# [2] Reconstitution of Nucleosome Core Particles from Recombinant Histones and DNA

*By* PAMELA N. DYER, RAJI S. EDAYATHUMANGALAM, CINDY L. WHITE, YUNHE BAO, SRINIVAS CHAKRAVARTHY, UMA M. MUTHURAJAN, and KAROLIN LUGER

## Introduction

The ability to prepare nucleosome core particles (NCPs), or nucleosomal arrays, from recombinant histone proteins and defined-sequence DNA has become a requirement in many projects that address the role of histone modifications, histone variants, or histone mutations in nucleosome and chromatin structure. This approach offers many advantages, such as the ability to combine histone variants and tail deletion mutants, and the opportunity to study the effect of individual histone tail modifications on nucleosome structure and function.

We have previously described comprehensive protocols for the expression and purification of histones, for the refolding of the histone octamer, and for the reconstitution and purification of crystallization-grade mononucleosomes.[1] The previously published version has now been amended, and steps that can be omitted or simplified if high degrees of purity and homogeneity are not an issue are indicated. The cloning strategies for the construction of plasmids containing multiple repeats of defined DNA sequences, and the subsequent large-scale isolation of defined-sequence DNA for nucleosome reconstitution, are described in detail. We also describe adapted procedures to prepare nucleosomes with histones from other species, and for the refolding and reconstitution of (H2A–H2B) dimers and $(H3–H4)_2$ tetramers. Methods to reconstitute nucleosomes from different histone subcomplexes are also described. A flow chart for all procedures involved in the preparation of ''synthetic nucleosomes'' is given in Fig. 1. Procedures described here are indicated in gray in Fig. 1.

## Cloning and Purification of Large Amounts of Defined-Sequence DNA

### *Cloning Strategy*

A general procedure to construct a plasmid containing multiple repeats of a given DNA sequence, based on published strategies,[2,3] and to purify large amounts of defined-sequence DNA fragments is outlined below.



0076-6879/04 $35.00

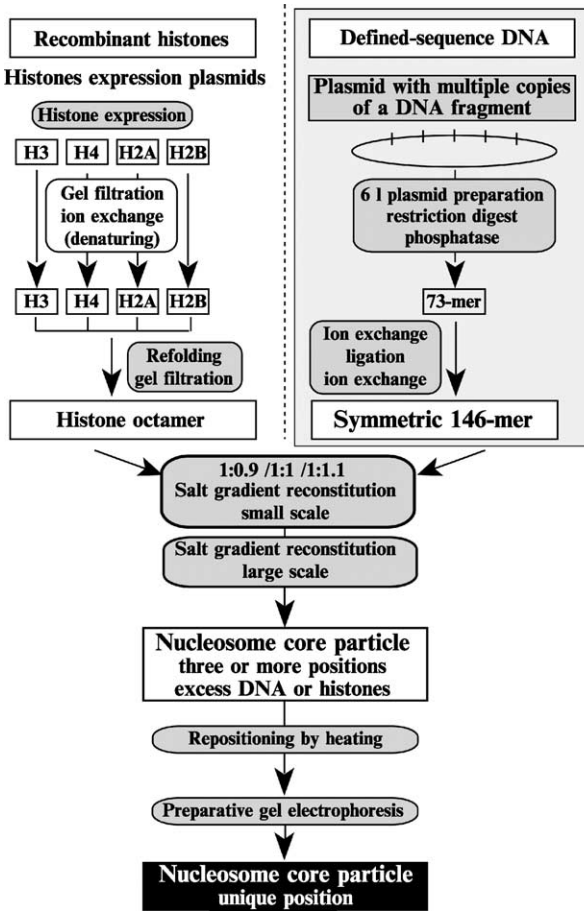


FIG. 1. Flow chart of methods used for preparation of components for nucleosomes. Procedures that are described in this chapter are shown in gray.

Figure 2 outlines the cloning strategy for fragments containing either the complete desired sequence (Fig. 2A), or one-half of a palindromic DNA fragment (Fig. 2B). Because of the recombination activities in most bacterial cells, long palindromic DNA fragments cannot be amplified, but must

[1] K. Luger, T. J. Rechsteiner, and T. J. Richmond, *Methods Enzymol.* **304,** 3 (1999).
[2] R. T. Simpson, F. Thoma, and J. M. Brubaker, *Cell* **42,** 799 (1985).
[3] T. J. Richmond, M. A. Searles, and R. T. Simpson, *J. Mol. Biol.* **199,** 161 (1988).

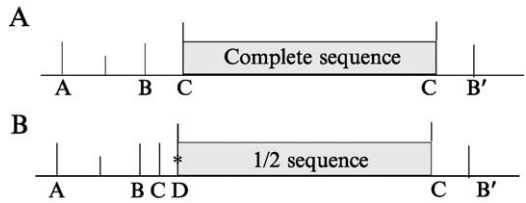


FIG. 2. Insert construction for the preparation of a defined-sequence DNA fragment. (A) Strategy for preparing an insert that encompasses the entire desired sequence (not suitable for palindromic sequences). (B) Strategy for designing inserts for ligation for palindromic (or partially palindromic) sequences. *, Site of large-scale ligation. Note that the two "halves" of the final product do not have to be identical if the restriction site for the final ligation step is chosen judiciously to prevent self-ligation (e.g., *Hinf*I). A, Unique site; B and B′, compatible cohesive ends; C, generates end(s) of actual fragments (large amounts needed); D, used for head–head ligation of two fragments; overhang can be chosen to allow or prohibit self-ligation.

be assembled by ligation of two halves. Figure 3 describes the strategy for duplication and outlines procedures for insert preparation. We use pUC-based vectors for these constructs.

In designing the cloning strategy for creating multiple DNA repeats, the DNA sequence of interest is flanked by restriction sites as shown in Fig. 2, where A is a unique site (e.g., *Kpn*I), B and B′ are sites for enzymes that are compatible, but nonidentical (e.g., *Bam*HI and *Bgl*II), and C is a site for an enzyme that is used to excise the fragment from the plasmid (e.g., *Eco*RV). Here, blunt ends are desirable. If the final DNA fragment is to be generated by large-scale ligation of two shorter fragments (e.g., if palindromic 146-bp DNA fragments are the desired end-product), restriction enzyme D should generate overhangs suitable for high-efficiency ligation. We used *Eco*RI for a perfectly palindromic 146-bp DNA fragment,[4] and a *Hinf*I site to generate 147-bp DNA fragments by ligation of two fragments.[5] Because large amounts of restriction enzymes cutting sites C and D will be used, economical considerations also come into play in the cloning strategy.

Digestion of the plasmid DNA with A and B creates a vector into which a fragment generated by A and B′ can be ligated, destroying the restriction site at the B–B′ junction (Fig. 3). Thus, with each cloning step, the number

[4] K. Luger, A. W. Maeder, R. K. Richmond, D. F. Sargent, and T. J. Richmond, *Nature* **389,** 251 (1997).

