[*This is a protocol for inclusion in CPMB. There are 7 figures and 2 tables. A PMC file is available]

Article Title

Reconstitution and purification of nucleosomes with recombinant histones and purified DNA

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Conflict of Interest Statement

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ABSTRACT:

Nucleosomes are substrates for a broad range of factors, including those involved in transcription, chromosome maintenance/reorganization, and enzymes that covalently modify histones. Given the heterogenous nature of nucleosomes *in vivo* (i.e. histone composition, post-translational modifications, DNA sequence register), understanding the specificity and activities of chromatin-interacting factors has required *in vitro* studies using well-defined nucleosome substrates. Here, we provide detailed methods for large-scale PCR preparation of DNA, assembly of nucleosomes from purified DNA and histones, and purification of DNA and mononucleosomes. Such production of well-defined nucleosomes for biochemical and biophysical studies is key for studying numerous proteins and protein complexes that bind and/or alter nucleosomes, and for revealing inherent characteristics of nucleosomes.

Basic Protocol 1: Generation of DNA fragment by large scale amplification via PCR

Basic Protocol 2: DNA and nucleosome purification using a Bio-Rad MiniPrep Cell/Prep Cell

Basic Protocol 3: Nucleosome reconstitution via linear gradient salt dialysis

KEYWORDS:

Nucleosome purification; Nucleosome reconstitution; large-scale PCR; histone octamer; DNA

INTRODUCTION:

Due to their central role as a regulators and carriers of epigenetic information for eukaryotic genomes, nucleosomes are widely studied and intersect with many systems. *In vivo*, nucleosome- and DNA-binding factors are faced with an incredible complexity of nucleosome substrates, arising from myriad combinations of histone and DNA modifications, variations in histone composition, and multiple positions of histones on DNA (Jiang & Pugh, 2009; Allis & Jenuwein, 2016; Atlasi & Stunnenberg, 2017). Whereas sequencing-based methods provide an increasingly clear picture of the genomic locations where nucleosomes, specific modifications, and bound factors are found *in vivo* (Jiang & Mortazavi, 2018; Klein & Hainer, 2020), *in vitro* studies are still required to reveal the biochemical characteristics and mechanisms of chromatin-interacting systems and their nucleosome substrates (Kornberg, 2000).

For *in vitro* biochemical and structural studies, mononucleosomes have been an essential reagent that has led to a better understanding of both the inherent properties of histone-DNA interactions as well as how nucleosomes can be recognized, manipulated, mobilized, and modified by a host of cellular factors (McGinty & Tan, 2015; Venkatesh & Workman, 2015; Zhou et al., 2018; Sundaram & Vasudevan, 2020). Nucleosomes present a natural barrier to transcription and, thus, how nucleosomes affect and are recognized by

transcriptional machinery continues to be a major area of interest. To allow RNA polymerases to transcribe through, nucleosomal DNA must be unwrapped from the histone core, temporarily disrupting canonical histone-DNA interactions (Kujirai & Kurumizaka, 2020). During such disruptive processes, nucleosomes intimately associate with histone chaperones, which assist in both histone eviction and deposition (Gurard-Levin et al., 2014; Liu et al., 2020). Nucleosomes also unwrap spontaneously in the absence of outside factors, providing transcription factors brief access to their otherwise buried binding sites (Li & Widom, 2004; Li et al., 2005; Wei et al., 2015). Illustrating the complex interplay between nucleosomes and transcription factors, nucleosomes can be partially unwrapped by transcription and pioneer factors (Li et al., 2005; Dodonova et al., 2020), yet rewrapping of nucleosomal DNA dramatically accelerates dissociation of some DNA-binding proteins (Luo et al., 2014). The intrinsic dynamics of DNA unwrapping can also be strongly influenced by DNA sequence (Ngo et al., 2015) and the substitution of particular histone variants (Bao et al., 2004; Stumme-Diers et al., 2018). Though DNA is often described as being tightly held in place by the histone core, nucleosomal DNA displays intrinsic translational positioning dynamics that are amplified and capitalized on by a number of systems, including chromatin remodelers (Clapier et al., 2017; Sabantsev et al., 2019), DNA repair machinery (Matsumoto et al., 2019), transcription factors (Rudnizky et al,. 2019), and viral integrases (Wilson et al., 2019). Nucleosomes also provide hubs for architectural factors, which – through substitution of histone variants that allow targeting of specific factors – are critical for nucleation of large cellular structures like the centromere (Yan et al., 2019). Nucleosomes are also subject to a rich diversity of post-translational modifications, which not only provide

unique environments for code readers, but also influence activities of downstream enzymes (Worden et al., 2019; Schmitges et al., 2011) and DNA dynamics of nucleosomes themselves (Simon et al., 2011; North et al., 2012; Bowman & Poirier, 2015). For these diverse systems, mononucleosomes have been instrumental for revealing biophysical and biochemical properties through numerous *in vitro* methods, including crystallography, cryo-electron microscopy (cryo-EM), single-molecule techniques (Förster resonance energy transfer (FRET) and force spectroscopy), mass-spectroscopy, NMR, cross-linking, footprinting, and other enzymatic, spectroscopic, and gel-based techniques.

For most *in vitro* studies, nucleosomes are made using positioning sequences that bias the placement of the histone octamer on DNA. Common positioning sequences used are the Widom 601 (Lowary & Widom, 1998), MMTV, 5S rDNA, and an alpha satellite derivative (Flaus, 2011). Even with strong positioning sequences, however, nucleosomes can occupy multiple positions on DNA, with each sequence typically giving a characteristic pattern of major and minor species. In addition to off-target species, nucleosomes reconstituted *in vitro* are often accompanied by free DNA and hexasomes, which lack one H2A/H2B histone dimer. Given the dependence of *in vitro* experiments on homogeneous preparations of nucleosomes, highly purified nucleosomes are often critical for obtaining structural, kinetic, and mechanistic insight into nucleosomes and chromatin-interacting factors.

Here, we describe methods for producing and purifying mononucleosomes in four basic steps. The first step is the production of double-stranded DNA fragments (145 to ~250 bp) via PCR (Basic Protocol 1). PCR-based amplification allows facile incorporation of biotin and

fluorescently-labeled DNA probes that enable basic nucleosome properties —such as positioning, DNA unwrapping, and complex formation— to be followed by numerous biochemical and single-molecule techniques. The second step is purification of DNA via acrylamide gel electrophoresis using a Bio-Rad MiniPrep or Prep Cell apparatus (Basic Protocol 2), and the third step is reconstitution of nucleosomes from purified histones and DNA using linear gradient salt dialysis (Basic Protocol 3). Finally, nucleosomes are purified using the same MiniPrep/Prep Cell apparatus as for DNA (Basic Protocol 2). The gel-based purification method described here allows mononucleosomes with distinct positions on DNA to be separated for subsequent biochemical and biophysical studies (Figures 1 and 2).

[*Figure 1 near here]

[*Figure 2 near here]

STRATEGIC PLANNING:

The protocol for nucleosome reconstitution (Basic Protocol 3) requires histone octamer and dimer to be purified ahead of time. Histones can either be expressed in inclusion bodies, purified separately and then refolded together (Luger et al., 1999), or may be co-expressed as soluble dimers, tetramers, or octamers (Anderson et al., 2010; Shim et al., 2012; Turco et al., 2015; Ivić et al., 2016). After purification and refolding, our lab concentrates histone octamer to 50–200 μM and H2A/H2B dimer to ~200 μM and then flash freezes small

aliquots (10-20 µl) in liquid nitrogen in buffer containing 2 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol.

When planning *in vitro* reconstitution of mononucleosomes, a major consideration is the DNA sequence to be used. Is a particular natural DNA sequence necessary, or can a nucleosome positioning sequence be used? With strong positioning sequences, nucleosomes reproducibly form in a limited number of distinct translational positions on DNA (Flaus, 2011). A popular nucleosome positioning sequence is the Widom 601 (Lowary & Widom, 1998), which strongly prefers a single unique octamer position on DNA. When a single translational position of a nucleosome is desired, nucleosomes made with positioning sequences produce the highest yields. A related question is whether the DNA needs to contain a particular element, such as a recognition sequence (as for a DNA-binding factor) or DNA modification (fluorescent labels or other chemical additions/alterations such as biotin, methylation, lesions, or single-stranded DNA gaps). If so, the accessibility of the DNA element will be affected by the rotational phasing and translational positioning of the DNA with respect to the octamer. Although the unique positioning of the Widom 601 sequence can be disrupted by sequence changes (for example, see Figure 2C), the 601 has allowed for placement of DNA-binding sites, DNA lesions, internal biotin moieties, and fluorescent dyes at defined locations on the nucleosome (Li et al., 2005; Matsumoto et al., 2019; Nodelman et al., 2017; Sabantsev et al., 2019;).

In some cases, it may be desirable to enrich for hexasomes, as these allow for production of asymmetric nucleosomes. Unlike nucleosomes, hexasomes lack one of the two H2A/H2B

dimers, which results in unwrapping of ~40 bp of DNA from the side lacking the dimer. Hexasomes can be transformed into nucleosomes with addition of H2A/H2B dimers, either before (Dao et al., 2020) or after purification (Levendosky et al., 2016; Levendosky & Bowman, 2019). Since the H2A/H2B dimer added to hexasomes need not be the same as the sole H2A/H2B dimer already present in the hexasome, the resulting nucleosomes can contain H2A/H2B dimers with distinct protein sequences (for example, a mutant or histone variant) or modifications (for example, a post-translational modification like ubiquitin or fluorescent dye) on the two sides of the nucleosome (Dao et al., 2020; Levendosky et al., 2016; Levendosky & Bowman, 2019; Sabantsev et al., 2019). Hexasomes migrate more rapidly than end-positioned nucleosomes in native acrylamide gels, yet for the Widom 601, the amount of separation between hexasome and end-positioned nucleosome depends on which side of the 601 contains the flanking DNA (Levendosky et al., 2016).

BASIC PROTOCOL 1: Generation of DNA fragment by large scale amplification via PCR

This protocol explains how to perform a large-scale PCR reaction and prepare the PCR product for subsequent purification. Here, a 10 ml PCR reaction is described that yields suitable amounts of a duplex DNA fragment for subsequent reconstitution into mononucleosomes. The ratio of reagents is similar to that used for a standard 100 µl reaction, but made in a larger volume master mix that is then distributed to individual tubes for the PCR reaction. Reactions are subsequently pooled and concentrated for purification (Basic Protocol 2). For biochemical studies, we routinely prepare 5-20 ml master mix reactions, which yield a total of 200-1200 µg DNA, or 40-60 µg of DNA per ml of the PCR master mix.

For the DNA template, we and many others typically use nucleosome positioning sequences such as the Widom 601 (Lowary & Widom, 1998), but any sequence may be used. For producing mononucleosomes, DNA fragments are typically between ~145 and ~250 bp in length, with ~145 bp being the minimal length needed for completely wrapping the histone octamer. Nucleosomes made with this minimal length, called nucleosome core particles, are often referred to as 0N0. With strong positioning sequences such as the Widom 601, nucleosomes can be given specific lengths of flanking DNA on one or both sides because the DNA sequence determines where the histone octamer will preferentially deposit (see Tan & Davey, 2011). With such strong positioning sequences, mononucleosomes can be made with specific lengths of flanking DNA on one or both sides, allowing one to create so-called end-positioned and centered mononucleosomes such as 80N0 or 40N40.

