turbid, which generally takes ~3–4 hours.

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5. Inoculate 100 mL of M9 minimal media culture containing antibiotics (50 ug/mL ampicillin and/or 12.5 ug/mL chloramphenicol) with 5 mL of the turbid culture. We typically inoculate a single 100 mL M9 minimal media culture per 5 bioreactor bottles
6. Grow the inoculated culture in a shaking incubator at 37 °C for 12–16 hours
7. Inoculate each 2 L bioreactor bottle containing 1.5 L of M9 minimal media and antibiotics (50 ug/mL ampicillin and/or 12.5 ug/mL chloramphenicol) with 15 mL of overnight culture that was inoculated and grown in steps 5 & 6.
8. Grow the large, inoculated cultures in a bioreactor at 37°C to an OD_{600}=0.4
9. Induce expression of the histone with the following IPTG (from a 1M IPTG stock) concentrations and induction duration:
   - H2A – 0.4 mM IPTG for 4 hours
   - H2B – 0.4 mM IPTG for 4 hours
   - H3 – 0.4 mM IPTG for 3 hours
   - H4 – 0.2 mM IPTG for 3 hours
10. Harvest cells by centrifugation 1,500 RCF at 25 °C in a swinging bucket rotor.
11. Resuspend each histone pellet with histone lysis buffer (15 mL per 1.5 L culture), and store frozen at −80 °C.

3.2.3 Histone Lysis and Extraction from Inclusion Bodies

1. Completely thaw the resuspended histone pellet at room temperature. This generally takes approximately one hour.
2. Dilute the resuspended cells with histone lysis buffer to a final volume of 160 mL.
3. Lyse the resuspended cells via sonication (QSonica Q500) on ice for three rounds of 10 seconds on and 50 seconds off (amplitude=90%).
4. Repeat the sonication procedure three times.
5. Transfer the 160 mL of cell lysate to 4 conical tubes.
6. Clear the cell lysate via high-speed centrifugation using a fixed angle rotor at 24,000 xG for 20 minutes at 25 °C.
7. Discard the supernatant and keep the pellet.
8. Resuspend each pellet with 25 mL of histone lysis buffer supplemented with 1% Triton X-100.
9. Centrifuge the resuspended pellet in a fixed angle rotor at 24,000 xG for 20 minutes at 25 °C.
10. Repeat step 8 and step 9.

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11. Discard the supernatant and resuspend each pellet in 25 mL of histone lysis buffer without Triton X-100.
12. Centrifuge the resuspended pellet in a fixed angle rotor at 24,000 xG for 20 minutes at 25 °C.
13. Discard the supernatant and consolidate each pellet into a beaker.
14. Add 1 mL of DMSO to the pellet and break the pellet up using a spatula.
15. Add a small stir bar to the beaker and stir the pellet for 30 minutes using a stir plate at 25 °C.
16. Add 30 mL of guanidinium buffer dropwise (in a continuous manner) to the pellet and DMSO mixture using a pasteur pipette.
17. Extract the histones from the pellet by stirring vigorously for 1 hour at 25 °C.
18. Centrifuge the solution containing extracted histones at 24,000 xG for 20 minutes at 25 °C.
19. Save the supernatant.
20. Perform a second round of histone extraction by repeating steps 14 through 19.
21. Combine the supernatant containing the extracted histones and dialyze against 8 M urea overnight (see note 9 and 10).

3.2.4 Histone Purification

1. Equilibrate 10 mL Q-sepharose Resin with 8M Urea.
2. Mix the supernatant containing the extracted histones with the equilibrated Q-sepharose resin (see note 11).
3. Incubate stirring for 30 minutes at 25 °C.
4. Add the slurry containing the extracted histones and Q-sepharose resin to a gravity-flow column.
5. Collect and save the Q-sepharose column flow-through containing the histone.
6. Wash the Q-sepharose resin with 40 mL of 8 M Urea and combine the wash with the flow-through from step 5.
7. Equilibrate 25 mL S-sepharose resin with 8 M Urea.
8. Mix the Q-sepharose flow-through and wash containing the histone with 25 mL of S-sepharose resin.
9. Add the slurry containing the extracted histones and S-sepharose resin to a gravity-flow column.
10. Wash the S-sepharose resin with 40 mL of 8 M Urea.
11. Elute the histone from the S-sepharose resin by addition of 50 mL of 8 M Urea containing increasing concentrations of NaCl. Collect each NaCl elution.