[5] C. A. Davey, D. F. Sargent, K. Luger, A. W. Maeder, and T. J. Richmond, *J. Mol. Biol.* **319,** 1097 (2002).

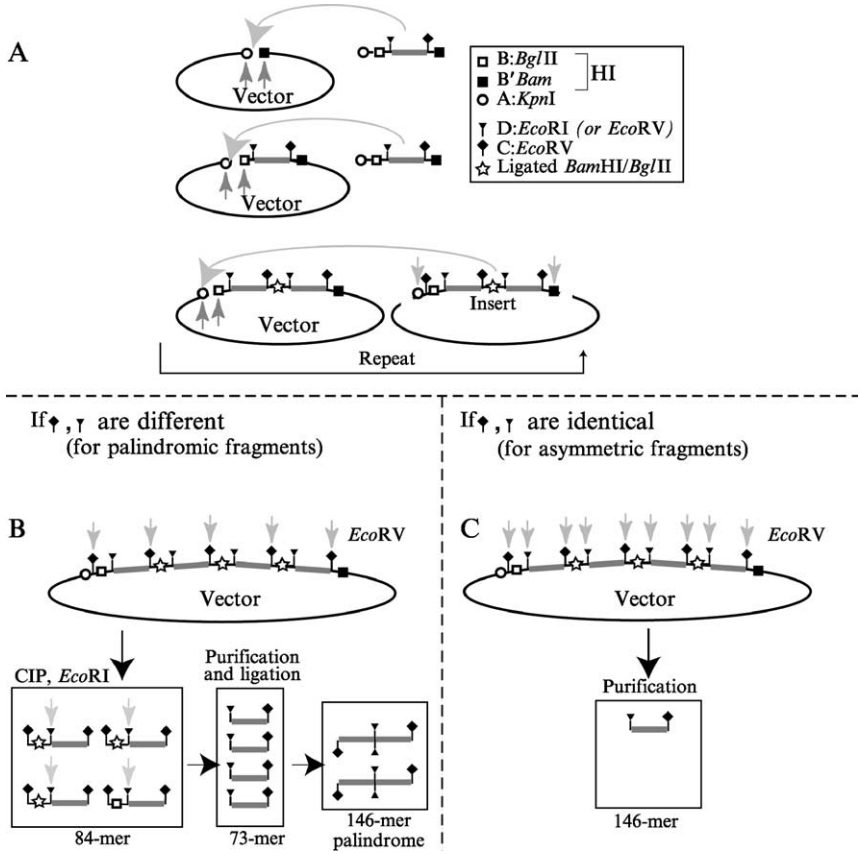


FIG. 3. Strategy for amplification and preparation of large amounts of inserts designed in Fig. 2. (A) Cloning and duplication strategy. Sites for restriction enzymes are indicated by symbols (see *inset* for legend). (B) Insert preparation from large-scale plasmid preparations (see text for details) for palindromic DNA fragments that undergo ligation. CIP, Incubation with calf intestine phosphatase. (C) Insert preparation for nonpalindromic DNA fragments that do not need to be self-ligated.

of inserts can be doubled. The individual steps for fragment insertion and amplification are described.

1. Synthesize and anneal pair(s) of suitable oligonucleotides (oligos). Follow standard cloning procedures to insert the fragment into a suitable high-copy plasmid via restriction sites A and B′.

2. Cut the plasmid containing the proper insert with restriction enzymes A and B (digest 1). Purify the vector DNA.

3. Cut the plasmid containing the insert with a second digest of restriction enzymes A and B′ (digest 2). Purify the insert DNA away from the plasmid vector and keep the insert generated by the digest.

4. Ligate the insert DNA (created by digest 2) with the vector DNA (created by digest 1).

5. Repeat steps 2–4: Each repetition will duplicate the number of previously present insert copies. Depending on the length of the insert, about 16 to 24 inserts can be obtained easily. Use HB 101 cells or other host cells that are RecA minus for plasmid amplification. The following statistics give the experimental amplification efficiencies found by our laboratory for each doubling cycle: $1 \rightarrow 2$, ~100% efficiency; $2 \rightarrow 4$, ~70% efficiency; $4 \rightarrow 8$, ~60% efficiency; $8 \rightarrow 16$, ~40% efficiency.

6. Assay for total size of the insert by digestion with restriction enzymes A and B′, and check for integrity of inserts by sequencing (early stages) and by cutting with C.

7. If efficiencies for duplication are low, try ligation of a 2-mer or 4-mer instead of duplication, to increase insert number.

### *Large-Scale Plasmid Purification*

This method has been adapted from the original alkaline lysis protocol described earlier.[6] It has been optimized for high yields and purity of pUC-based plasmids, containing 24 × 146 bp (or 84-bp) inserts.

#### *Equipment*

Centrifuge
37° incubator/water bath
TSK-DEAE column
Orbital shaker
12 wide-bottom 4-L Fernbach flasks

#### *Buffers and Reagents*

Alkaline lysis solution I: 50 m*M* glucose, 25 m*M* Tris-HCl (pH 8.0), 10 m*M* EDTA (pH 8.0)
Alkaline lysis solution II: 0.2 *N* NaOH, 1% (w/v) sodium dodecyl sulfate (SDS)
Alkaline lysis solution III: 4 *M* potassium acetate, 2 *N* acetic acid
Ampicillin (100-mg/ml stock solution, sterile filtered)
Calf intestine alkaline phosphatase (CIAP; Roche Molecular Biochemicals, Indianapolis, IN)

[6] J. Sambrook and D. Russell, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.

CIA: Chloroform–isoamyl alcohol (24:1, v/v)
*Eco*RI (~100,000 U/ml)
*Eco*RV (~100,000 U/ml)
100% ethanol, ice cold
Isopropanol
Calbiochem Miracloth (EMD Biosciences, San Diego, CA)
4 *M* NaCl, autoclaved
3 *M* Sodium acetate (pH 5.2), autoclaved
PAGE [10% polyacrylamide, 0.2 × Tris–borate–EDTA (TBE)]
40% PEG 6000, autoclaved
Phenol, Tris–EDTA (TE) equilibrated
RNase A (DNase free[6])
TE 10/0.1: 10 m*M* Tris-HCl (pH 8.0), 0.1 m*M* Na-EDTA; autoclaved
TE 10/50: 10 m*M* Tris-HCl (pH 8.0), 50 m*M* Na-EDTA; autoclaved
T4 DNA ligase (200,000 U/ml)
Terrific broth (TB): 1.2% (w/v) Bacto Tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol. Adjust autoclaved and cooled medium to a final concentration of 17 m*M* $KH_2PO_4$ and 72 m*M* $K_2HPO_4$

*Plasmid Purification*

1. Inoculate each of four 5-ml precultures containing TB (or 2× TY; see Histone Expression and Purification, below) and ampicillin (100 μg/ml) with a colony from a freshly transformed plate. Shake for 3–4 h at 37°. Transfer all precultures to a 500-ml flask containing 100 ml of 2× TY and ampicillin (100 μg/ml), and incubate for 2–3 h at 37° until turbid. Do not grow to saturation. Transfer equal amounts of the preculture to 12 Fernbach flasks containing 500 ml of TB and ampicillin at 100 μg/ml. Incubate under vigorous shaking for 16–18 h at 37°. Harvest cells by centrifugation in 500-ml centrifuge bottles. Fresh weight yields ~125 g of cells. Cells should be processed immediately for optimal yields.