For the primers, we recommend that they match the template for at least 28-30 nucleotides. Primers offer an easy means for introducing sequence alterations and chemical moieties at defined locations. For most gel-based applications, a fluorescent probe at the 5' end (e.g. /56FAM/ for IDT primers) allows nucleosomes to be easily visualized. Other common primer modifications include biotinylation, useful for tethering DNA/nucleosomes to streptavidin-coated quartz slides for single-molecule studies, and Cy3 and Cy5 dyes for bulk or single-molecule FRET (Li et al., 2004; Yang & Narlikar, 2007; Blosser et al., 2009).

Materials:

Ultrapure water (e.g. Milli-Q purified)

10x NEB ThermoPol buffer (see Reagents and Solutions)

100 mM $MgSO_4$

10 mM dNTPs (2.5mM of each nucleotide; Thermo Fisher Scientific, cat #10297018)

100 µM DNA primer, forward direction (custom ordered from, for instance, Integrated DNA Technologies)

100 µM DNA primer, reverse direction

100 ng/µl DNA template

Taq polymerase (e.g. NEB cat #M0273; or produced in-house and diluted such that 10 µl/ml of PCR master mix gives optimal activity)

Agarose (Bio-Rad cat #16500500)

100 bp ladder (NEB cat #N3231)

6x DNA loading buffer (see Reagents and Solutions)

40% sucrose (w/v; sterile filtered; Fisher #S5-500)

Conical tubes, 15 ml (Falcon Conical tubes cat #352196)

0.2ml PCR 8-tube strips (USA Scientific cat #1402-4700)

Thermocycler (preferably with ≥ 96 tube capacity, e.g. Bio-Rad T100)

Agarose gel electrophoresis system, e.g.

Gel box: Bio-Rad Mini-Sub Cell GT Cell

Gel tray: Subcell GT UV Mini-gel tray 7x10 cm (Bio-Rad #1704435)

1.5 mm 8-well comb (Bio-Rad #1704462)

Power supply (e.g. Bio-Rad PowerPak Basic #1645050)

Refrigerated Centrifuge (e.g. Sorvall Legend XTR with swing bucket rotor for concentrators)

Spin concentrators, 10 kDa MWCO concentrators

- 0.5 ml capacity, Amicon Ultra-0.5ml (cat # UFC501096)
- 4 ml capacity, Amicon Ultra-4 (cat #UFC801024)
- 15 ml capacity, Amicon Ultra-15 (cat #UFC901024)

Microcentrifuge (e.g. Eppendorf 5424), at 4˚C (in cold room)

Protocol Steps

Test and optimize PCR conditions in 100 µl single reactions

1. Perform a few small-scale PCR reactions. These test reactions will ensure that, before moving on to a larger scale, all components produce sufficient DNA. In thin-walled 0.2 ml tubes, make several 100 µl test reactions using the amounts (and order) of each reagent given in Table 1.

   One component that can have a significant effect is the concentration of $MgSO_4$, which we suggest varying in test reactions. The amount of Taq can also be titrated to optimize best yield of target product while keeping unwanted products to a minimum.

[* Table 1 near here]

2. Run the following PCR program for the 100 µl test reactions:

Initial step:	1 min	95˚C (denaturation)
40 cycles:	30 s	95˚C (amplification)
	30 s	55˚C
	1 min	72˚C
Final step:	10 min	72˚C (elongation)

3. Evaluate the quantity and quality of product by analyzing PCR reactions on a 1.5% agarose gel in 1x TBE. Load 10 µl PCR sample plus 1x DNA loading buffer. Successful test reactions should yield a single DNA product of correct size at approximately 0.5 µg/lane (estimated by eye, using the ladder as a reference).

Perform large scale reaction

4. Once the conditions are optimized, prepare a 10 ml PCR master mix. In a 15 ml conical tube, first combine all reagents except Taq polymerase, in the order given in Table 1. Mix thoroughly by inverting tube multiple times and/or vortexing.

   This reaction may be scaled from 5 ml to 20 ml, depending on the tube capacity of the thermocycler (e.g. 96 vs 384 wells) and amount of DNA product desired. If more DNA is required, PCR can be repeated consecutively in volumes equal to the thermocycler capacity. We have prepared 100-140 ml of PCR in 20 ml batches. We expect final purified yields of DNA to be 40-60 µg per ml of PCR master mix.

5. Next, add Taq polymerase to PCR mix.

   Since the Taq is stored in high concentrations of glycerol, use a wide-bore pipet tip or, alternatively, cut off the bottom third of a (sterile) pipet tip with a clean razor blade. This will allow for more accurate and rapid pipetting of Taq. Add Taq into the PCR master mix, gently pipetting up and down until no residue remains

on the pipette tip. Additionally, since the Taq will settle to the bottom of the master mix, thoroughly mix by inverting the tube ~30 times. Failure to completely mix will lead to poor yields.

6. Aliquot master mix into 0.2 ml thin-walled strip tubes. For a 10 ml reaction in a 96 well thermocycler, aliquot 105 µl per tube. Cap tubes and place in thermocycler.
7. Run the PCR program given in step 2 above.

   This will take approximately 2.25 hours.

8. Once the thermocycler program has finished, transfer the PCR product from each tube to a fresh 15 ml conical tube.

   At this stage, the sample can be stored at -20˚C for later processing.

9. *Optional:* Run 10-40 µl PCR product on agarose gel along with 100 bp ladder to confirm that the amount and size of the major product is as expected.

Prepare DNA for purification

10. Using spin concentrators, concentrate the PCR product to these target volumes:
    - For MiniPrep Cell = 50-60 µl
    - For Prep Cell, 28 mm column = 100 µl
    - For Prep Cell, 37 mm column = 600 µl-1 ml

With large amounts of PCR (>40 ml), the Taq enzyme can start precipitating and interfere with concentration. If this occurs, remove the sample to 1.5 ml microfuge tubes and give a 1 min hard spin to pellet the Taq.

When using spin concentrators, spin below the maximum recommended g-force to avoid sample loss. We routinely use an Amicon Ultra-4 (for 5-20 ml) or Amicon Ultra-15 (for 20 ml or more). To reach a final volume of 50 µl, we typically use Amicon Ultra-0.5ml spin concentrators. When the sample has been reduced to the desired volume, transfer to a fresh microfuge tube, and then rinse the concentrator membrane. For the MiniPrep Cell, rinse the membrane with up to 20 µl, and for the Prep Cell (28 mm column), with up to 50 µl. Since the rinse will increase volume, for the MiniPrep Cell, we recommend using 40% w/v sucrose to wash the membrane. For the Prep Cell, we normally use eluate for rinsing.

11. Add 40% w/v sucrose to the DNA sample to achieve a final concentration of 6%. Vortex and then pulse-spin in microfuge. Final volumes for DNA purification should be in the range listed:
    - For MiniPrep Cell = ~80 µl
    - For Prep Cell, 28 mm column = ~180-250 µl
    - For Prep Cell, 37 mm column = ≥ 1.2 ml
12. Samples can be stored on ice for same-day purification (Basic Protocol 2), or at -20˚ C if purification will be carried out one or more days later.

BASIC PROTOCOL 2: *DNA and nucleosome purification using a Bio-Rad MiniPrep Cell/Prep Cell*

After production of a DNA fragment (Basic Protocol 1), the next step is to purify the desired DNA by electrophoresis through a native acrylamide gel column. Note that the same procedure is also used after reconstitution of nucleosomes (Basic Protocol 3); therefore, here we describe the purification protocol for both DNA and nucleosomes, noting differences where appropriate. DNA and nucleosomes migrate through gels based on their size, shape, and charge. For DNA, purification by native acrylamide gel allows separation of different sized PCR products and primers, as well as DNA fragments digested from plasmids containing multiple copies of nucleosome positioning sequences. Purified DNA will enable more accurate concentration measurements needed to attain good yields from nucleosome reconstitutions. For mononucleosomes, gel-based purification enables separation of nucleosomes with distinct positions of the histone core, hexasomes, and free DNA. Due to differences in shape, mononucleosomes with extra DNA on one side (e.g. 0N80) migrate more quickly than when DNA is distributed on both sides (e.g. 20N60 or 40N40) (Meersseman et al., 1992).

The purification protocol described here utilizes either a MiniPrep Cell or Model 491 Prep Cell apparatus, manufactured by Bio-Rad. The user polymerizes acrylamide in a central glass tube, and then electrophoreses a sample through this gel column. The MiniPrep/Prep Cell apparatus is designed to recover samples once they elute off the column, with a channel for

buffer to carry samples from the base of the column to an external fraction collector (Figure 3). In this way, a complex mixture can be fractionated based on its migration speed through the gel.

The main consideration for whether the MiniPrep Cell or Prep Cell should be used is the amount of sample to be purified. As a rough guideline, the MiniPrep Cell should be used for nucleosome reconstitutions in the range of 1 nmol or less (Figure 1) or ≤ 10 ml PCR of DNA. For larger amounts, the Prep Cell should be used to avoid overloading, which will result in poorer resolution. The Prep Cell has two different diameter columns, 28 mm and 37 mm. For the 28 mm gel column, we routinely purify 2.5-11 nmol (reconstitutions containing 300 µg-1.5 mg of DNA) of reconstituted nucleosomes. For the 37 mm gel column, we have successfully purified nucleosomes from reconstitutions containing 17 nmol (2 mg of DNA) of a mixed species nucleosome sample, to as high as 74 nmol (10 mg of DNA, originating from a plasmid harboring a 601 array) of a well-behaved 601 nucleosome sample (Figure 2). When using both small and large diameter gel columns of the Prep Cell, a central cooling core is present when the acrylamide gel is poured. The cooling core is used in conjunction with a recirculation pump to keep the gel cooled during polymerization and during electrophoresis of the sample. Due to the addition of the cooling core/recirculation pump, as well as other design differences, the Prep Cell has a more involved set up than the MiniPrep Cell. For the Prep Cell, the acrylamide gel should be allowed to polymerize overnight and, therefore, two days should be allotted for purification. For the MiniPrep Cell, overnight polymerization is also recommended for nucleosome purifications; however, a

shorter polymerization is often sufficient for DNA purifications, allowing smaller scale DNA purifications to be carried out in one day.