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- 8 M urea, 100 mM NaCl
- 8 M urea, 200 mM NaCl
- 8 M urea, 300 mM NaCl
- 8 M urea, 400 mM NaCl
- 8 M urea, 500 mM NaCl
- 8 M urea, 600 mM NaCl
- 8 M urea, 700 mM NaCl

12. Run a sample of each NaCl elution on a 12% SDS-PAGE gel with a protein ladder.
13. Pool fractions containing histone protein based on the molecular weight of each histone. A representative gel for the purification of each individual histone is shown in Fig. 2.
14. Pool the purified histone fractions and dialyze against 4 L of water. Exchange the water for fresh water 5 times, with at least two of those exchanges lasting overnight.
15. Aliquot into the purified histone fractions into 50 mL conical tubes and flash freeze using liquid nitrogen.
16. Lyophilize the frozen, purified histone.
17. Aliquot the lyophilized histone and store at −20°C.

3.3 Generation of H2A/H2B dimer and H3/H4 tetramer

After purifying each individual histone, histone octamers or histone H2A/H2B dimers and H3/H4 tetramers can be generated, purified, and stored for rapid reconstitution of NCPs. Below, we outline the salt-dialysis method for refolding H2A/H2B dimers and H3/H4 tetramers. We prefer refolding H2A/H2B dimers and H3/H4 tetramers instead of histone octamers due to the increased yield of in-tact complexes.

3.3.1 Refolding of H2A/H2B dimer

1. Resuspend the lyophilized H2A and H2B in guanidinium buffer to a final concentration of 2 mg/mL.
2. Incubate the resuspended H2A and H2B in guanidinium buffer at room temperature for 2 hours.
3. Determine the concentration of resuspended H2A and H2B spectroscopically using the following theoretical molar extinction coefficients:
   - H2A: 4,470 $M^{-1}cm^{-1}$
   - H2B: 7,450 $M^{-1}cm^{-1}$
4. Mix equimolar amounts of H2A and H2B in dialysis tubing (3,000 Da MWCO).

5. Dialyze against 1 L of high salt refolding buffer (ice cold, 4 °C) three times, for a minimum of 8 hours each exchange.

3.3.2 Refolding of H3/H4 tetramer

1. Resuspend lyophilized H3 and H4 in guanidinium buffer to a final concentration of 2 mg/mL.
2. Incubate the resuspended H3 and H4 in guanidinium buffer at room temperature for 2 hours.
3. Determine the concentration of resuspended H3 and H4 spectroscopically using the following theoretical molar extinction coefficients:
   - H3: 4,470 $M^{-1}cm^{-1}$
   - H4: 5,960 $M^{-1}cm^{-1}$
4. Mix equimolar amounts of histone H3 and H4 in dialysis tubing (3,000 Da MWCO).
5. Dialyze against 1 L of high salt ice cold (4 °C) refolding buffer three times, with at least two of those exchanges overnight (see note 12).

3.3.2 Purification of H2A/H2B dimer and H3/H4 tetramer

1. Concentrate the refolded H2A/H2B dimer and H3/H4 tetramer to ~100 μM (see note 13).
2. Purify H2A/H2B dimer or H3/H4 tetramer over a Superdex 200 Increase 10/300 GL gel filtration column.
3. Confirm the purity and stoichiometry of the H2A/H2B dimer or H3/H4 tetramer by running an SDS PAGE gel of the S200 gel filtration fractions (Fig. 3, see note 14).
4. Combine fractions containing purified H2A/H2B dimer or H3/H4 tetramer and concentrate using a centrifugal filter unit (10,000 Da MWCO) to at least 100 μM.
5. Mix the concentrated H2A/H2B dimer and H3/H4 tetramer with an equal volume of 100% glycerol and store indefinitely at −20 °C.

