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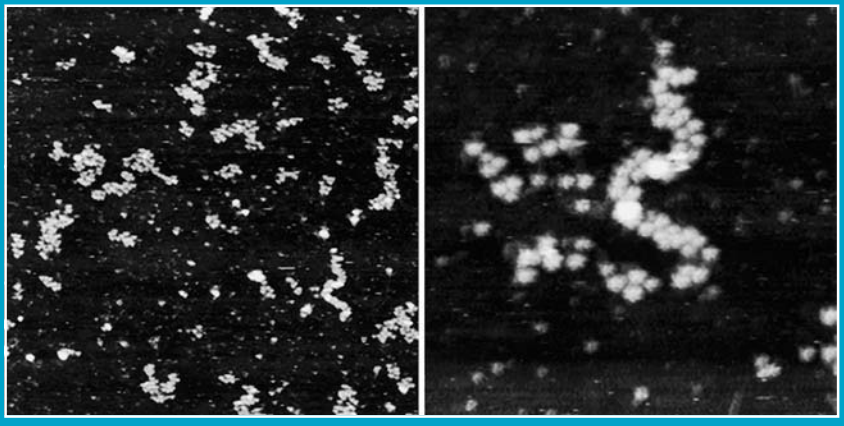

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Expression and Purification of Recombinant Histones and Nucleosome Reconstitution

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1. Introduction

In vitro studies on nucleosome core particles (NCPs) and nucleosomes have generally been limited to the use of histone proteins isolated from chromatin. Numerous reliable and well-established methods have been described of obtaining single histone proteins in significant quantity (e.g., **refs.** ***1*** and ***2***, and references therein). Briefly, the histone complexes (histone octamer, or histone tetramer and histone dimer) are isolated from "long chromatin," which is extracted from nuclei. The histone complexes can be further fractionated into individual histone proteins. This approach suffers from several disadvantages. First, the procedure is time-consuming and depends on the availability of fresh tissue or blood from the organism of choice. Second, histone proteins isolated from natural sources are often degraded by contaminating proteases ***(3)***. Third, histone isotypes and posttranslational modifications of histone proteins give rise to heterogeneity. The extent of heterogeneity and modification strongly depend on the type and developmental state of the tissue from which chromatin is isolated and can vary significantly between different batches. Fourth, and most important, only naturally occurring histone proteins can be obtained by this method.

The availability of large amounts of naturally occurring mutants, or of new site-directed mutants of the highly conserved histone proteins, will be extremely valuable in our attempts to reconcile the observed functions and biophysical properties of the NCP with the recently determined atomic structure ***(4)***. The ability to express all four histone proteins in bacteria has allowed us to develop a method for the mapping of nucleosome position to base pair

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resolution *(5)* and has been instrumental in the structure determination of the NCP at high resolution *(4)*. In comparison to yeast expression systems, yields are high, protease activity is low, and purification does not rely on the presence of histidine-tags or other fusions *(6,7)*.

This section describes the overexpression of histones H2A, H2B, H3, and H4, both as full length proteins and corresponding trypsin-resistant "globular domains" (as defined in *[8]*). A simple and efficient purification protocol yields large amounts of homogenous protein in denatured form. The methods for refolding and purification of histone octamer and for assembly and purification of nucleosome core particles using 146 bp of DNA are described, together with a protocol for a high-resolution gel shift assay to monitor the purity and homogeneity of the final core particle preparation.

2. Materials

2.1. Histone Expression

1. pET-histone expression plasmids *(2)* and transformation-competent cells of the expression strain BL21(DE3) pLysS *(9)*.
2. 2X TY-AC media: 16% (w/v) bacto-tryptone, 10% (w/v) yeast extract, and 5% (w/v) NaCl, supplemented with 100 µg/L ampicillin and 25 µg/L chloramphenicol.
3. AC agar plates: 10% (w/v) bacto-tryptone, 5% (w/v) yeast extract, 8% (w/v) NaCl, and 1.5 % (w/v) Agar, supplemented with 100 µg/L ampicillin and 25 µg/L chloramphenicol.
4. IPTG: 0.4*M* Isopropyl-ß-D-thiogalactopyranoside in water; pass through 0.2-µm sterile filter, store frozen in aliquots.
5. Wash buffer: 50 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 1 m*M* Na-EDTA, 1 m*M* benzamidine. Shortly before use, add 5 m*M* 2-mercaptoethanol.