2. Resuspend cells from 6 liters of cell culture in a total of 360 ml of alkaline lysis solution I by passage through a 10-ml plastic pipette. Redistribute the cells equally back into the six centrifuge bottles. Add 120 ml of alkaline lysis solution II to each bottle. Mix by shaking vigorously at least 20 times, until the thick translucent suspension is completely free of any clumps of cells. Incubate on ice, and shake repeatedly for a total of 10 to 20 min. Break up large clumps that still remain after such treatment by passage through a 10-ml disposable plastic pipette.

3. Carefully pour 210 ml of ice-cold alkaline lysis solution III down the side of each bottle. Mix by inverting and swirling 10 times and incubate on ice for 20 min. This step is critical because plasmid DNA is renatured,

whereas chromosomal DNA precipitates. Viscosity is reduced dramatically during this step. Low yields, or large amounts of chromosomal DNA in the plasmid preparation, may result if mixing is done too slowly.

4. Centrifuge at 10,000*g* for 20 min at 4°. Warm the rotor to 20° by running empty at 8000*g* for 15 min. Pour the supernatant through Miracloth to remove remaining precipitate, and add 0.52 volume of isopropanol. Let stand at room temperature for 15 min.

5. Centrifuge at 10,000*g* for 30 min at 20° to collect the precipitate. Air dry for 30 min to 1 h. Using a clean spatula, distribute pellets between two 30-ml centrifuge tubes. Use 5 ml of TE 10/50 to rinse out centrifuge bottles, and adjust each tube to a final volume of 20 ml. Mix the DNA into a homogeneous solution, and then add 120 $\mu$l of RNase A (10 mg/ml) (an RNase A stock of 1.2 Kunitz units/$\mu$l should be diluted to 1:100 in relation to the final reaction, ~0.01 Kunitz unit/$\mu$l reaction mix) and incubate at 37° overnight. The pellets should have dissolved completely. (Store at −20° as necessary.)

6. If the suspension is viscous, dilute with TE 10/50 buffer to up to twice the volume. Extract each 20 ml of suspension with 10 ml of phenol. Centrifuge at 27,000*g* for 20 min at 20°. The DNA will be in the upper, aqueous phase, separated from the phenol phase by a thick white interphase. Repeat two more times or until the interface is clear. Extract the aqueous phase with 10 ml of CIA. Spin for 5 min (12,000*g*, 20°). Transfer the aqueous phase into a 50-ml centrifuge tube and adjust to a final volume of 30 ml with TE 10/50.

7. Precipitate plasmid DNA by adding one-fifth of the original volume of 4 *M* NaCl (to give 0.5 *M* NaCl) and two-fifths of 40% PEG 6000 [to give 10% (w/v) PEG 6000]. Mix at 37° for 5 min and incubate on ice for 30 min.

8. Centrifuge at 3000*g* in a swinging-bucket tabletop centrifuge for 20 min at 4°. Decant the supernatant, which contains RNA. Dissolve the pellets in a total of 15 ml of TE 10/0.1 (overnight at room temperature or for less time at 37°). Check both fractions by agarose gel electrophoresis. Fractionation should be complete, and there should be no traces of RNA visible in the plasmid fraction.

9. Extract two times with 10 ml of CIA to remove PEG. Ethanol precipitate DNA by addition of a 1/10 volume of 3 *M* sodium acetate (pH 5.2) and 2.5 volumes of 100% cold absolute ethanol. Pellet the DNA, dissolve in 10 ml of TE 10/0.1 by incubating for 1 to several hours at 37°, and determine the total concentration. Yields are usually between 150 and 200 mg.

*Purification of Insert.* Experimental details in this section depend on the restriction sites that were chosen in the design of the plasmid. Given

the large amounts of DNA present, restriction digests can routinely be performed at plasmid concentrations of 1 mg/ml. Most restriction enzymes are more efficient under these conditions. Optimize reaction conditions before proceeding with large-scale digestions. Below we give conditions that were used for isolation of the palindromic 146-bp DNA fragment derived from human $\alpha$-satellite DNA that is routinely used for crystallography.[4]

1. The insert is excised with *Eco*RV, at a concentration of 1 mg/ml plasmid, in sterile 50-ml centrifuge tubes. Use 30 units of *Eco*RV per nanomole of *Eco*RV site. Incubate at 37° for at least 16 h, and then check for completion by gel electrophoresis on 10% polyacrylamide gels (0.2× TBE). If the digest is not complete, add 50% more restriction enzyme and incubate for another 15 h. Check the digest as described above.

2. Separate the excised *Eco*RV fragment from the linearized plasmid by PEG precipitation. Add 0.192 volume of 4 *M* NaCl and 0.346 volume of 40% PEG 6000. Incubate on ice for 1 h and spin down the vector DNA at 27,000*g* and 4° for 20 min. Precipitate the *Eco*RV fragment contained in the supernatant by the addition of 2.5 volumes of 100% cold ethanol. Air dry the DNA briefly (~10 min) and dissolve in 5 ml of TE 10/0.1.

3. Determine the concentration. Check both precipitated PEG supernatant and PEG pellet on a 1% agarose gel and PAGE as described above (run series of 1:10 dilutions). There should be no cross-contamination between the two fractions. Yields should be close to 90% (i.e., if the fragments encompass 40% of the entire plasmid, ~40 mg of excised fragment should be obtained 100 mg of plasmid). *Note:* This procedure will *not* work for DNA fragments with sticky ends.

4. If the cloned fragment represents the entire sequence, either use as is (after phenol extraction and ethanol precipitation), or purify further by ion-exchange chromatography. If further cutting and ligation are required, proceed with step 5.

5. Dephosphorylate *Eco*RV fragment by combining *Eco*RV fragment (1 mg/ml) with calf intestine alkaline phosphatase (CIAP, 1 U/nmol of DNA end; Roche), using the conditions given by the manufacturer. Incubate at 37° for 24 h, and then add 50% of the original amount of CIAP and incubate for another 24 h at 37°. Complete phosphorylation is essential, because self-ligation of the blunt ends during subsequent steps needs to be avoided. If in doubt, perform a small-scale assay for blunt-end ligation. None should occur if dephosphorylation is complete.