3.4 Reconstituting nucleosomes containing DNA damage

NCP reconstitution is done via a salt dialysis method using the damaged DNA, H2A/H2B dimer, and H3/H4 tetramer previously purified. After nucleosome reconstitution, the nucleosomes containing DNA damage are purified by sucrose gradient ultracentrifugation to separate free linear DNA and other subnucleosomal species, which makes them suitable for quantitative *in vitro* experiments.

3.4.1 Nucleosome reconstitution

1. Thaw the damaged DNA, H2A/H2B dimer, and H3/H4 tetramer on ice.

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2. Determine the concentration of the damaged DNA spectroscopically using the theoretical molar extinction coefficient for the specific DNA substrate.

3. Dilute the damaged DNA to a concentration of 2.5 μM using high salt buffer.

4. Determine the concentration of H2A/H2B dimer and H3/H4 tetramer spectroscopically using the following theoretical molar extinction coefficients:

   - H2A/H2B dimer: 11,920 $M^{-1}cm^{-1}$
   - H3/H4 tetramer: 20,860 $M^{-1}cm^{-1}$

5. Dilute the H2A/H2B dimer to 5 μM and H3/H4 tetramer to 2.5 μM using high salt buffer.

6. Mix the damaged DNA, H2A/H2B dimer, and H3/H4 tetramer in a 1:2:1 molar ratio and place in dialysis tubing (10,000 Da MWCO).

7. Place the dialysis tubing in a beaker containing 300 mL of high salt buffer and equilibrate for 30 minutes.

8. Dilute the high salt buffer with no salt buffer stepwise to the following concentrations:

   - 1.5 M NaCl, 150 mL of no salt buffer, 2 hours
   - 1 M NaCl, 150 mL of no salt buffer, 2 hours
   - 0.75 M NaCl, 300 mL of no salt buffer, 2 hours
   - 0.5 M NaCl, 300 mL of no salt buffer, 2 hours
   - 0.25 M NaCl, 1200 mL of no salt buffer, overnight
   - 0.125 M NaCl, 2400 mL of no salt buffer, 2 hours

3.4.2 Nucleosome Purification

1. Remove the reconstituted nucleosome from the dialysis tubing and place in a conical tube.

2. Concentrate the nucleosome down to ~0.5 mL using a centrifugal filter unit (10,000 Da MWCO).

3. Heat-shock the nucleosome at 55 °C for 30 minutes (see note 15). A representative gel of the nucleosome prior to purification can be seen in Fig. 4a.

4. Make six 10 – 40% sucrose gradients in 38.5 mL Beckman Coulter Ultra Centrifuge tubes using a Biocomp Gradient Master 108 (see note 16).

5. Layer the 0.5 mL of damaged nucleosome to the top of a sucrose gradient.

6. Ultracentrifuge the sucrose gradients at 125,000 xG for 40–42 hours in an SW 32 rotor at 4°C.

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7. Fractionate the sucrose gradient containing nucleosome by pulling 1 mL from the top of the tube and storing in 1.5 mL eppendorf tubes.

8. Measure the absorbance at 260 nm for each fraction to identify those that contain nucleic acids and run the fractions containing nucleic acid on a Native PAGE gel (5% 59:1 acrylamide:bis-acrylamide and 0.2X TBE). A representative chromatogram of the nucleosome fractions after sucrose gradient ultracentrifugation can be seen in Fig. 4b.

9. Pool the fractions that contain purified nucleosome and buffer exchange 5 times into 1X TE using a centrifugal filter unit (10,000 Da MWCO). A representative gel of the purified nucleosome containing DNA damage can be seen in Fig. 4c.

10. Concentrate the nucleosome to at least 1 μM and store at 4°C (see note 17 and 18).

4. Notes

1. The glucose, $MgSO_4$, $CaCl_2$, and vitamin solution are added to the M9 media after autoclaving as they can precipitate and/or degrade during autoclaving.