[*Figure 3 near here]

Materials:

30% acrylamide:bis-acrylamide (60:1) solution (see Reagents and Solutions)

10x TBE (see Reagents and Solutions)

Ultrapure water (e.g. Milli-Q)

Tetramethylethylenediamine (TEMED; Fisher #BP150-20)

10% ammonium persulfate (APS; see Reagents and Solutions; Bio-Rad #161-0700), freshly prepared

Water-saturated 1-butanol (see Reagents and Solutions)

0.5x TBE (see Reagents and Solutions), filtered and chilled

- 1 L for MiniPrep Cell
- 3 L for Prep Cell

DNA Elution Buffer (see Reagents and Solutions)

- 500 ml for MiniPrep Cell
- 1 L for Prep Cell

Nucleosome elution buffer (RB Zero, see Reagents and Solutions)

- 500 ml for MiniPrep Cell
- 1 L for Prep Cell

For analysis of DNA-alone fractions:

Agarose (Bio-Rad #16500500)

100 bp ladder (NEB #N3231)

6x DNA loading buffer (see Reagents and Solutions)

1x TBE

0.25x TBE (native PAGE running buffer for analyzing nucleosome-containing fractions)

40% sucrose (w/v; sterile filtered; Fisher #S5-500)

MiniPrep Cell (Bio-Rad #1702908) or Model 491 Prep Cell (Bio-Rad #1702926)

Recirculating pump (only for Prep Cell; Bio-Rad #1702930)

Parafilm

Graduated cylinders

- 10 ml for MiniPrep Cell
- 50 ml for Prep Cell, 28 mm column
- 100 ml for Prep Cell, 37 mm column
- 1 and 2 L, for buffer preparation

Plastic syringes

- 2x 10 ml, for pouring MiniPrep column and for system assembly
- 2x 60 ml, for pouring Prep Cell column and for system assembly
- 5 ml for loading Prep Cell

Tygon tubing

Tube fittings

- Barbed/barbed connectors (PVDF 1-2mm Gilson #F1179941)
- female luer-to-hose barbed adaptor (e.g. Cole-Palmer)

PFA tubing

- 1/16 inch inner diameter for pouring 28 mm Prep Cell column
- 1/16 inch outer diameter for loading Prep Cell

Microfuge tubes, 1.5 ml (e.g. Fisher 05-408-129)

Spatula (Fisher #21-401-10)

Gel loading tips, round, 0.5mm thick, 1-200 µl (for loading MiniPrep Cell column; Costar #4853)

Fraction collector (e.g. Bio-Rad 2110 or Gilson FC203B)

Fraction collector tubes

- 0.6 ml capless, for MiniPrep Cell (e.g. Fisher #02-681-451)
- 2 ml capless, for Prep Cell (e.g. Fisher #02-681-453)

Peristaltic pump for fraction collector (e.g. Gilson Minipuls 3)

Tubing for peristaltic pump (e.g. 1.5mm PVC tubing #F117942, for Gilson Minipuls 3)

Power supplies

- For Prep Cell: 10 W capability (e.g. Bio-Rad PowerPac HV, cat #1645056)
- For MiniPrep Cell: <5 mA capability (e.g. Bio-Rad PowerPac HV, cat #1645056)
- For analyzing fractions (e.g. Bio-Rad PowerPak Basic, cat #1645050)

Spin concentrators, 10kDa MWCO concentrators

- 0.5 ml capacity, Amicon Ultra-0.5ml cat # UFC501096
- 4 ml capacity, Amicon Ultra-4 cat #UFC801024
- 15 ml capacity, Amicon Ultra-15 cat #UFC901024

Refrigerated Centrifuge (e.g. Sorvall Legend XTR with swing bucket rotor for concentrators)

Microcentrifuge, refrigerated (e.g. Eppendorf 5424 in cold room, or Beckman Microfuge 20R)

For analysis of DNA-alone fractions: Agarose gel electrophoresis system, e.g.

Gel box: Bio-Rad Mini-Sub Cell GT Cell

Gel tray: Subcell GT UV Mini-gel tray 15x10cm (Bio-Rad #1704416)

30x 1.5mm well comb #1704449

For analysis of nucleosome-containing fractions: Acrylamide gel electrophoresis system, e.g.

Bio-Rad MiniProtean III system

Short plates (Bio-Rad #1653308)

1.5 mm spacer long plates (Bio-Rad #1653312)

10 well 1.5mm combs (each well is ~4.5 mm wide)

Spectrophotometer (e.g. Varian Cary 50 UV-Vis spectrophotometer)

Protocol Steps

Set up acrylamide gel column

1. In the afternoon the day before purification, prepare acrylamide solution.
   - For the MiniPrep Cell, and using a 30% acrylamide:bis-acrylamide (60:1) stock solution, make 10 ml of a 5.5% to 7% acrylamide:bis-acrylamide (60:1) solution in 0.5x TBE. Vacuum degas solution for 20 min. Prepare fresh 10% APS (100 µl) and chill on ice.
   - For the Prep Cell, and using a 30% acrylamide:bis-acrylamide (60:1) stock solution, make 30 ml (for 28 mm column) or 60 ml (for 37 mm column) of a 5.5% to 7% acrylamide:bis-acrylamide (60:1) solution in 0.5x TBE. Vacuum degas solution for 20 min. Prepare fresh 10% APS (200 µl) and chill on ice. Fill a syringe with water-saturated butanol and attach tubing so that it is ready to overlay once gel is poured.

For nucleosome purifications, use a 7% acrylamide gel, which helps separate hexasomes and mis-positioned nucleosome species. For DNA purifications, a 5.5-6 % acrylamide gel is sufficient.

For nucleosome purification, acrylamide columns should be not more than 12-18 hours old when sample is applied to the column. For DNA purifications, the column is routinely poured the day before as well, but for the MiniPrep Cell, the column can also be poured 3-5 hours before use. Also, MiniPrep Cell columns, stored at 4˚C, can be used for DNA purifications up to 3-4 days later.

2. Prepare the column to pour the acrylamide solution.

- For MiniPrep Cell, securely cover bottom of two or three glass columns with parafilm.

A casting stand may also be used. However, only one casting stand is included with the MiniPrep Cell system, and it is helpful to pour an extra column as a backup. Double wrap the bottom of each gel tube, winding the parafilm several times just above the gray gasket portion to make a seal. Place upright on benchtop pressing down on the gel tube to flatten the parafilm.

- For the Prep Cell, attach the glass column to the casting stand and place the cooling core inside, according to the manufacturer's instructions. Attach the buffer recirculation pump tubing to the cooling core and recirculate with chilled

ultrapure water. Once the outside of the glass gel column is cool to the touch (~15 min), the column is ready for pouring the acrylamide solution.

Make sure that the casting stand is level, using the built-in bubble level.

3. Polymerize and pour acrylamide gel.

- For the MiniPrep Cell column, transfer gel solution (10 ml) to a small beaker. Add 25 µl of 10% APS, followed by 2.5 µl TEMED. Quickly but gently mix by swirling. Draw gel solution into a 10 ml syringe. Quickly attach the tubing to the syringe and fill the gel column to the desired height (5-7 cm).
- For the 28 mm Prep Cell column, transfer the gel solution to a beaker and add 75 µl of 10% APS and 7.5 µl TEMED; draw gel solution into a 60 ml syringe. Quickly attach the tubing to the syringe and fill the gel column to the desired height (5-7 cm).
- For the 37 mm Prep Cell column, transfer the gel solution to a beaker and add 150 µl 10% APS and 15 µl TEMED. Directly pour the acrylamide solution into the glass column from the beaker to the desired height (5-7 cm).

We typically use 1:1 ratios of APS to TEMED, and concentrations of 0.025–0.033% APS and TEMED. For gels (e.g. native gels) exposed to air, Bio-Rad recommends doubling the amount of TEMED (1:2 of APS:TEMED; see Bio-Rad Tech Note 1156).

In general, a higher column will give better separation, but there will be more diffusion of sample. We generally work within a gel height range of 5-7 cm for DNA and nucleosomes. A 5.5 cm Prep Cell column is often sufficient to separate a well-positioned nucleosome (e.g. 601) from hexasomes and free DNA; however, if there are other minor species running closely together on a native gel, then a taller gel may be needed. Also, when loading the upper end of recommended amounts for the particular prep cell system, a taller column of 6-7 cm will give more time for species to separate.

Try not to introduce air bubbles when drawing gel solution into syringe. Note that there is some shrinkage when the acrylamide polymerizes, so the gel should be poured a couple millimeters above desired height.

4. Immediately after pouring gel, gently tap the column on the benchtop to release air bubbles at the bottom of the column. Tap (~10 times) until bubbles are no longer seen rising from the base. Overlay with water-saturated 1-butanol to a height of ~3 mm.

- For the MiniPrep Cell, use a p200 to add 60-100 µl of butanol.
- For the Prep Cell column, use a syringe with attached tubing to add 0.5 - 1 ml (for 28 mm column) or 1.5 - 2 ml (for 37 mm column) of butanol.

Gently stream butanol down the side of the glass column so as not to disturb the gel surface. The butanol should be added as soon as possible after the gel is poured into the column.

5. Allow acrylamide columns to polymerize at room temperature.
    - For the MiniPrep Cell, after 30 min, cover the top of the glass tube with parafilm and gently place upright in a beaker in the cold room for overnight storage.
    - For the Prep Cell, allow the gel column to polymerize on the benchtop overnight at room temperature with water recirculation. An example of the setup is shown in Figure 4A. The beaker of recirculating water can be placed in a partially filled ice bucket to keep the water chilled for a couple of hours.
    - Prepare the 0.5x TBE (used as running buffer for MiniPrep/Prep Cells) and either DNA or nucleosome elution buffer, depending on your use (add DTT fresh just before use). Sterile filter and store in the cold room.

[*Figure 4 near here]

Set up the MiniPrep/Prep Cell

6. The next morning, decant the butanol. Gently and extensively rinse the surface with deionized water to flush away any traces of butanol.
7. Prepare column for assembly
    - For the MiniPrep Cell, gently remove parafilm from the base of the column.
    - For the Prep Cell, carefully detach the gel column from the casting stand. If necessary, use a spatula to carefully break the seal between the gasket and the casting stand.
8. Inspect the column. Ensure the top and bottom surfaces of the gel column are level and devoid of air pockets/bubbles. For the Prep Cell, ensure the top surface of the

gel adheres to the inside wall of the glass column. Shortly after inspection, add a few ml of running buffer to the top of the column to keep surface hydrated.

The top surface affects the shape of the front moving through the gel and, therefore, a flat surface provides the best separation. If the base of the column has an air pocket, this may not impact DNA purification, but should not be used for nucleosome purification. For the Prep Cell, the process of removing the column from the casting stand can compromise the integrity of the gel. In particular, excessive manipulation can lead to an air gap between the glass tube and the acrylamide gel. An air gap that does not reach the top surface of the gel will not impact the run. However, an air gap that extends to the top surface will result in the sample running down the side of the column and sample loss.

9. Assemble the MiniPrep Cell/Prep Cell system, according to the manufacturer's instructions (see Internet References below). See Figure 5 for an overview of MiniPrep Cell assembly.

Extra care should be taken to avoid bending the stiff tubing exiting the MiniPrep Cell support frit/elution manifold base, as it can be prone to breaking, which then requires replacement of the support frit.