6. Inactivate the CIAP by extracting the DNA solution two times with 50% of the original volume of phenol–CIA (1:1 mixture) and then ethanol precipitate by addition of a 1/10 volume of 3 *M* sodium acetate (pH 5.2)

and 2.5 volumes of cold ethanol. Spin down the precipitated DNA at 3000*g* (swinging bucket tabletop centrifuge), air dry the pellet briefly, and dissolve in 5 ml of TE 10/0.1.

7. To create cohesive ends for self-ligation, use *Eco*RI at 20–30 U/nmol of *Eco*RI site (substrate concentration, 1 mg/ml) and incubate at 37° for at least 15 h. Check completion of the digest by PAGE. Make sure the digestion is complete before proceeding with the next step.

8. FPLC purify the fragment by chromatography over a TSK-DEAE column (the sample can be loaded directly, or it can be ethanol precipitated to reduce the volume). Ethanol precipitate the FPLC fractions (no need to add salt), air dry the pellet briefly, and dissolve it in ~5 ml of TE 10/0.1 or 1× ligation buffer (see below). Yields are typically 85% of the starting amount.

9. Perform a small-scale ligation to test whether ligation can be driven to completion and to assess whether phosphorylation of *Eco*RV ends was complete. The latter should be visible in the formation of a ladder as a result of blunt-ended tail–tail ligation of the *Eco*RV fragments. Use ~0.5 U of ligase per microgram of fragment, at a substrate concentration of 1 mg/ml, under conditions as given by the manufacturer. Incubate at room temperature for at least 15 h, and check completion of ligation by PAGE. Add more ligase if necessary.

10. If necessary, purify ligated from unligated fragments by ion-exchange chromatography on a TSK-DEAE column (or another ion-exchange column of similarly high resolution). This separation depends strongly on the DNA sequence and must be optimized individually.

## Histone Expression and Purification

These procedures, which utilize expression vectors for *Xenopus laevis* histones[7] have been described extensively.[1] We have since used this protocol to express and purify various H2A and H3 histone variants from different species (e.g., Suto *et al.*[8]), and histones from yeast (White *et al.*[9]; also see Wittmeyer *et al.*[10]), *Drosophila,* and mouse. All these histones have been subcloned in untagged form into the pET vector series (Novagen,

[7] K. Luger, T. J. Rechsteiner, A. J. Flaus, M. M. Waye, and T. J. Richmond, *J. Mol. Biol.* **272,** 301 (1997).
[8] R. K. Suto, M. J. Clarkson, D. J. Tremethick, and K. Luger, *Nat. Struct. Biol.* **7,** 1121 (2000).
[9] C. L. White, R. K. Suto, and K. Luger, *EMBO J.* **20,** 5207 (2001).
[10] J. Wittmeyer and T. Formosa, *Methods Enzymol.* **262,** 415 (1995).

Madison, WI). Histidine-tagged histones are also purified in the same way. In some cases, codon usage has been optimized for *Escherichia coli,* and the time after induction as well as the bacterial strain have been optimized for each case. In some cases, better results are obtained with BL21(DE3) strains that compensate for poor codon usage. All expressed proteins are invariably expressed in insoluble form and isolated from the insoluble fraction obtained after cell lysis (inclusion bodies).

*Equipment*

Dialysis tubing (6- to 8-kDa cutoff, 2.5- to 4-cm flat width)
Ion-exchange column, TSK SP-5 PW resin material
Lyophilizer
Orbital shaker
Peristaltic pump
Sephacryl S-200 high-resolution gel-filtration column (5 × 100 cm; Pharmacia, Uppsala, Sweden)
6 wide-bottom Fernbach flasks (4 L)
Tissumizer (Tekmar, Cincinnati, OH) or sonicator for cell lysis

*Buffers and Reagents*

Ampicillin (100-mg/ml stock solution, sterile filtered)
2-mercaptoethanol (2-ME)
BL21(DE3)pLysS, BL21(DE3)pLysS Codonplus or BL21(DE3) cells, competent
Isopropyl-$\beta$-D-thiogalactopyranoside (IPTG)
Centrifuge tubes, 50 ml
Chloramphenicol stock solution, 25 mg/ml in ethanol
Dimethyl sulfoxide (DMSO)
Glucose
Lysozyme
Liquid nitrogen
SDS–PAGE equipment: Standard equipment, 18% SDS gels
TYE agar plates: 1.0% (w/v) Bacto Tryptone, 0.5% (w/v) yeast extract, 0.8% (w/v) NaCl, 1.5% (w/v) agar, ampicillin (100 $\mu$g/ml), and chloramphenicol (25 $\mu$g/ml)
2× TY: 1.6% (w/v) Bacto Tryptone, 1.0% yeast extract, 0.5% NaCl, with antibiotics and 0.1% glucose
Unfolding buffer: 6 *M* guanidinium-HCl, 20 m*M* Tris-HCl (pH 7.5), 5 m*M* dithiothreitol (DTT)
Wash buffer: 50 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, 1 m*M* benzamidine, 1 m*M* 2-ME

*Histone Expression*

1. Transfect BL21(DE3)pLysS cells with 0.1 to 1 $\mu$g of the pET-histone expression plasmid and plate on TYE agar plates with ampicillin (100 $\mu$g/ml) and chloramphenicol (25 $\mu$g/ml). Incubate at 37° overnight. For best and most reproducible results, a new transformation should be done each night for the protein that is expressed the next day. For some histones, BL21(DE3)pLysS Codonplus (RIL) or BL21(DE3) cells will give better results.

2. Expression conditions depend on the histone in question and should be optimized individually. For most histones, conditions given in Luger *et al.*[7] are adequate.

3. Inoculate each of four preculture tubes (4 ml of 2× TY with antibiotics and 0.1% glucose) with one colony from the culture plate. Incubate in a shaker at 37°.

4. When preculture tubes appear slightly turbid (2–3 h), add the contents of all four tubes to a flask containing 100 ml of 2× TY with appropriate antibiotics and glucose. Incubate in a shaker at 37°. For most reproducible results, do not let precultures grow to saturation.

5. When the 100-ml flask has reached an $OD_{600}$ of ~0.4, distribute the contents evenly into six wide-bottom Fernbach flasks containing 1 liter each of 2× TY medium and appropriate antibiotics and glucose. Incubate in a shaker at 37° until the $OD_{600}$ reaches about 0.4. Induce expression by addition of IPTG to a final concentration of 0.2–0.4 m*M*.

6. After 2 h, harvest the cells at room temperature and resuspend the cell pellets in a total of 35 ml of wash buffer. Flash freeze in liquid nitrogen and store at −20° in a 50-ml centrifuge tube.