2. The DNA damage can be placed on either I-strand or J-strand depending on the desired location in the nucleosome. If moving the DNA damage to the J-strand, we suggest changing the J-strand to three oligos and the I-strand to two oligos

3. Avoid placing the THF or other DNA damage within 5 bp of any T4-DNA ligase sites. If unavoidable, the reactions will result in a significantly reduced yield of full-length ligated 147 bp DNA.

4. We perform each individual reaction with 10 nmols of each individual oligo, which should theoretically yield 10 nmols of final ligated product (assuming 100% yield). An average prep size is ~50–100 nmols, or 5–10 individual reactions.

5. The ligation reaction generally does not reach 100% completion. If desired, an additional 5uL of T4 DNA ligase can be added after the first 24 hours to ensure as much of the DNA substrate has been ligated as possible.

6. Concentrating the extracted DNA to 10 μM enables efficient annealing of the I- and J-strand oligos in the subsequent annealing step.

7. The purified damaged DNA should be stored in aliquots that match the average prep size of the downstream nucleosome reconstitution. We typically perform 5 nmol nucleosome reconstitutions.

8. We have stored damaged DNA substrates for up to a year without issues. After long-term storage, we suggest checking the quality of the DNA on an agarose gel before proceeding to nucleosome reconstitution.

9. Urea can spontaneously degrade into isocyanic acid, which reacts with lysine and arginine side chains to form carbamylation [78]. To prevent carbamylation

of histones during purification, 8M urea solutions are made fresh and de-ionized with a Dowex Amberlite resin.

10. The extracted histones should be dialyzed against enough 8M Urea to bring the concentration of guanidinium-HCl below 250 mM. This step is critical for the subsequent purification of the histone via ion-exchange chromatography as the histones will not bind the S-sepharose resin above 300 mM NaCl.

11. The Q-sepharose is used to bind contaminating proteins from the histone extraction step.

12. It is not uncommon to see precipitant forming in the dialysis membrane after the first 24 hours spinning in the high salt buffer. This is often a result of impurities in the purification of the individual histones.

13. The H2A/H2B dimer and H3/H4 tetramer is concentrated to no more than 100 μM to ensure separation from contaminants during gel filtration.

14. It's not uncommon to see minor contaminates in the dimer or tetramer. In our experience, these can be removed at subsequent purification steps, and do not significantly affect reconstitution of the nucleosome.

15. Purifying the damaged DNA via denaturing PAGE can lead to an excess of either the I- or J-strand. Excess I- or J-strand ssDNA will readily form histone octamers wrapped by single-stranded DNA [79]. The heat-shock at 55 °C will ensure the nucleosome is properly positioned on the 601 DNA, while also denaturing any ssDNA-histone octamer complexes that are difficult to purify via sucrose gradient ultracentrifugation.

16. Another method for purifying nucleosomes is via electrophoresis using a Bio-Rad Model 491 Prep Cell [80].

17. The nucleosome concentration is determined spectroscopically by diluting 2x with 4 M NaCl and measuring the absorbance at 260 nM. Theoretical extinction coefficients should be determined using the damaged DNA sequence.

18. The purified nucleosomes should be stored at 4°C in 1x TE at a concentration of at least 1 μM. The purified nucleosomes should be stable for at least 1 month. For longer term storage (>1 month), we suggest storing the purified nucleosomes at 4°C in 1x TE at a concentration of at least 10 μM.