The trickiest part of the Prep Cell setup is purging the air out from around the fluted gasket of the elution chamber (attached to the gel tube assembly). Fill a 60 ml syringe with elution buffer and attach the elution buffer outlet tubing. Use the syringe to fill the tubing with elution buffer and, while holding onto the plunger of the

syringe, attach the tubing to the elution buffer port of the cooling core. Lean the assembly towards yourself such that the elution buffer inlet port on the lower chamber cap is farthest away from you and at the highest point (see Figure 6). Holding the syringe, push the elution buffer into the central port of the cooling core. As the area within the elution chamber fills with elution buffer, air bubbles will be evacuated through the elution buffer feedline and then into the elution buffer reservoir. Often, there will remain bubbles trapped around the gray gasket within the lower chamber cap. To remove these, carefully reintroduce air into the chamber, to merge with any remaining air bubbles, by pulling on the plunger of the syringe. This will draw air from the elution buffer reservoir in the upper chamber back through the elution buffer feedline and into the elution chamber. Once all the air bubbles are merged, immediately reverse and push the air out of the lower chamber cap. Repeat the process to remove any remaining air bubbles. For stubbornly trapped air bubbles, these can be coaxed out by more quick reversals of the syringe plunger.

[*Figure 5 near here]

[*Figure 6 near here]

10. Fill the bottom chamber of the MiniPrep/Prep Cell apparatus with chilled 0.5x TBE running buffer.

The lower buffer chamber should be filled with buffer to a height ~ 1 cm above the top of the acrylamide gel. This will help keep the gel chilled during the run.

At this point the system should be transferred to a flat surface in the cold room.

11. Fill the central chamber on the top portion of the MiniPrep/Prep Cell apparatus with chilled 0.5x TBE running buffer (Figures 3 and 5J).
    - For the MiniPrep Cell, fill to ~1.5 cm from the rim. Sometimes, in the process of filling the inner chamber, an air bubble can get trapped at the top of the glass column. In this case, use a clean p1000 tip to dislodge the bubble.
    - For the Prep Cell, prior to adding running buffer to the top inner chamber, attach tubing from the recirculation pump to the cooling core. This step should be done in the cold room where the run will occur. Also, connect the tubing from the other side of the recirculation pump to the port on the lower buffer chamber, and open the stopcock. Start the recirculation pump (Bio-Rad recommended flow rates are 80-100 ml/min).
12. Fill the outer elution buffer chamber with elution buffer (DNA elution buffer or RB Zero) (Figure 3).

    If any 0.5x TBE running buffer was spilled into the elution buffer chamber, remove any contaminating running buffer before adding the elution buffer.

    Be sure to save some of the elution buffer to serve as a blank for an $OD_{260\text{-}310}$ *absorbance reading following MiniPrep Cell/Prep Cell purification.*

13. Connect the elution buffer tubing to the peristaltic pump, which leads to the fraction collector. An overview of the Prep Cell with accompanying equipment is shown in Figure 4B.

Before connecting, wash the pump and fraction collector tubing with elution buffer. While the syringe is still attached to the elution buffer tubing, push a little buffer through the elution tubing to dislodge any bubbles that might have entered the elution buffer feedline prior to filling outer the chamber with elution buffer. Next, remove the syringe from the elution buffer tubing and attach to the peristaltic pump tubing, such that the flow will be directed from the MiniPrep Cell/Prep Cell to the fraction collector.

14. Pre-run MiniPrep/Prep Cell gel columns for ≥ 20 min.
    - For the MiniPrep Cell, run at 1 W constant power.
    - For the Prep Cell, run at 10 W constant power.

    For the MiniPrep Cell, the volts should be in the 250-300 V range, and for the Prep Cell, the volts are typically ~350 V (28 mm column) or ~250-270 V (37 mm column). If the volts are much higher than expected (> 400 V), there are several possible causes that should be resolved before sample is loaded. Possible causes are: (i) trapped air bubble around the region of the frit; (ii) clogged elution frit; (iii) incorrect buffer in the upper inner and/or lower chamber of the apparatus; or (iv) improper alignment of the electrodes on the upper and lower chambers.

15. When the pre-run is finished and the sample is ready to load, pause the power supply and remove the lid.

16. For nucleosome samples only, save 1 µl of load for gel analysis. Transfer the 1 µl of load to a tube containing 99 µl of elution buffer, gently mix, and store in ice bucket for use in step 22.

The load sample will help identify desired peak species as well as unwanted species.

Purify DNA or nucleosome sample

17. Load the sample.
    - For the MiniPrep Cell, load using a p200 with a round, 0.5 mm thick, gel-loading tip.
        - **For DNA purifications**, the load volume can be in the 60-100 µl range.
        - **For nucleosome purifications**, load volume should be as low as manageable (≤ 50-60 µl range).
    - For the Prep Cell, load using a small syringe fitted with stiff tubing (e.g. PFA, 1/16 inch outer diameter, tubing can be long enough to hold 200 µl sample) so that the end of the tubing can extend through the guide tube to ~3 cm above the top of the gel. Slowly eject the sample so that it gently settles on the gel surface. Target load volumes are ~180 - 200 µl (for the 28 mm column) or 0.5 - 1 ml (for the 37 mm column).

    For DNA purifications, there is more flexibility in the load volumes used. For nucleosome purifications, however, for which much higher resolution is generally required, most optimal results will be obtained with smaller load volumes.

In addition to PCR-generated DNA (Basic Protocol 1), the Prep Cell can also be used to purify mononucleosome-sized DNA fragments that have been digested from plasmids harboring arrays of nucleosome positioning sequences.

Be sure that the sample contains a final concentration of ~6% sucrose.

18. Reconnect the top lid and restart power supply to start the run.
19. Turn on the peristaltic pump for the fraction collector. Suggested flow rates for the pump are:
    - 100 µl/min for the MiniPrep Cell column
    - 500-600 µl/min for the 28 mm column of the Prep Cell
    - 800-900 µl/min for the 37 mm column of the Prep Cell

    At the start, no fractions are collected, and the elution buffer should be set to go to waste. Maintaining the flow of the elution buffer prior to fraction collection helps to clear away contaminants eluting from the gel prior to fraction collection.
20. Prepare gels for analyzing fractions.
    - **For DNA purifications**, pour a 1.5% agarose gel with 1xTBE and 1 µg/ml ethidium bromide, in a wide tray with 25-30 wells.
    - **For nucleosome purifications**, pour 7% native acrylamide gels sufficient for loading 40 samples (e.g. four gels with 10 lanes/gel). Prepare and chill 0.25x TBE running buffer (2 L for two gel boxes). See Figures 1 and 2.

For routine nucleosome preps, four native acrylamide gels are typically sufficient, but for larger scale preps, or for those containing slowly migrating samples (e.g. centered mononucleosomes), six or more gels may be necessary.

21. Start collecting fractions. For fluorescently-labeled DNA/nucleosome, the sample can be visually followed as it migrates down the gel column. Begin collecting fractions when it reaches the 1.5 - 2 cm mark. The delay before starting the fraction collector should be ~1.5 hr for DNA and ~3 hr for nucleosomes with 60-80 bp extranucleosomal DNA.

- For the MiniPrep Cell, collect 3 min fractions in 0.6 ml capless tubes.
- For the Prep Cell, collect 2 min fractions in 2 ml capless tubes.

For the MiniPrep/Prep Cell with a 6.0% acrylamide gel column, the leading edge of the migration band for a 225 bp DNA sample migrates ~3 cm/hr. For mononucleosome-sized DNA fragments (e.g. 145-225 bp), the DNA sample should be entirely eluted within ~3 hours after loading.

For the MiniPrep and Prep Cell with a 7% acrylamide gel column, the leading edge of the migration band for end-positioned nucleosome samples (e.g. 0N80) migrates ~1.0 cm every 45 min, whereas that of a centered nucleosome (e.g. 40N40) migrates ~1.0 cm/hr. We have noted slower migration rates for the 37 mm Prep cell gel column. For a 6 cm gel column, total elution time is roughly ~6.5 hr for an end-positioned 0N60 nucleosome and ~9 hr for a centrally positioned 30N30 nucleosome.

When purifying nucleosomes, two distinct bands are often observed on the gel column, with the faster migrating species corresponding to free DNA and the slower species corresponding to nucleosomes.

For unlabeled samples, it may be possible to visualize a concentrated sample on the Prep Cell as a front moving down the gel, due to a difference in refractive index. The setup described here does not depend on an inline UV detector. However, if available, a detector can assist in determining which fractions to analyze by gel.

22. Analyze fractions.

- **For DNA purifications:** collect up to 40 fractions and analyze by agarose gel.
    - Set up the 1.5% agarose gel (prepared in step 20) in 1x TBE running buffer.

    We often take every other or every third fraction for the first few and last few fractions. The majority of the peak should elute within ~12 fractions.

        - For the MiniPrep Cell, mix 3 µl of each fraction with 3 µl 6x DNA loading buffer.
        - For the Prep Cell, mix 8-10 µl of each fraction with 3µl of 6x DNA loading buffer.
    - Load entire 6 µl sample in each well.
    - Load 100 bp ladder in the center lane of the gel.
    - Run the gel at 150 V for 30 min and visualize on a UV box.

- **For nucleosome purifications:** collect 80-100 fractions and analyze by native PAGE (Figures 1 and 2).
    - Pre-run 7% native acrylamide gels (prepared in step 20) in 0.25x TBE running buffer at 100 V for ~20 min in the cold room.
    - Prior to loading, use a 20 ml syringe to flush wells with 0.25x TBE.
    - Load native gels while gel is running. For each gel, include a load sample in lane 1. Mix 2 µl of the diluted 100 µl load sample (step 16) with 2 µl 12% sucrose and load 2.5 µl/lane. You can prepare the samples for loading during the pre-run.
        - For the MiniPrep Cell, mix 2 µl of each fraction with 2 µl 12% sucrose and load 2.5 µl per lane.
        - For the Prep Cell, mix 5.5 µl of each fraction with 1.2 µl 40% sucrose and load 5.5 µl per lane. This volume still allows bands to be resolved for a 1.5 mm thick gel where width of each well is about 4.5 mm.

    More fractions should be taken for centered nucleosomes, or those of large scale. Gel samples can be taken for every third fraction for the first 15 fractions, followed by every other fraction for the remaining fractions. If sample is concentrated enough and fluorescently labeled, it may be visible in the fractions.

    - After loading, allow gels to continue to run at 100 V for 1 hr 45 min.

- Analyze gels.
  - If fluorescently labeled, scan gel on Typhoon5 Imager (GE; Figure 2A-C).
  - If nucleosome is not fluorescently labeled, remove short plate from gel and, using a razor blade, cut off the bottom corner corresponding to the side that has the load sample. Place the gel in a gel dish with 50 ml of 0.5x TBE and 1 µg/ml ethidium bromide. Place the dish on a rotator plate, gently shake for 5 min, and then visualize on UV box (Figure 2D).

23. Pool peak fractions into a 15 or 50 ml conical tube and place on ice.

The desired material typically corresponds to the most enriched species in the load. If scanning on the Typhoon5, reduce sensitivity (photomultipler tube, PMT) as necessary so that nucleosome bands are not saturated and individual nucleosome species can be clearly differentiated.

24. Concentrate pooled fractions using a spin concentrator.

- **For DNA purifications**, concentrate to roughly ~200 µl (for a 10 ml PCR) or ~400 µl (for a 20 ml PCR). Determine the concentration by measuring $OD_{260-310}$, using ultrapure water or elution buffer as a blank. Aim for ~20-30 µM concentration. Following the concentration step, measure the volume of the DNA sample to determine the total yield in micrograms.

This target DNA concentration will give flexibility in setting up nucleosome reconstitutions, where the target final concentration is 6 µM (see Protocol 3).