*Note.* Cells expressing histone proteins (especially H4) are prone to lysis and should be centrifuged at room temperature. For the same reason, it is not recommended (or necessary) that the cell pellet be washed. Resuspend the cells well before freezing, as this will improve lysis on thawing. The cell suspension can be stored at −20 or −70°.

*Inclusion Body Preparation*

1. Lyse the cell suspension by thawing at 37°.

2. Pour the cell extracts into 250-ml centrifuge bottles. At this point, the cells should be viscous. If the cell suspension is still watery, then full lysis has not occurred. In this case, or if no pLysS plasmid has been present, add lysozyme to a concentration of 1 mg/ml and incubate on ice for 30 min. Repeated freeze–thaw cycles also facilitate lysis. Bring the total volume to 100 ml.

3. Blend the cell extracts with the Tissumizer to reduce viscosity. Blend until viscosity is reduced; avoid overheating of sample. A sonicator can also be used with similar results.

4. Spin at $4^{\circ}$ for 20 min at 12,000*g*. Pour off the supernatant and resuspend the tight, solid pellet with 75 ml of wash buffer containing 1% Triton X-100. If the pellet is ''spongy,'' sonicate/blend (Tissumizer) again. Spin for 20 min as described previously.

5. Repeat once as described above and once with wash buffer without Triton X-100. The drained pellet can be stored for a limited time at $-20^{\circ}$.

### *Histone Purification*

A two-step purification procedure yielding up to 1 g of highly pure histone protein from 6 liters of induced cells has been described previously.[1] The purification protocol involves gel filtration and HPLC/ion-exchange chromatography under denaturing conditions. If purity is not a major concern, one of the chromatography steps (usually the ion-exchange chromatography) can be omitted. The gel-filtration column can be scaled down accordingly if only small amounts of histones are purified. The purified proteins can be stored as lyophilisates for extended periods of time, to be used in refolding reactions as described subsequently.

## Refolding of Histone Octamer

All possible combinations of recombinant *Xenopus laevis* full-length and globular domain histone proteins, as well as histone octamers from other species, or containing histone variants, can be refolded to functional histone octamers according to a previously described protocol.[1] The method works best for 6 to 15 mg of total protein; the limiting factor here is the size of the gel-filtration column. Much smaller samples can be prepared when using an analytical column. Some applications require the preparation of H2A–H2B dimers and $(H3–H4)_2$ tetramers. The same protocols can be used for refolding and purification of these histone subcomplexes.

### *Equipment*

Dialysis tubing (6- to 8-kDa cutoff, 2.3-cm flat width)

HiLoad 16/60 Superdex 200 HR preparation-grade gel-filtration column (Pharmacia), equipped with UV detector and fraction collector

SDS-PAGE equipment: Standard equipment, 18% SDS gels

Concentration device: Devices suitable for up to 25-ml volumes [e.g., Centricon centrifugal filter devices; Amicon Bioseparations (Millipore, Bedford, MA)]

*Buffers and Reagents*

Purified and lyophilized histones (3- to 4-mg aliquots)
Unfolding buffer: 6 *M* guanidinium chloride, 20 m*M* Tris-HCl (pH 7.5), 5 m*M* DTT. Needs to be made fresh for good refolding efficiency
Refolding buffer: 2 *M* NaCl, 10 m*M* Tris-HCl (pH 7.5), 1 m*M* Na-EDTA, 5 m*M* 2-ME

*Histone Octamer Refolding*

1. Dissolve each histone aliquot to a concentration of approximately 2 mg/ml in unfolding buffer. Unfolding should be allowed to proceed for at least 30 min and for no more than 3 h. Determine the concentration of the unfolded histone proteins by measuring absorbance of the ''undiluted'' solution against unfolding buffer at 276 nm (remove any undissolved particulate matter by centrifugation, if necessary). Extinction coefficients can be obtained (see Table I for full-length *Xenopus* and yeast histones) or calculated (for histones from other species or histone variants) using the following Web site: http://ca.expasy.org/tools/protparam.html. *Note:* Using correct extinction coefficients is essential for good yields in refolding.

2. Mix histone proteins to exactly equimolar ratios and adjust to a total final protein concentration of 1 mg/ml, using unfolding buffer. Dialyze at 4° against at least three changes of 600 ml of refolding buffer (at least 6 h each; the second or third step should be overnight). Histone octamer should always be kept at 0–4° to avoid dissociation.

3. Remove any precipitated protein by centrifugation. Concentrate to a final volume of approximately 1 ml, using the concentration device. Histone octamers refolded with tailless histones often stick to the filter membrane of the concentration device and take a much longer time to concentrate. Make sure the octamer solution is mixed (pipette up and down) to avoid clogging filtration devices.

4. Load samples onto the gel-filtration column previously equilibrated with refolding buffer as described.[1] High molecular weight aggregates will elute after about 45 ml, histone octamer at 65 to 68 ml, $(H3–H4)_2$ tetramer at about 72 ml, and histone (H2A–H2B) dimer at 84 ml (Fig. 4).

5. Check the purity and stoichiometry of the fractions by 18% SDS–PAGE. Dilute sample by a factor of at least 2.5 before loading onto the gel to reduce distortion of the bands resulting from the high salt concentration.

TABLE I
MOLECULAR WEIGHTS AND MOLAR EXTINCTION COEFFICIENTS ($\varepsilon$) FOR FULL-LENGTH *Xenopus laevis* AND *Saccharomyces cerevisiae* HISTONE PROTEINS

| Histone | Full-length *Xenopus* Histone | | Full-length *S. cerevisiae* histone | |
|---|---|---|---|---|
| | Molecular weight | $\varepsilon$ (cm/*M*), 276 nm | Molecular weight | $\varepsilon$ (cm/*M*), 276 nm |
| H2A | 13,960 | 4050 | 13,858 | 4350 |
| H2B | 13,774 | 6070 | 14,106 | 7250 |
| H3 | 15,273 | 4040 | 15,225 | 2900 |
| H4 | 11,236 | 5400 | 11,237 | 5800 |