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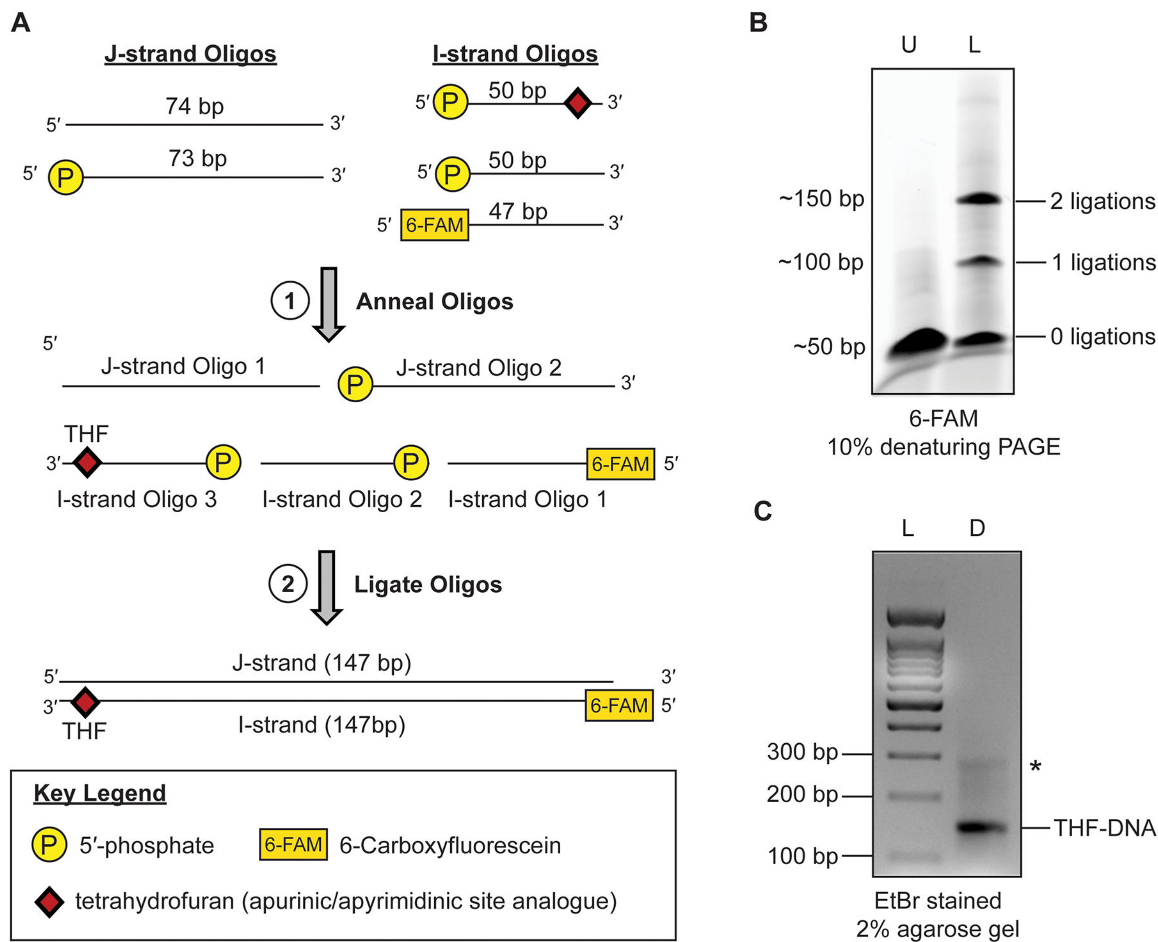

Fig 1.

Generation and purification of damaged DNA substrates using the ligation-based method. (a) Diagram for the individual oligos generation of damaged DNA substrates. (b) A representative 10% denaturing PAGE gel for the ligation reaction of. Lane ‘U’ is the starting unligated reaction and lane ‘L’ is the ligated reaction. The DNA weas visualized using the 6-FAM label. (c) A representative 2% agarose gel of the purified and annealed damaged DNA substrate. Lane ‘L’ is a 100bp DNA ladder and lane ‘D’ is the purified and annealed damaged DNA. The DNA was visualized with ethidium bromide. The * denotes a minor contamination that is commonly seen after purification of the ligated DNA.