- **For nucleosome purifications**, concentrate to ~150-200 µl (for the MiniPrep Cell) or ~400-600 µl (for the 28 mm Prep Cell) and ~800µl (for the 37 mm Prep Cell). Determine the concentration by measuring DNA absorbance at $OD_{260\text{-}310}$, using elution buffer as a blank. Nucleosomes are typically concentrated to 2 µM-50 µM depending on the experimental constraints; 2-7 µM is adequate for most biochemical studies, whereas higher concentrations are often needed for structural studies. If sample is too concentrated for experiments, use elution buffer to dilute to desired concentration range.

These suggested target volumes for concentrating are meant as rough guidelines for routine purifications, and can differ for individual preparations and experimental needs (e.g. single molecule vs cryo-EM).

Nucleosome samples should be concentrated immediately after pooling peak fractions and not left diluted overnight. For concentrations under 1-2 µM, addition of 0.1 mg/ml BSA (C_f) can help stabilize nucleosomes. If freezing nucleosome samples (see next step), and in order to not waste sample, take only a single concentration measurement of the nucleosome sample to ensure a good concentration range has been reached, since addition of glycerol for freezing (step 25) will require a further concentration reading(s). We store all of our

nucleosomes frozen. If stored on ice, nucleosomes should be used within a couple of weeks.

25. Freeze purified samples.
    - Store **DNA samples** at -20˚C.
    - For **nucleosome samples**, add 100% sterile-filtered glycerol to a final concentration of 20%. To aid pipetting 100% glycerol, cut off the bottom third of a sterile yellow tip (p200) using a sterile razor blade. Mix the glycerol gently and thoroughly throughout the sample.
    - Aliquot 6-10 µl of purified nucleosome into prechilled 0.6 ml microcentrifuge tubes (on ice), and flash freeze in liquid nitrogen. Store at -80˚C.

Given the volume change from glycerol addition, we typically remeasure nucleosome concentration after thawing to obtain an accurate final reading. Nucleosomes are routinely frozen one day after nucleosome purification. When thawing an aliquot for use, nucleosomes are generally only used for that day's experiments and not refrozen.

BASIC PROTOCOL 3: *Nucleosome reconstitution via linear gradient salt dialysis*

Using purified DNA and refolded histones, nucleosome reconstitutions by linear gradient salt dialysis have been well described (Luger et al., 1999; Dyer et al., 2004). We present it here for the sake of convenience to the reader, as it is a necessary step between PCR

production (and purification) of DNA and nucleosome purification. This method begins by mixing DNA and histone octamer in 2 M salt buffer, and allowing DNA and histones to form stable associations by slow reduction in salt concentration through salt gradient dialysis. During dialysis, the salt concentration is slowly lowered through the introduction of a low salt buffer and concurrent removal of the high salt/low salt mixture. Buffer exchange/removal is achieved with a dual channel peristaltic pump, such that one tubing introduces low salt buffer into the beaker containing the nucleosome sample, whereas the other removes the high salt/low salt mixture to a waste container (Figure 7). As the salt concentration is reduced, the histone tetramer first deposits on DNA, followed by the histone dimers (Hansen et al., 1991; Andrews & Luger, 2011), ultimately resulting in a characteristic left-handed supercoil wrapping of DNA into a nucleosome. When the volume of the initial DNA/histone mixture is greater than ~2.5 ml (assuming a starting concentration of ~6 µM DNA, corresponding to >15 nmol DNA, or >2 mg of a 225 bp fragment, here considered large scale), a standard dialysis bag is used during gradient dialysis. For smaller volumes, microfuge caps are used as dialysis buttons, each holding ~150 µl of the sample.

This protocol requires purified histone octamer and dimer, which can be produced as previously described (See Strategic Planning and (Luger et al., 1999; Dyer et al., 2004)). When using histones from certain species, such as *Xenopus laevis*, it is not uncommon to obtain hexasomes as well as nucleosomes, even when starting with purified histone octamers (Levendosky et al., 2016). To reduce hexasome formation, some researchers add excess H2A and H2B when refolding histone octamers (Shahian & Narlikar, 2012). In this

protocol, excess H2A/H2B dimer is included during the nucleosome reconstitution stage to minimize hexasomes, as previously recommended by others (Gaykalova et al., 2009).

[*Figure 7 near here]

After the last step of this protocol, the user should have a concentrated nucleosome sample that can then be purified following Basic Protocol 2.

Materials

RB-High, 400 ml or 1 L (see Reagents and Solutions)

RB-Low, 2 or 3 L (see Reagents and Solutions)

Purified DNA, mononucleosome-sized fragment (Basic Protocols 1 and 2)

Histone octamer, purified (50-200 µM)

Histone H2A/H2B dimer, purified (~200 µM)

Milli-Q-filtered water

1 M Tris-HCl, pH 7.5, 0.22 µm filtered, 1L (Sigma #T1503)

4 M KCl, 0.22 µm filtered, 50ml (Sigma #P3911)

500 mM EDTA, pH 8.0, 0.22 µm filtered, 500ml (Sigma #E9884)

500 mM DTT (Sigma #D9163)

10 mg/ml Bovine serum albumin (BSA)

Graduated cylinders (500 ml and 2 L)

For smaller-scale (<2.5 ml) nucleosome reconstitutions:

- 1.5 ml microfuge tubes

- Razor blade
- Bunsen burner

For large-scale (>2.5 ml) nucleosome reconstitutions:

- Dialysis clips
- Float buoy

Dialysis tubing, 3.5 kDa or 6-8 kDa MWCO (e.g. Spectra/Por 6-8kDa MWCO #132655 or Spectra/Por 3.5kDa MWCO #132724)

Glass beakers (500 ml and 2 L or 1 L and 3 L)

Peristaltic pump with two channels (e.g. Gilson Minipuls 3)

Tubing for peristaltic pump

Tygon tubing

Tube fittings

Serological pipets, plastic, 2 ml

Ring stand

Stir plate/stir bar

Protocol Steps

Prepare buffers for nucleosome reconstitution

1. Make RB-High and RB-Low buffers on the day of the reconstitution (see Reagents and Solutions).
    - For a reconstitution < 10 ml, prepare 400 ml of RB-High buffer and 1 L of RB-Low buffer.

- For > 10 ml, prepare 1 L of RB High buffer and 3 L of RB Low buffer.

Transfer buffers to beakers and place in cold room.

If you anticipate having 1.5 – 2.5 ml of starting DNA/histone mixture, which would require > 10 dialysis caps (step 5), transfer the 400 ml of RB-High buffer into a larger beaker (1 L) to give more room.

Although we increase the volume of reconstitution buffers for > 10 ml reconstitutions, Luger and Richmond use 400 ml RB-High buffer for large- and small-scale reconstitutions (Luger et al., 1999; Dyer et al., 2004).

2. Set up the peristaltic pump and buffers as shown in Figure 7.

Prepare sample for nucleosome reconstitution

3. Determine the amount of purified DNA (from Basic Protocol 2) to be used in the nucleosome reconstitution. If not done previously, measure the DNA concentration using a spectrophotometer. Using water as a blank, measure the concentration of a 100- to 500-fold dilution of a purified DNA stock. For duplex DNA, use an $A_{260\text{-}310}$ = 1.0 to correspond to 50 μg/ml.

DNA is typically the more limiting reagent and, therefore, the amount of DNA used will determine how much histone octamer is required and the volume of the reconstitution.

4. Calculate the volume of the nucleosome reconstitution based on amount of DNA:

    a. Calculate the molecular weight (MW) of the DNA fragment.

    DNA MW (Da) = # bp x 650 Da/bp

    b. Convert the concentration of DNA in µg/ml to µM.

    e.g., for a 100,000 Da MW, 100 µg/ml = 1 µM

    c. Assuming that all of the DNA is used for a 6 µM reconstitution, calculate the total volume of reconstitution:

    X µl total reconstitution = $(Volume_{purified\ DNA})$ x $(Concentration_{purified\ DNA})$/(6 µM)

    e.g., for 450 µl of a purified DNA sample at 17 µM, the total reconstitution volume would be 1275 µl.

5. Based on volume of reconstitution, determine volume of histone octamer and dimer stocks to use, assuming a final concentration of 5.95 µM histone octamer and 1.8 µM histone dimer.

    e.g., a total reconstitution volume of 1275 µl would require 40.4 µl of a 188 µM purified histone octamer sample, and 11.5 µl of a 200 µM purified histone dimer sample.

6. Based on total reconstitution volume of sample, determine whether the reconstitution will be carried out with dialysis tubing (>2.5 ml) or using microcentrifuge caps as dialysis buttons (<2.5 ml) (Vary et al., 2004). Each cap holds

≤ 150-160 µl. Prepare micro-dialysis buttons by cutting the caps of the tubes off using scissors. Then, using a hot razor blade (held with forceps over a Bunsen burner), slice off the top ~0.5 cm of the 1.5 ml microfuge tubes (described by Vary et al., 2004). This top portion will serve as a ring to fasten the membrane to the cap. For smaller reconstitutions, caps made from 0.6 ml tubes may be used instead.

Cutting the plastic tubes with a hot razor blade produces fumes, so this step should be done in a hood. Prepare a few extra caps then needed in case one of the rings does not fit.

7. Soak dialysis membrane in ultrapure water. If using caps, use scissors to cut open the dialysis tubing so it is a flat sheet. Cut dialysis membranes into ~2.5 cm x 2.5 cm squares, one for each cap, and leave in ultrapure water until needed.
8. Calculate the volumes of buffer components needed for nucleosome reconstitution based on the concentrations given in Table 2.
9. Pipette components in Table 2, in the order listed, into a 1.5 ml – 15 ml tube. Prior to addition of histones, vortex the sample. Add histone octamer and dimer to the reconstitution, and gently and thoroughly mix.
10. Set up samples for dialysis
    - For smaller reconstitutions (< 2.5 ml total volume), place caps to be used for dialysis on clean benchtop. Working with 3-4 caps at a time and using a p200, pipette ~150 µl into each cap (final cap will have less volume). Then, immediately lay a square of dialysis membrane over the cap containing the

reconstitution sample. Immediately seal the dialysis tubing to the cap using the top sliced-off portion of the tube. Place each filled cap in an ice bucket (with the dialysis membrane facing up) while preparing remaining caps. Trim dialysis tubing if too large.

- For larger reconstitutions (> 2.5 ml total volume), transfer sample to dialysis tubing, using plastic clips to seal the bag. Attach float buoy to one end.

[* Table 2 near here]

Perform linear salt gradient dialysis

11. Transfer caps or dialysis bags containing reconstitution sample for linear gradient dialysis to a beaker containing RB-High buffer, pre-chilled in the cold room. Place the caps in the beaker, with dialysis membrane face down. Keep stir plate on a low setting so as not to disturb the caps, but allowing sufficient mixing of buffer.

12. Position the tubing into the beaker for linear gradient dialysis. Start peristaltic pump at a rate of 1.2-1.5 ml/min. Reconstitutions are typically completed in ~24 hours.

    For large scale reconstitutions (1 L of RB High and 3L of Rb Low), use a 1.5 ml/min flow rate, which may take ~33 hours.