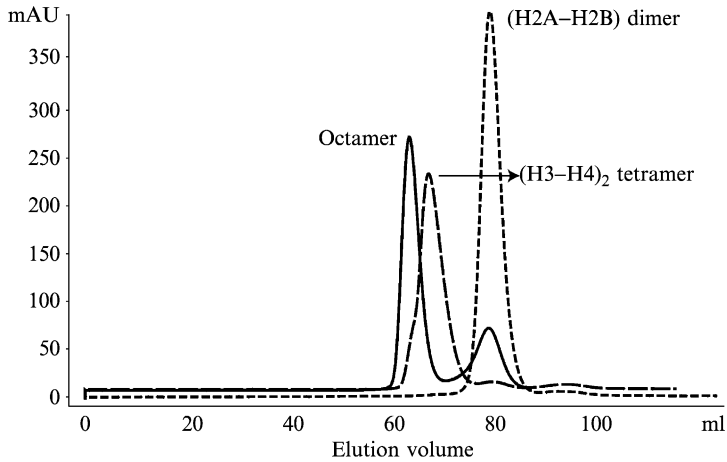


Fig. 4. Elution profile of histone subcomplexes from a Superdex S-200 gel-filtration column. See text for details. Histone octamer (solid line) elutes first, in accordance with its molecular weight (108,500). A small excess of H2A and H2B is apparent in the formation of a small dimer peak, which can be separated from the main peak by this method. In contrast, $(H3–H4)_2$ tetramer (MW, 53,000) elutes close to the octamer peak (dashed line). Note the small shoulder indicative of some octamer-like assemblies formed by $(H3–H4)_2$ tetramer. H2A–H2B dimer (MW, 27,000; dotted line) elutes last.

If octamer contains globular H3 histone, be aware that globular histone H3 comigrates with full-length H4, and only two bands will be seen on the gel.[7]

6. Pool fractions containing octamer and concentrate, using the concentration device, to 3–15 mg/ml. Determine the concentration of the octamer spectrophotometrically. Extinction coefficients can be

approximated by adding up those of individual histones (times two). Yields of pure histone octamer are usually between 50 and 75% of the input material (yields may be lower for octamer containing tailless histones).

7. Octamer can be stored on ice (for short-term storage), or at $-20^{\circ}$ as a 50% (v/v) glycerol solution (for long-term storage). Octamer containing globular histones usually is stable only for a few months and can often form higher order aggregates on storage for longer periods of time. Octamer stored on ice can be used as such for nucleosome reconstitution. Concentrations of octamer in 50% glycerol are extremely inaccurate, and pipetting of accurate amounts is difficult. When stored in glycerol, dialyze octamer overnight at $4^{\circ}$ against refolding buffer before use for nucleosome reconstitution and redetermine the concentration spectrophotometrically.

## Reconstitution of Nucleosome Core Particles

*In vitro* (and *in vivo*) reconstitution of nucleosomes relies on the sequential binding of one $(H3–H4)_2$ tetramer and two H2A–H2B dimers onto the DNA. This may be achieved in two different ways. Salt gradient deposition[3,11] relies on the fact that $(H3–H4)_2$ tetramers bind at higher salt concentrations than do H2A–H2B dimers. Chaperone-assisted assembly makes use of specific histone–chaperone complexes that ensure the ordered addition of histone complexes onto the DNA.[10]

We have previously described a detailed method for the large-scale assembly of NCPs, using salt gradient deposition.[1] Here we describe three methods to reconstitute nucleosomes. Microscale reconstitutions (1 $\mu$g) are routinely done with radiolabeled DNA. Small-scale reconstitutions (25–100 $\mu$g) are used to carefully titrate histones and DNA to optimize yields for subsequent large-scale reconstitutions (0.5–4 mg).

We note that identical methods can be used with refolded and purified H2A–H2B dimers and $(H3–H4)_2$ tetramers instead of octamers, with essentially the same results (Fig. 5). However, special care must be taken to combine H2A–H2B dimer, $(H3–H4)_2$ tetramer, and DNA at a molar ratio of 2:1:1, because excess dimers and tetramers can lead to the formation of aggregate or of nonnative nucleosome species. These species are apparent by their relatively intense staining with ethidium bromide as compared with Coomassie Brilliant Blue, and by the inability to reposition to the energetically favored position by ''heat shifting.''

[11] J. O. Thomas and P. J. G. Butler, *J. Mol. Biol.* **116,** 769 (1977).

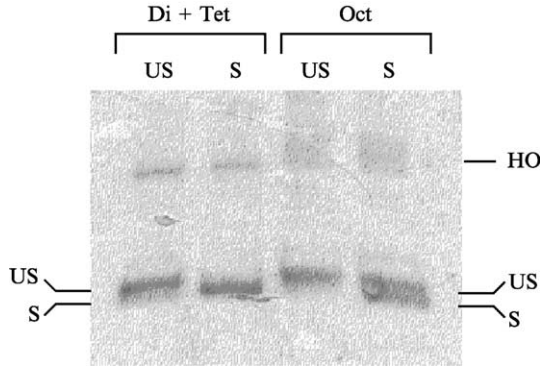


FIG. 5. Nucleosome core particles formed with DNA and octamer, or with (H2A–H2B) dimers and $(H3–H4)_2$ tetramer, are identical. High-resolution gel-shift assays (see text for details) demonstrate that the final nucleosome core particle product is independent of the type of histone subcomplexes used for assembly. US and S, samples before and after a 2-h incubation at 37°, respectively; HO, higher order aggregates, which are easily removed by preparative gel electrophoresis (Fig. 6). The gel was stained with Coomassie Brilliant Blue.

*Equipment*

Dialysis tubing (6- to 8-kDa cutoff, 1 or 2.3-cm flat width); dialysis membrane cut into a circle with a radius of 3 cm

Concentration device: Devices suitable for up to 25-ml volumes [e.g., Centricon centrifugal filter devices from Amicon Bioseparations (Millipore); Vivaspin devices from ISC Bioexpress (Kaysville, UT)]

Microdialysis devices that hold total volumes of 5–350 $\mu$l [e.g., dialysis buttons from Hampton Research (Laguna Hills, CA)]

Reconstitution apparatus with connected tubing, as introduced in Luger *et al.*[1]

Peristaltic pump with a double pump head, capable of maintaining a constant flow rate of 1–6 ml/min [e.g., Econo pump (Bio-Rad, Richmond, CA)]

Prep Cell apparatus: Model 491 Prep Cell (Bio-Rad) with a standard power supply, connected to a UV detector (e.g., Econo UV monitor; Bio-Rad), a fraction collector (e.g., model 2110 fraction collector; Bio-Rad), a chart recorder (e.g., model 1327 Econo recorder; Bio-Rad), and equipped with a peristaltic pump

Standard PAGE apparatus, 5% polyacrylamide gels (acrylamide–bisacrylamide 59:1, 0.2× TBE, 10 cm × 10 cm × 1.5 mm)

*Buffers and Reagents*

0.2× TBE (Prep Cell electrophoresis buffer) (2000 ml)
4 *M* KCl or 5 *M* NaCl stock solution
CCS (long-term storage buffer): 20 m*M* potassium cacodylate (pH 6.0), 1 m*M* EDTA
Purified octamer (3–15 mg/ml) or dimers and tetramers; DNA (3–6 mg/ml)
Reconstitution and storage buffers (make and prechill buffers at 4°):
RB-high (reconstitution buffer): 2 *M* KCl, 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 1 m*M* DTT (400 ml)
RB-low (reconstitution buffer): 0.25 *M* KCl, 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 1 m*M* DTT (2000 ml)
TCS (short-term storage buffer, Prep Cell elution buffer): 20 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 1 m*M* DTT

Make sure all buffers are made fresh. During reconstitution of NCP with octamer containing globular histones, make the above-described buffers with 5 m*M* DTT (instead of 1 m*M* DTT).