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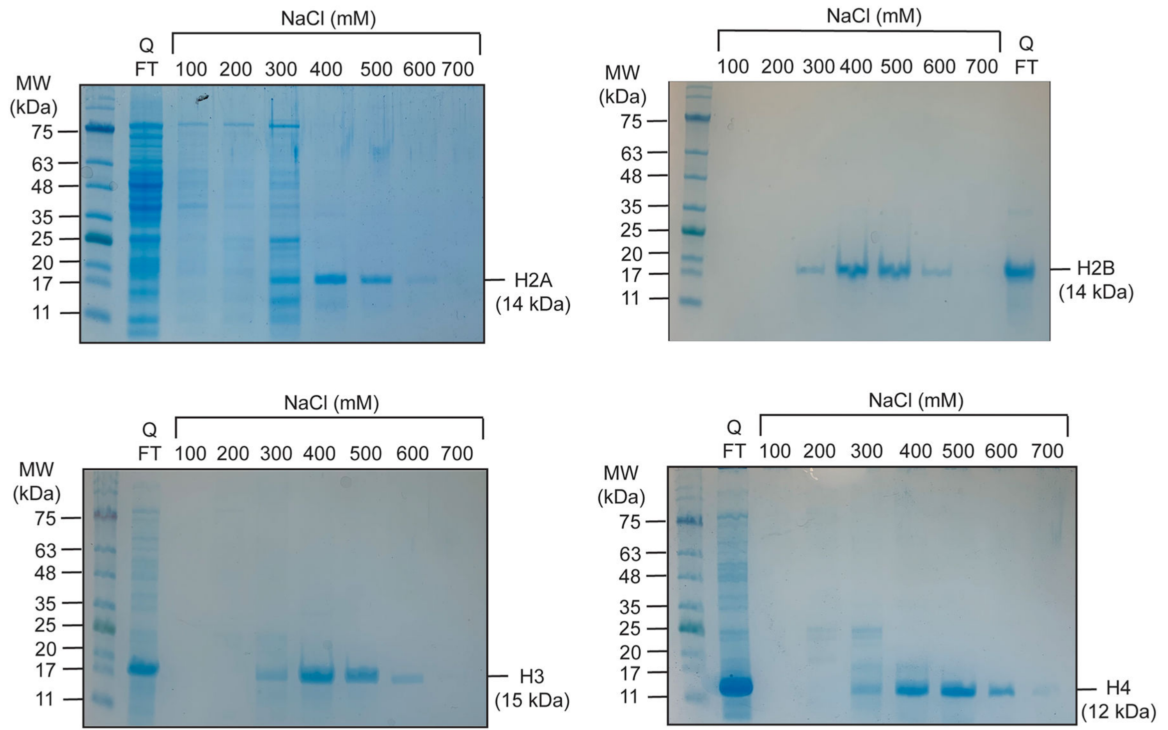

Fig 2.

Representative 12.5% SDS-PAGE gels for the purification of histone H2A (top left), H2B (top right), H3 (bottom left), and H4 (bottom right). The proteins were visualized by coomassie blue staining.