13. Upon completion of dialysis, transfer the reconstitution sample to a beaker with RB Zero (400 ml – 1 L depending on size of reconstitution; see recipe). Allow to dialyze overnight or for a minimum of 3-5 hours.

We typically time the reconstitution such that the nucleosome purification can be carried out the day after gradient dialysis is finished.

14. Remove sample from dialysis. If using caps, first gently dab the outside of the membrane with a Kimwipe to remove any buffer (Vary et al, 2004). Then, using a p200 with a sterile tip, pierce the membrane and transfer sample to a fresh microfuge tube.
15. Concentrate the nucleosome sample in an Amicon 10 kDa MWCO spin concentrator at 4˚C. Reduce volume to recommended load volumes (Basic Protocol 2, step 17). Add 40% w/v sucrose to a final concentration of 6%. Mix gently and thoroughly. If there are air bubbles, give a brief spin in a cold room microfuge.
16. Continue to nucleosome purification in Basic Protocol 2.

REAGENTS AND SOLUTIONS:

30% Acrylamide:Bis-acrylamide (60:1) gel solution, 200 ml

60 g acrylamide (Bio-Rad #161-0101)

1 g of bis-acrylamide (Bio-Rad #161-0201)

Add acrylamide powder to a 250 ml beaker containing 50 ml of purified water. Add water to bring closer to appropriate volume. Once dissolved, transfer to a graduated cylinder and fill to 200 ml with water.

Filter (0.22 µm), then vacuum degas for 30 min.

Store in a foil-wrapped bottle at 4˚C for up to 6-8 months.

CAUTION*: Acrylamide is toxic. Wear gloves and a lab coat when working with acrylamide solutions, and additionally wear a mask when working with acrylamide powder.*

7% acrylamide gel solution, 50 ml (for four native PAGE gels for analyzing fractions)

11.7 ml of 30% (60:1) acrylamide:bis-acrylamide gel solution

2.5 ml 10x TBE

35.8 ml of ultrapure water

Prepare fresh and vacuum degas for 20 min prior to polymerization.

To polymerize, add 500 µl 10% APS, swirl, and then 50 µl TEMED; mix thoroughly by swirling. Immediately pour gels and let set for a minimum of three hours at room temperature before use.

CAUTION*: Acrylamide is toxic. Wear gloves and a lab coat when working with acrylamide solutions.*

1.5% agarose gel, 200ml

3.0 g agarose (Bio-Rad #16500500)

200 ml 1x TBE

Mix all reagents, and then microwave until agarose is fully dissolved. Add 200 µl of 1 mg/ml ethidium bromide and swirl to mix. Pour into gel tray in a hood. Immediately place comb, and remove any bubbles using a clean pipette tip. Allow 45 minutes to solidify. Once ready, place tray in electrophoresis system and fill tank with 1x TBE until gel is just submerged. Carefully remove comb.

CAUTION: *Ethidium bromide is toxic. Wear gloves and a lab coat when handling solutions containing ethidium bromide.*

10% Ammonium Persulfate (APS), 1ml

Weigh out 100 mg of APS directly in a microfuge tube.

Add 1 ml of ultrapure water.

Vortex until dissolved.

Prepare fresh, keep on ice until use.

Butanol, water-saturated, 50 ml

25 ml 1-butanol (Fisher #A399-500)

25 ml ultrapure water

Mix well, let separate. Use top (butanol) layer for overlaying acrylamide columns.

6x DNA loading buffer

300 mM Tris-HCl, pH 7.8

60 mM EDTA

36% (v/v) Glycerol

0.12% (w/v) SDS

0.06% (w/v) Bromophenol Blue

0.06% (w/v) Xylene Cyanol FF dye

Store at room temperature; good for several years.

10 mM dNTPs, 10 ml

We purchase a set of dNTPs, 100 mM (250 µl) each (Thermo Fisher Scientific #10297018). Pipette each 250 µl dNTP into a 15 ml conical tube. Add 9 ml of Milli-Q water, then mix thoroughly by

vortexing. Each [dNTP] will be 2.5 mM, equal to a total [dNTP] of 10 mM. Place on ice, and aliquot. Store at -20˚C for up to 6 months in a freezer that does not undergo freeze/thaw cycles.

DNA Elution Buffer, 500 ml

10 mM Tris-HCl, pH 7.5

1 mM EDTA, pH 8.0

Use Milli-Q water to bring up to final volume, mix, and filter (0.22 µm).

Prepare the morning of purification or the day before.

Histone H2A/H2B dimer, purified

Purify as described (Luger et al., 1999; Dyer et al., 2004).

Determine protein concentration using a spectrophotometer, with an extinction coefficient based on the protein sequences being used. Aliquot and flash freeze at a final concentration of ~200 µM in 10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol.

Store in -80˚C.

Histone octamer, purified

Purify as described (Luger et al., 1999; Dyer et al., 2004).

Determine protein concentration using a spectrophotometer, with an extinction coefficient based on the protein sequences being used. Aliquot and flash freeze at a final concentration of 50-200 µM in 10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol.

Store in -80˚C.

RB High (Luger et al., 1999)***, 400 ml or 1 L***

10 mM Tris-HCl, pH 7.5

2 M KCl

1 mM EDTA, pH 8.0

1 mM DTT

Use Milli-Q water to bring up to final volume.

Prepare on the day of nucleosome reconstitution.

RB Low (Luger et al., 1999)***, 2 L or 3 L***

10 mM Tris-HCl, pH 7.5

200 mM KCl

1 mM EDTA, pH 8.0

1 mM DTT

Use Milli-Q water to bring up to final volume.

Prepare on the day of nucleosome reconstitution.

RB Zero (Luger et al., 1999)***, 400 ml – 1 L (nucleosome reconstitution) or 500 ml – 1 L (nucleosome purification)***

10 mM Tris-HCl, pH 7.5

1 mM EDTA, pH 8.0

2.5 mM KCl

1 mM DTT

Use Milli-Q water to bring up to final volume, mix, then filter (0.22µm).

Prepare up to 1 day before, but add DTT just before use.

40% Sucrose (w/v), 40 ml

Place 16 g of sucrose in a 50 ml conical tube, fill with Milli-Q water to just under 40 ml. Vortex until sucrose is completely dissolved. Transfer to a 50 ml graduated cylinder and bring volume up to 40 ml with Milli-Q water. Sterile filter (0.22µm) into sterile 50 ml conical tube.

Store at 4˚C for up to 1 year. Sucrose can become contaminated, so always check for contamination before using.

10x TBE, 1 L

55 g Boric Acid (Fisher #A73-500)

108 g Trizma Base (Sigma #T1503)

40 ml EDTA, pH 8.0 (Sigma #E9884)

Dissolve in 700 ml Milli-Q water.

Fill to 1 L with Milli-Q water.

Autoclave.

10x TBE can be prone to precipitation, however, this appears to be less likely if stored unopened for several months at room temperature. Once opened the solution will be good for many months; however, do not use if precipitation occurs.

0.5x TBE, 1 L or 3 L

In a 1 L graduated cylinder, add 950 ml Milli-Q water and 50 ml autoclaved 10x TBE.

Cover with parafilm and mix thoroughly.

Sterile filter (0.22 µm) into 1 L bottles.

Store in cold room for up to several months.

0.25x TBE, 2 L

In a 2 L graduated cylinder, add 1950 ml cold Milli-Q water and 50 ml autoclaved 10x TBE.

Cover with parafilm, mix thoroughly, and transfer to the cold room.

Prepare fresh.

10x ThermoPol buffer (NEB), 50 ml

200 mM Tris-HCl, pH 8.8 @25˚C

100 mM $[NH_4]_2SO_4$

100 mM KCl

20 mM $MgSO_4$

1% (v/v) Triton X-100

Use Milli-Q water to bring up to final volume.

Sterile filter (0.22 µm) and aliquot into 1.5 ml microfuge tubes.

Store at -20˚C for up to one year (preferably in a non-freeze thaw freezer).

COMMENTARY

BACKGROUND INFORMATION:

For the *in vitro* production of mononucleosomes consisting of a single sequence, DNA can be obtained either through digestion of plasmids harboring arrays of nucleosome positioning sequences —usually carried out for large-scale applications like crystallography (Dyer et al., 2004; Palmer et al., 1995)— or via PCR. Compared to large-scale plasmid preparations, generating DNA via PCR is much faster and less laborious, since DNA derived from plasmids requires amplification of DNA in bacteria, plasmid purification, and then

digestion with restriction enzymes to release the desired fragment. PCR is also much more flexible than plasmid-based purifications, as changes to the sequence or DNA length often only require a different template and/or different primers. Another advantage of PCR-based amplification is that generating DNA with functional groups on one or both ends (e.g. FAM, Cy3, Cy5, biotin) simply requires using appropriately modified primers. Such modifications are instrumental for visualizing or manipulating nucleosomes or DNA in gel- or solution-based procedures.

Primers also allow site-specific introduction of base modifications that can alter duplex DNA: to create single-stranded DNA gaps, uracils can be included in the primer sequence, which can then be site-specifically cleaved using the USER enzyme (NEB) (McKnight et al., 2011). Single-stranded DNA gaps have been used to reveal where chromatin remodelers act on nucleosomal DNA (Schwanbeck et al., 2004; Saha et al., 2005; Zofall et al., 2006; McKnight et al., 2011; Ranjan et al., 2015). Single-stranded DNA gaps do not seem to interfere with nucleosome formation and, therefore, provide a useful tool for revealing where nucleosome-interacting factors require integrity of one or both DNA strands for proper recognition/action.

For *in vitro* nucleosome reconstitution, histones can be deposited onto DNA either using histone chaperones such as Nap1 (Vary et al., 2004; Fujii-Nakata et al., 1992), or by shifting from a high salt to a low salt solution. The salt reduction can be accomplished stepwise through dilution (Lorch et al., 1987) or by linear gradient dialysis, where the initial high salt dialysis buffer is continuously exchanged with lower salt buffer (Luger et al., 1999). In our lab, we exclusively use linear gradient salt dialysis to reconstitute all nucleosomes.

When reconstituted *in vitro*, mononucleosomes can occupy multiple positions on DNA, and often are accompanied by hexasomes and free DNA. Mononucleosomes can be purified with one of three methodologies. One method is to bind nucleosomes to an ion-exchange column (Source 15Q [GE Healthcare] or TSKgel DEAE-5PW [Tosoh Bioscience]), allowing separation from free DNA and aggregates (Luger et al., 1999; McGinty et al., 2016). To our knowledge, ion-exchange columns have not been documented to resolve nucleosomes at distinct positions on DNA. Another common method is fractionating nucleosome species by glycerol or sucrose gradient centrifugation, in which the nucleosome sample is layered on top of a gradient and ultracentrifuged for about 18 hours (Phelan et al., 2000; Lee et al., 1999; Kagalwala et al., 2004). This method offers the advantage of simultaneously purifying multiple nucleosomes in a single ultracentrifugation run, yet, in our opinion, does not provide the same high degree of resolution in separating nucleosome species as another common option, native acrylamide gel electrophoresis. This third method separates nucleosome/DNA species by electrophoresis through native acrylamide gels. In this method, the desired products can either be excised and extracted from gel slices (O'Donohue et al., 1994; Pennings, 1999; Längst et al., 1999), or collected as they elute off the end of a gel column, using a MiniPrep or Prep Cell, as described here. Native acrylamide gels offer excellent resolution in separating mononucleosome species with different positions of the histone along the DNA (Meersseman et al., 1992). The MiniPrep/Prep Cell systems are quite versatile, handling a range of load amounts, where a single purification can yield ample material for multiple types of biochemical and biophysical experiments. We have used these

systems to purify DNA and mononucleosomes of varying DNA lengths (145-245 bp), distinct placements of the histone core, and different nucleosome positioning sequences.