*Microscale reconstitution*

Microscale reconstitution is a good method for reconstitution with radiolabeled DNA.[12,13] If performed at ambient temperatures, only one species will be observed by gel electrophoresis, and the ''heat-shifting'' step (see below) can be omitted. At 4°, different translational positions are observed, and heat shifting can be studied.

1. Mix 1 μg of radioactively labeled DNA in ~9 μl of 2 *M* NaCl (bringing the NaCl concentration to 2 *M* with 5 *M* NaCl), and then add the appropriate amount of octamers.
2. Incubate for 30 min at ambient temperature, then add an equal volume (10 μl) of 10 m*M* Tris-HCl, pH 7.6, and incubate for 1 h either at 4° or at room temperature.
3. The following additions of 10 m*M* Tris-HCl, pH 7.6, are each for 1 h: 5 μl (→0.8 *M* NaCl); 5 μl (→0.67 *M* NaCl); 70 μl (→0.2 *M*); and 100 μl (→0.1 *M;* optional).
4. Analyze by native PAGE as described below, and autoradiograph the gel.

[12] J. M. Gottesfeld, C. Melander, R. K. Suto, H. Raviol, K. Luger, and P. B. Dervan, *J. Mol. Biol.* **309,** 625 (2001).
[13] J. M. Gottesfeld and K. Luger, *Biochemistry* **40,** 10927 (2001).

*Small-Scale Reconstitution of NCP*

Small-scale reconstitution works well for amounts of NCP 25 and 500 $\mu$g. Multiple setups can be dialyzed in one vessel. The efficiency of reconstitution of octamers containing different histones (e.g., full-length histones, globular histones, and histone variants) into NCPs can vary, mainly because of inaccuracies in the concentration of histone octamer. If reconstitutions are performed with refolded H2A–H2B dimer and $(H3–H4)_2$ tetramers, titration of the three components is essential. An excess of DNA results in nonnative nucleosomal species that cannot subsequently be removed, whereas an excess of histones results in low yields. Hence, small-scale reconstitutions are recommended to establish the relative molar ratios of DNA to octamer [or of DNA, H2A–H2B dimer, and $(H3–H4)_2$ tetramer] before a large-scale experiment.

1. Titrations are performed by varying the molar ratio of DNA to histone complexes. A typical experiment using histone octamer includes three small-scale setups of 0.9:1.0, 1.0:1.0, and 1.1:1.0 DNA-to-octamer ratios in a volume of 100 $\mu$l or smaller. If H2A–H2B dimer and $(H3–H4)_2$ tetramers are used for reconstitution, the three components must be titrated accurately. Make sure to adjust to 2 *M* KCl or NaCl before adding histone octamer. The final DNA concentration is between 0.2 and 0.7 mg/ml (ideally, 0.7 mg/ml).

2. Use the reconstitution method and apparatus described below for large-scale reconstitution. The last dialysis step may be omitted if time is critical. Alternatively, stepwise dialysis against subsequent changes of more dilute buffers can be used. Dialyze at 4° (or at ambient temperatures; see above) against 300 ml each of TCS buffer containing 2, 0.85, 0.65, and 0.2 *M* KCl (at least 90 min per step).

3. Remove the contents from the dialysis buttons, incubate one-third each for 2 h at 37 and 55°, respectively (spin frequently to collect condensation), and leave one-third on ice.

4. Analyze the products by using the high-resolution gel-shift assay described earlier[1] and later in the chapter. Sample that has not been heat treated is run as a control. For subsequent large-scale reconstitutions, choose conditions that (1) contain only a small (if any) excess of free DNA and (1) are completely shifted to a single position.

*Large-Scale Reconstitution*

Large-scale reconstitution and purification of up to 4 mg of nucleosomes require about 5 days and involve the following steps.

1. Octamer and DNA are mixed at 2 *M* KCl. Make sure to adjust the salt concentration of the DNA to 2 *M,* using 4 *M* KCl. Always add histone octamer last. The mixture can incubate at 4° while the dialysis apparatus is being set up. Adjust to a final DNA concentration of 0.7 mg/ml with RB-high.

2. Set up the dialysis apparatus at 4° as described in Luger *et al.*,[1] but calibrate the peristaltic pump to a flow rate of 1.5 ml/min. This reduces the reconstitution time from 36 to 18 h. Transfer the sample to a dialysis bag and start dialysis against 400 ml of RB-high at 4° under constant stirring. Make sure the dialysis bag also spins vigorously to ensure mixing of the contents. The apparatus is set up in such a manner that the pump continuously replaces buffer from the dialysis vessel with RB-low.

3. After the gradient has finished, dialyze for at least 3 h against 400 ml of RB-low. If the samples are to be stored without any further purification, dialyze against an appropriate low-salt buffer (include DTT and 0.1 m*M* buffered cacodylic acid) and store at 4°. If samples will be further purified, dialyze against TCS buffer and store at 4° until the next step.

*High-Resolution Gel Shift and Heat Shifting of NCPs*

Reconstitution on longer DNA fragments usually results in a heterogeneous population of NCP with respect to the position of the DNA on the histone octamer. Surprisingly, this also holds true for DNA fragments with a limiting length of 146 bp, even if presumed ''strong positioning sequences'' are used. A simple heating step (37–55° for 20–180 min) results in a uniquely positioned NCP preparation for DNA 145 to 147 bp in length. Repositioning can be monitored by a high-resolution gel shift assay described below (e.g., Fig. 5). Incubation time and temperature necessary for repositioning depend on the sequence and the length of the DNA fragment, and must be checked individually for each combination of DNA fragment and histone octamer. For example, *Xenopus laevis* full-length histone octamer with the 146-bp fragment derived from the 5S RNA gene of *Lytechinus variegatus* is heated for 30 min at 37° for a complete shift, whereas other sequences might require as long as 2 h at 55°.

1. Prerun a 5% polyacrylamide gel (10 × 8 × 0.15 cm gel: 5% polyacrylamide; 59:1 acrylamide to bisacrylamide; 0.2 × TBE) for at least 1 h at 4° and 150 V.

2. Mix the buffer from the two chambers and redistribute. This significantly improves the resolution. Alternatively, use a gel apparatus in which the contents of the upper and lower chambers are recirculated continuously.