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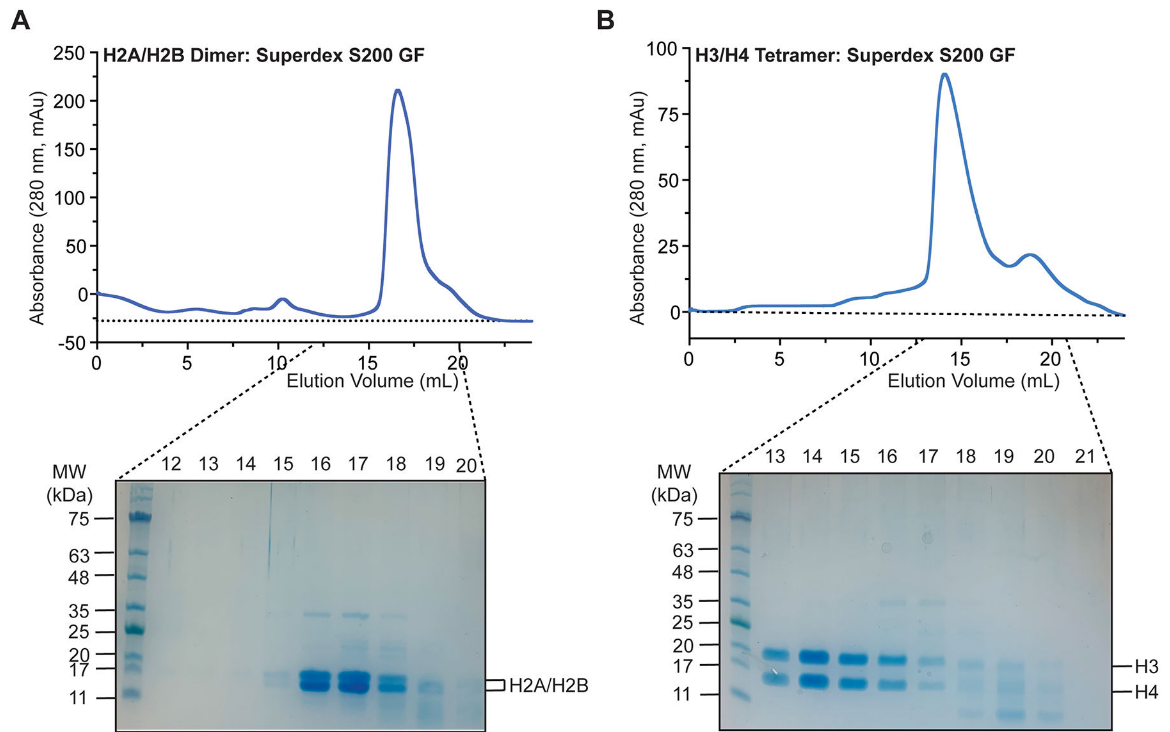

Fig 3.

Purification of H2A/H2B dimer and H3/H4 tetramer. (a) Representative H2A/H2B dimer chromatogram from a Superdex S200 gel filtration run. The resulting fractions were resolved on a 12.5% SDS-PAGE gel and visualized by coomassie blue staining. (b) Representative H3/H4 tetramer chromatogram from a Superdex S200 gel filtration run. The resulting fractions were resolved on a 12.5% SDS-PAGE gel and visualized by coomassie blue staining.

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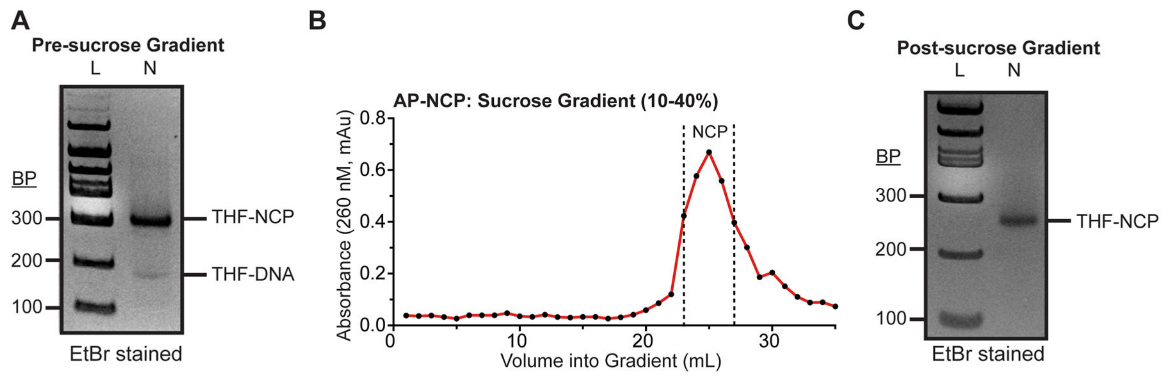

Fig 4.

Generation and purification of nucleosomes containing site-specific DNA damage. (a) A representative 6% Native PAGE (59:1) gel of the reconstituted nucleosome before purification (b) A representative chromatogram of the nucleosome after fractionation from a 10–40% sucrose gradient. (c) A representative 6% Native PAGE (59:1) gel of the reconstituted nucleosome after purification.