CRITICAL PARAMETERS:

When setting up the MiniPrep Cell/Prep Cell for purification, two important parameters to consider are load volume and gel height. In general, keeping the load volumes as low as possible, so that the band entering the gel is < 1 mm, will help in achieving maximum separation. Increasing the height of the gel column will also aid in the separation of unwanted species, however, there is a trade-off in the time required to complete the purification. The migration behaviors of nucleosome/DNA species observed by native PAGE will parallel that on the MiniPrep Cell/Prep Cell and, therefore, one should consider what migration rate and alternative products might be expected from the nucleosome construct. For a Widom 601 nucleosome, we often pour a 5 cm Prep Cell column for a centered 40N40 nucleosome, but for an end-positioned 80N0 nucleosome, we might use a 6.0-6.5 cm column. For DNA purifications, where fewer closely migrating species are expected, a 5 cm column is often sufficient. Also, increasing the flow rate of the elution buffer up to 1 ml/min for the Prep Cell can also increase separation to some degree, but will also dilute the fractions to a greater extent.

TROUBLESHOOTING:

Basic Protocol 1: PCR of DNA

1) **Undesired products or smearing of PCR product.** Possible causes include: (i) concentration of Taq is too high, (ii) the plasmid DNA template is impure, (iii) the Taq or primer stock is contaminated (e.g. with another template or primer), (vi) there is an issue with the primer design. Running control reactions by titrating Taq and $MgSO_4$, as well as checking PCR components with a different plasmid or Taq prep, can be helpful in pinpointing problems.

2) **Poor Yields from PCR.** Yields lower than expected can arise from various sources. Insufficient mixing of components in PCR master mix is not uncommon. To identify the issue, perform test reactions, titrating one component while keeping the others constant. Recommended components to titrate include Taq, $MgSO_4$, DNA template, and primers. Perform control reactions by testing components with other templates, primers, and Taq. Repeated freezing and thawing of dNTPs may lead to lower yields. If possible, check sample in another thermocycler to rule out issues with a particular PCR machine. While users may check the theoretical annealing temperature, we have found that annealing temperatures of 55˚C generally work well for primers that are 28-30 bases in length.

Basic Protocol 2: MiniPrep/Prep Cell purifications of nucleosomes and DNA

1) **Uneven bands.** During purification over the MiniPrep Cell/Prep Cell, fluorescently-labeled nucleosome and free DNA separate into individual bands over the course of the run. Hexasomes can appear as a distinct third band that migrates between the two, just ahead of the nucleosome peak. These bands should be flat, not wavy or deformed. Uneven bands can be due to trapped air pockets present during pouring/polymerization, which can be avoided (on subsequent runs) by vacuum degassing acrylamide solutions and tapping column to dislodge air bubbles after pouring. For the Prep Cell system, wavy bands may also be caused from a temperature gradient over the gel. Running the system in the cold room in conjunction with buffer recirculation through the cooling core and lower buffer chamber should keep temperatures uniform across the gel.

2) **Poor separation of nucleosome from other reconstituted products.** To maximize separation between different species, make sure load volumes are kept low, such that the volume can just spread over the entire surface of the column. Overloading of sample can be avoided by splitting sample into multiple runs or switching to a wider diameter column if possible. Increased separation can also be achieved by making a taller gel column. We generally find that optimization within a 5-7 cm gel height to be sufficient. Another option is to increase the flow rate of the elution buffer to upper end of recommendations (100 µl/min for MiniPrep Cell and 1 ml/min for Prep Cell).

3) **High voltage during the MiniPrep Cell/Prep Cell run.** In our experience, the most common reason for high voltage when using the MiniPrep Cell is a clogged elution frit, which will necessitate replacement. To avoid this, filter and vacuum degas running buffer

solutions of 0.5x TBE. Always keep frits wet and stored along with the dialysis membrane at 4˚C in 0.5x TBE. A simple reason for high voltage can be misalignment of upper and lower portions of the Prep Cell, so first check to see if this is the cause. High voltage can also be a sign of trapped air that was not adequately purged from the base of the gel column/elution chamber. If sample has not yet been loaded, disassembly and reassembly of the system may help solve the problem. Another possible cause can be improperly made gel solutions or running buffer.

4) **Loss of sample.** Sample loss is uncommon, but one possibility is for the load to travel down the side of the gel column due to an air gap between the top of the acrylamide gel and either the gel column or cooling core. To prevent this, handle the gel column gently during setup and when attaching tubing to the cooling core. Another possibility for sample loss could be cracked elution tubing of the MiniPrep Cell, at the point of attachment to the bottom of the support frit. Sample loss could also result from using a perforated dialysis membrane (6 kDa MWCO), as the membrane retains molecules above 6 kDa from escaping into the lower buffer chamber.

Basic Protocol 3: Nucleosome Reconstitution

1) **Loss of sample in dialysis.** If using microfuge caps as dialysis vessels, sample loss can occur by inadvertently ripping the membrane when placing the ring onto the microfuge cap. This will often not be evident until samples are retrieved at the end of the reconstitution. To avoid loss of sample, attach ring to cap by placing it on at an angle. The dialysis membrane is

more likely to rip if the ring is pushed straight down on the cap. To avoid loss of sample from a dialysis bag, always clip one end and test by temporarily filling with ultrapure water. Hold tubing for a short period to ensure there are no leaks before placing sample in bag.

2) **Large amounts of free DNA or higher aggregate species.** Remeasure DNA and histone stocks being used in the reconstitution. For determining octamer concentration, use recommended wavelength and extinction coefficients for histones pertaining to the particular species (Luger et al., 1999; Dyer et al., 2004; Klinker et al., 2014). If histone concentrations are much lower than expected, significant amounts of free DNA will be observed; if higher than expected, higher order species/aggregates will be observed. Titrations of DNA and histones can help alleviate these issues (Luger et al., 1999; Dyer et al., 2004).

3) **Issues with the pump.** If the pump stops during the gradient dialysis, restart from where it left off. Even with a dual-head pump, the flow rates of the two sets of tubing can be different. This can be due to differences in wear or different tension between each tubing and pump head. If difficult to control, ensure that the tubing with the slower rate is the one adding RB Low, to ensure that the main beaker does not overflow. Also, position the tubing that removes buffer from the main beaker close to the surface, so that a constant volume is maintained.

ANTICIPATED RESULTS:

Using *Xenopus* histones and the Widom 601 positioning sequence, nucleosomes can be made and, in a single run on the MiniPrep/Prep Cell, purified in amounts ranging from 100 picomoles to 10's of nanomoles. Although molecular weight markers are not typically used with native acrylamide gels, purified nucleosomes can be inferred from slower migration compared to naked DNA (Figures 1 and 2). Nucleosomes can also be evaluated for their histone content by SDS-PAGE analysis. Although the Widom 601 sequence produces few off-product nucleosome species, nucleosome preparations may also contain significant amounts of hexasomes if histone ratios are off. However, nucleosomes and hexasomes can still be completely separated using the MiniPrep/Prep Cell systems (Figure 2B). Based on our experience with a 601 variant that produces a distribution of nucleosome positions, even closely migrating species can be separated, though yields from reconstitutions are much lower (Figure 2C). Yields tend to range from 38-55% when using the canonical Widom 601. Nucleosomes produced and purified using these protocols should be of sufficient quality for crystallography (Luger et al., 1997), high-resolution footprinting (Schwanbeck et al., 2004), site-specific cross-linking (Nodelman et al., 2017), nucleosome sliding and binding assays (McKnight et al., 2011), single-molecule FRET (Sabantsev et al., 2019), and cryo-EM (Matsumoto et al., 2019).

TIME CONSIDERATIONS:

The entire procedure described here – starting with PCR and ending with purified nucleosomes – takes roughly four to five days.

Basic Protocol 1: A single run of PCR (typically 10 ml) takes 4 hr: PCR setup (45 min); PCR program (2.25 hr); pooling PCR sample/concentrating PCR reaction (1 hr).

Basic Protocol 2: DNA purifications can be completed within 6 hr, whereas nucleosome purifications typically take 11-12 hr.

Specifically:

DNA purification: Setup of apparatus/pre-run/concentration of sample (1-1.5 hr); passage through the Prep Cell (3 hr); loading and running fractions on agarose gel (45 min); pooling and concentrating (45 min).

Nucleosome purification: Setup and pre-run of apparatus/concentration of sample (1-1.5 hr); passage through the Prep Cell (6-9 hr); loading and running fractions on native gels (2 hr); scanning (15 min); pooling and concentrating (1 hr).

Setup of the Prep Cell is more involved than the MiniPrep Cell, and for first time users, we recommend practicing the steps involved in setting it up before the day of a nucleosome purification.

Basic Protocol 3: Nucleosome reconstitutions require 1.5 to 2 days: Setup of buffers and nucleosome reconstitution sample (2 hr); linear gradient dialysis (24 hr); dialysis into Rb-Zero (3 hr to overnight).

Since DNA purifications can be completed in half a day, it is possible to do PCR in the morning, purify the DNA in the afternoon, and setup a nucleosome reconstitution in the evening. Another way to increase time efficiency is to purify two different nucleosome or DNA samples in parallel on two Prep Cell systems. Given the long day required for nucleosome purifications, it is prudent to work as efficiently as possible by using downtime to prepare for later steps. For example: set up the MiniPrep Cell/Prep Cell the morning of the purification while samples are concentrating; pour gels for fraction analysis immediately after loading sample onto gel column; and begin preparing and running the first three gels for analyzing fractions as soon as enough samples are available. It is helpful to have a dedicated set of syringes with attached tubing ready for both pouring gel columns and setup for the MiniPrep/Prep Cell. Also, retrieving samples from RB Zero dialysis the day before purification and leaving on ice overnight will save time.

Also, an important time consideration is the sample run time on the MiniPrep/Prep Cell systems. If working with a centered nucleosome (e.g. 40N40), the run time will be significantly longer compared to a related end-positioned nucleosome (0N80) run on a

column of the same gel height. For a canonical 601 sequence, where the major product should be greatly enriched, a 5-5.5 cm gel height is usually sufficient for the Prep Cell system; a higher column can add time to the day without added gain in separation. If working with positioning sequences that give rise to other significant species, a higher 6-7 cm column is often helpful.