3. Shortly before loading the samples, the wells should be rinsed well with 0.2 × TBE.

4. Load 3–4 pmol of NCP [mixed in sucrose to a final concentration of 5% (v/v) sucrose for gel loading] in no more than 10 μl. Traces of bromphenol blue can be added for easier loading.

5. Run the gel at 150 V for a suitable length of time, or until bromphenol blue has reached the bottom of the gel.

6. Stain the gel first with ethidium bromide. Note that free DNA is stained significantly better by ethidium bromide than is DNA bound to the histone octamer. Subsequent staining with Coomassie Brilliant Blue sometimes gives better resolution on slightly overloaded gels because of the limited sensitivity of Coomassie Brilliant Blue compared with ethidium bromide; however, free DNA will not be evident in Coomassie Brilliant Blue staining.

*Purification of NCP by Preparative Gel Electrophoresis*

The method relies on the differential migration of free DNA, NCP, and high molecular weight aggregates on nondenaturing polyacrylamide gels. It gives rise to highly pure NCP preparations suitable for crystallization, is only marginally affected by covalent modification of the histones, and can be used for nucleosomes that are unstable at slightly elevated salt concentrations. The method works well for amounts between 200 μg and 2 mg. If larger amounts are purified, perform two runs with the same gel. For best results, the gel should be no more than 24 h old.

Given below are conditions that have been optimized for the purification of NCP containing 146 bp of DNA. Conditions for preparative gel electrophoresis should be optimized by analytical nondenaturing gel electrophoresis (see earlier), following the guidelines given in the instruction manual for the model 491 Prep Cell (Bio-Rad). In our hands, the correlation between analytical and preparative gels has been excellent, and the described protocol works well to separate nucleosomes from free DNA and aggregates. Note that the ratio between acrylamide and bisacrylamide, the length of the gel, and the elution speed can greatly alter the relative mobility and the separation of the components (Fig. 6). The choice of elution buffer and electrophoresis buffer may also influence the relative mobility of the different species. Improved resolution between different peaks is often a tradeoff with high dilution of the sample. For example, we are able to partially separate shifted from unshifted nucleosomes using longer gels (7.5 cm instead of 5 cm). Figure 6A shows fractions from preparative gel electrophoresis using a standard 5-cm gel, and Fig. 6B shows the same sample run on a 7.5-cm gel. Note the improved separation

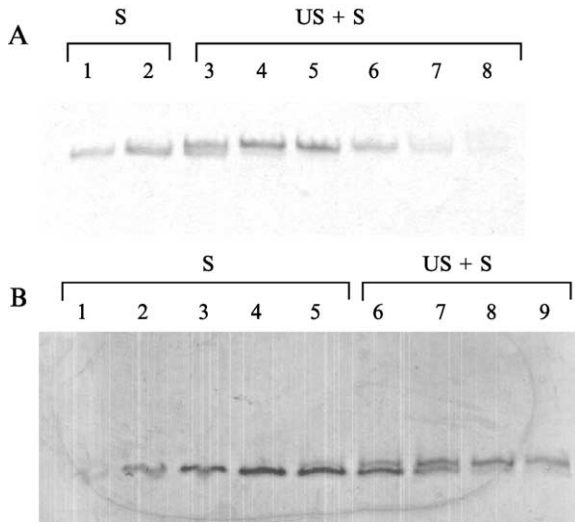


FIG. 6. Preparative gel electrophoresis is capable of separating different nucleosome species. Fractions from a 5-cm (A) or 7.5-cm (B) preparative gel are analyzed by high-resolution gel-shift assay. Note that the separation between shifted and unshifted (S and US, respectively) species is improved, although still incomplete, when a longer gel is used. This improved separation is not apparent when analyzing the $OD_{260}$ chromatogram of the eluting material (not shown). Gels were stained with Coomassie Brilliant Blue.

between unshifted and shifted nucleosome core particles, which cannot be obtained by any other method. However, high dilution of NCP during purification using longer gels might result in a partial dissociation of DNA and octamer, and thus a 5-cm gel is the correct choice for most applications.

1. Prepare 20 ml of a 5% polyacrylamide gel mixture (same conditions as that for smaller 5% gels), and pour a cylindrical gel with an outer radius of 28 mm, an inner radius of 19 mm, and a height of 50 mm. Polymerize overnight at room temperature while recirculating water through the cooling core, and assemble the apparatus at 4° according to instructions given in the manual for the model 491 Prep Cell. Connect to the power supply, UV detector, fraction collector, and peristaltic pump. Use the circular dialysis membrane (see Materials). Prerun the gel under constant recirculation of the buffer for 90 min in 0.2 × TBE (2000 ml) at 4° and at a power of 10 W. Record a baseline at 260 nm, using TCS buffer (TCS buffer used during reconstitution can be reused for this purpose) as elution buffer.

2. Concentrate NCP (in TCS buffer) to a maximum of 600 μl for a 4-mg reconstitution. Mix with sucrose to a final concentration of 5% (v/v)

and load on the preparative gel, using a syringe with an attached piece of tubing. Electrophoresis is carried out at constant power of 10 W, and the complex is eluted at a flow rate of 1.0 ml/min with TCS buffer as the elution buffer and 0.2× TBE as the electrophoresis buffer. Record the elution at an OD of 260 nm, and collect fractions of appropriate size (0.7 to 2 ml). Free DNA will appear first, followed by NCP, and finally, higher molecular weight aggregates.

3. Analyze fractions on a 5% nondenaturing gel and pool peak fractions corresponding to NCP. Concentrate NCP immediately to at least 1 mg/ml.

4. Dialyze (23-mm dialysis bags; MWCO, 6000–8000 Da) semiconcentrated NCP against CCS buffer (400 ml for 3 h at 4° each time—change the buffer three times).

5. Finally, concentrate NCP to the desired final concentration (typically, 6–10 mg/ml for crystallization purposes) and store at 4°. NCP purified by this method can be stored up to several months.

## Acknowledgment

We thank Joel Gottesfeld (Scripps Research Institute) for developing the protocol for microscale reconstitution.

# [3] Preparation and Crystallization of Nucleosome Core Particle

*By* B. Leif Hanson, Chad Alexander, Joel M. Harp, and Gerard J. Bunick

## Structural Biology and Chromatin Studies

The last half-decade has seen the development of experimentally determined atomic position models for the nucleosome core particle (NCP), based on palindromic DNA engineered from a human X chromosome alphoid satellite DNA repeat.[1] Modifications of that structure, such as NCP with variant histones and other DNA sequences, are being studied. One can anticipate that future diffraction-based studies of chromatin will range from further DNA and histone variants of the basic NCP structure to gene

[1] J. M. Harp, E. C. Uberbacher, A. Roberson, and G. J. Bunick, *Acta Crystallogr. D. Biol. Crystallogr.* **52,** 283 (1996).





0076-6879/04 $35.00