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Luger et al., 1999. *An essential resource for nucleosome production. Describes the expression and purification of recombinant histones, refolding and purification of histone octamer, reconstitution of nucleosomes using linear gradient salt dialysis, and purification using the Bio-Rad Model 491 Prep Cell or a DEAE ion-exchange column.*

Dyer et al., 2004. *Updates and extends methods described in* (Luger et al., 1999)*. Includes protocols for creating and purifying DNA arrays of nucleosome positioning sequences, histone expression and purification, refolding and purification of histone octamer, nucleosome reconstitution, and nucleosome purification using the Bio-Rad Model 491 Prep Cell.*

INTERNET RESOURCES:

MiniPrep Cell Instruction Manual (catalog number 170-2908). Available at http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006032.pdf

Model 491 Prep Cell Instruction Manual. Available at http://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1702925.pdf

Acrylamide Polymerization: A Practical Approach (Bio-Rad Tech Note 1156). Available at http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_1156.pdf

FIGURE LEGENDS:

Figure 1. An example of nucleosome purification results using the MiniPrep Cell. Shown (numbered) are fractions resolved on 7% native acrylamide gels, scanned for Cy5 on a Typhoon5 Imager.

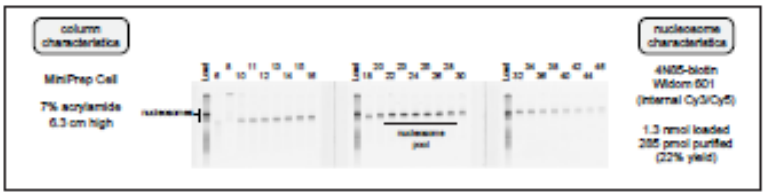

Figure 1
Nodelman, 2020

Figure 2. Examples of nucleosome purification results using the Prep Cell. Panels (A) and (B) show purifications using the 28 mm diameter column, and (C) and (D) show the elution profiles from the 37 mm diameter column. All fractions (numbered) were resolved on 7% native acrylamide gels, and either detected using a fluorophore (FAM for panels A and C, and Cy5 for B) using a Typhoon5 Imager, or stained with ethidium bromide and imaged on a UV box (D). In panel (B), the reconstitution used a mixture of purified histone dimer and tetramer (rather than histone octamer), with a reduced amount of H2A/H2B dimer to obtain both hexasome and nucleosome from a single prep. In panel (C), the nucleosome positioning sequence was a variant of the canonical Widom 601, containing a 1 bp insertion on the TA-rich side, 21 bp from the nucleosome dyad (Winger et al., 2018). This sequence change disruptions the strong positioning of the Widom 601, giving rise to several distinctly positioned species.

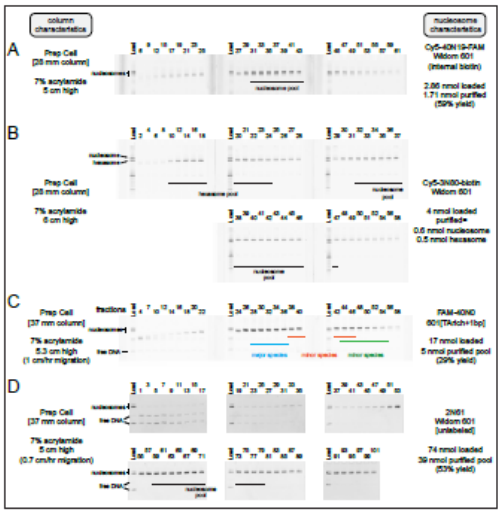

Figure 2
Nodelman, 2020

Figure 3. Schematic diagrams of the MiniPrep Cell and Prep Cell, indicating the flow of buffer and samples (reproduced with permission from Bio-Rad).

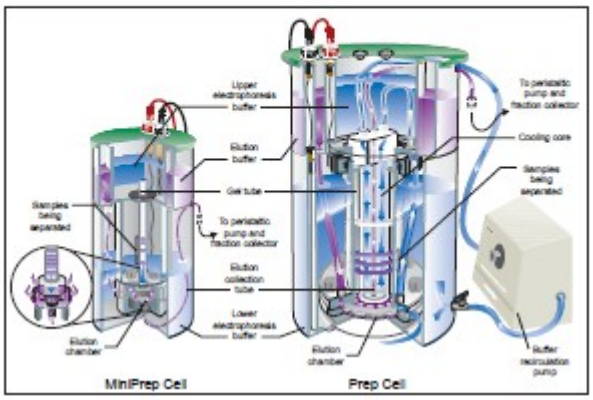

Figure 3
Nodelman, 2020

Figure 4. Overview of the Prep Cell. (A) Setup for polymerization of acrylamide gel, with buffer recirculation through a central cooling core. (B) Key equipment used for purification: (1) Prep Cell apparatus; (2) recirculation pump; (3) peristaltic pump; (4) fraction collector; (5) power supply. The MiniPrep Cell has the same setup but without the recirculation pump.

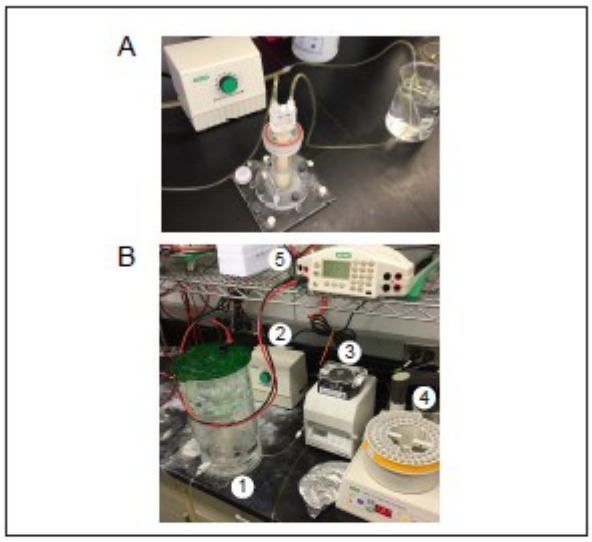

Figure 4
Nodelman, 2020

Figure 5. Overview of MiniPrep Cell assembly. (A) Parafilmed gel column after initiation of acrylamide gel polymerization and layering of butanol. (B) Polymerized acrylamide gel in glass column, after attachment to the elution manifold top. After gentle but thorough rinsing of gel top to remove butanol, this portion is briefly set aside while the elution manifold base is prepared. (C, D) Adding the dialysis membrane and then the support frit to elution manifold base, using a syringe to introduce elution buffer after each addition. (E, F) Attaching the elution manifold top onto the elution manifold base. Align the plastic screws on the manifold top with the holes in the manifold base. (G) After attachment, air bubbles should be removed by flushing with elution buffer using the attached syringe. After inserting gel column into the upper assembly (H), electrophoresis buffer should be added to the lower chamber to a volume that covers the height of the acrylamide gel column (I) and then added to the center reservoir of the upper chamber assembly (J). (K) Addition of elution buffer to outer chamber. (L) Fully assembled MiniPrep Cell, ready for pre-running in the cold room.

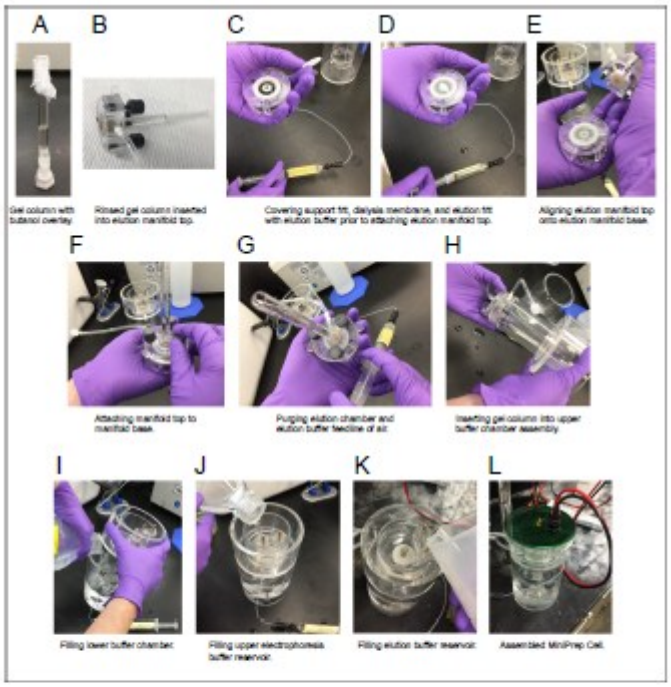

Figure 5
Nodelman, 2020

Figure 6. Purging air bubbles from the elution chamber of the Prep Cell. White arrows indicate the direction of buffer flow. Dashed arrows indicate paths for air bubble removal.

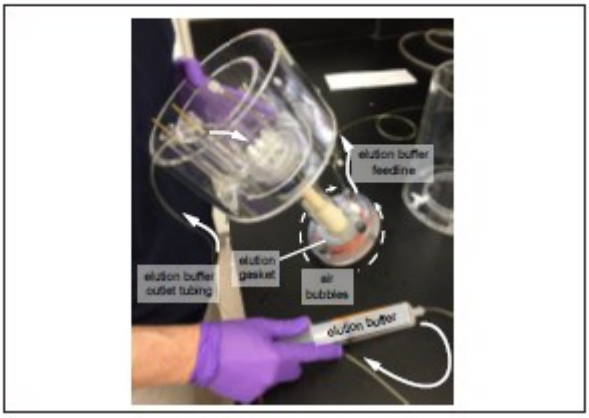

Figure 6
Nodelman, 2020

Figure 7. Schematic diagram of a nucleosome reconstitution setup.

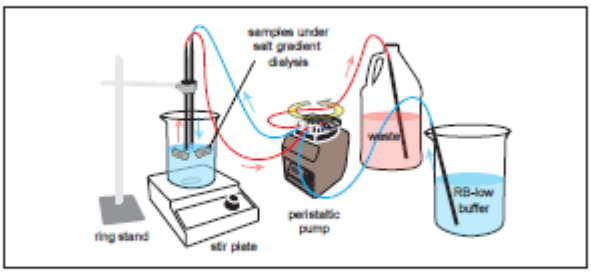

Figure 7
Nodelman, 2020

TABLES:

Table 1. Concentrations and volumes of reagents for PCR reactions.

Component	Final concentration	Single test reactions	Master mix
Ultrapure water	-	63-65 µl	6300 µl **
10x Thermopol Buffer	1x	10 µl	1 ml
100 mM $MgSO_4$	0, 1, 2 mM	0, 1, 2 µl	200µl **
100 ng/µl plasmid template	2 ng/µl	2 µl	200µl
100 µM primer forward	1 µM	1 µl	100 µl
100 µM primer reverse	1 µM	1 µl	100 µl
10 mm dNTP mixture	200 µM	20 µl	2 ml
Taq polymerase (100x)	1x	1 µl	100 µl
Vol_f		100µl	10ml

***We typically use 2 mM $MgSO_4$, but have occasionally needed to adjust this parameter. If different amounts are used, be sure to adjust amount of water accordingly.*

Table 2. Concentrations of reagents needed for a nucleosome reconstitution.

Component	Final concentration
Purified DNA	6 µM*
Ultrapure water	Add to reach final volume
4 M KCl	2 M
1 M Tris-HCl, pH 7.5	10 mM
500 mM EDTA, pH 8.0	1 mM
500 mM DTT	1 mM
Histone Octamer	5.95 µM
Histone Dimer	1.8 µM

**Nucleosomes can also be successfully reconstituted at concentrations < 6µM.*