2.2. Histone Purification

1. Wash buffer: as in **Subheading 2.1., item 5**.
2. TW buffer: wash buffer with 1% (v/v) Triton X-100.
3. Unfolding buffer: 7*M* guanidinium HCl, 20 m*M* Tris-HCl, pH 7.5, 10 m*M* DTT. Pass through 0.4-µm filters before use.
4. Amberlite MB3 or similar ion exchange resin for batch deionization of urea stock solutions.
5. SAU-1000: 7*M* urea (deionized), 20 m*M* sodium acetate, pH 5.2, 1 *M* NaCl, 5 m*M* 2-mercaptoethanol, 1 m*M* Na-EDTA. Pass through 0.4-µm filters before use.
6. SAU-200: 7*M* urea (deionized), 20 m*M* sodium acetate, pH 5.2, 0.2*M* NaCl, 5 m*M* 2-mercaptoethanol, 1 m*M* Na-EDTA. Pass through 0.4-µm filters before use.
7. SAU-600: 7*M* urea (deionized), 20 m*M* sodium acetate, pH 5.2, 0.6*M* NaCl, 5 m*M* 2-mercaptoethanol, 1 mM Na-EDTA. Pass through 0.4-µm filters before use.
8. Gel filtration column XK-50 (Pharmacia, Uppsala, Sweden), packed with Sephacryl S-200 high-resolution gel filtration resin (Pharmacia). Gel bed: 5-cm diameter, 75-cm height.

9. An HPLC system equipped with a TSK SP-5PW HPLC column, 2.15 cm × 15.0 cm (Toyo Soda Manufacturing Company, Tokyo, Japan).
10. Dialysis tubing, molecular weight cutoff 6–8 kDa, widths 5 cm and 2.5 cm. Prepare according to the supplier and rinse thoroughly with distilled water before use.
11. Standard SDS-PAGE equipment; 18% SDS gels for analysis of protein fractions ***(10)***.

2.3. Histone Octamer Reconstitution

1. Unfolding buffer: as in **Subheading 2.2.3.**
2. Refolding buffer: 2*M* NaCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* Na-EDTA, 5 m*M* 2-mercaptoethanol.
3. Gel filtration column HiLoad 16/60 Superdex 200 prep grade (Pharmacia), equipped with UV-detector and fraction collector; at 4°C (*see* **Note 7**).
4. Standard SDS-PAGE equipment (*see* **Subheading 2.2.**, **step 11**).
5. Concentration device suitable for 1–25 mL volumes (e.g., Sartorius ultrathimble, Sartorius AG, Göttingen, Germany; or Centricon 10, Amicon AG, Beverley, MA).

2.4. Nucleosome Core Particle Reconstitution

1. Purified histone octamer at a concentration of at least 0.75 mg/mL, in refolding buffer.
2. DNA of length greater than 138 bp, with a known concentration (at least 3 mg/mL).
3. A peristaltic pump with a double pump head, capable of maintaining a flow rate of approx 2–6 mL/min (e.g., Gilson Minipuls 3 peristaltic pump, equipped with tubing with 2.5 mm inner diameter; Gilson Medical Electronics SA, Villier-leBel, France); or two peristaltic pumps.
4. A reconstitution flask with connected tubing, as shown in **Fig. 1**.
5. Buffers for reconstitution:
   RB-high: 2*M* KCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* DTT
   RB-low: 0.25*M* KCl, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* DTT.

2.5. Nucleosome Core Particle Purification

2.5.1. Purification by HPLC-Ion Exchange Chromatography

1. TES-250: 0.25*M* KCl, 10 m*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA.
2. TES-600: 0.6*M* KCl, 10 m*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA.
3. A HPLC apparatus equipped with a TSK DEAE-5PW HPLC column, 2.15 × 15.0 cm, or a TSK DEAE-5PW HPLC column, 7.5 × 75 mm (Toyo Soda Manufacturing); preferrably at 4°C.

2.5.2. Purification by Preparative Gel Electrophoresis; High-Resolution Gel Shift Assay

1. Model 491 Prep Cell (Bio-Rad Laboratories, Richmond, CA) with a standard power supply, connected to a UV detector and a fraction collector, and equipped with a peristaltic pump.
2. Gel running buffer: 0.20X TBE (1X TBE: 89 m*M* Tris-HCl, 89 m*M* boric acid, and 2.5 m*M* EDTA).

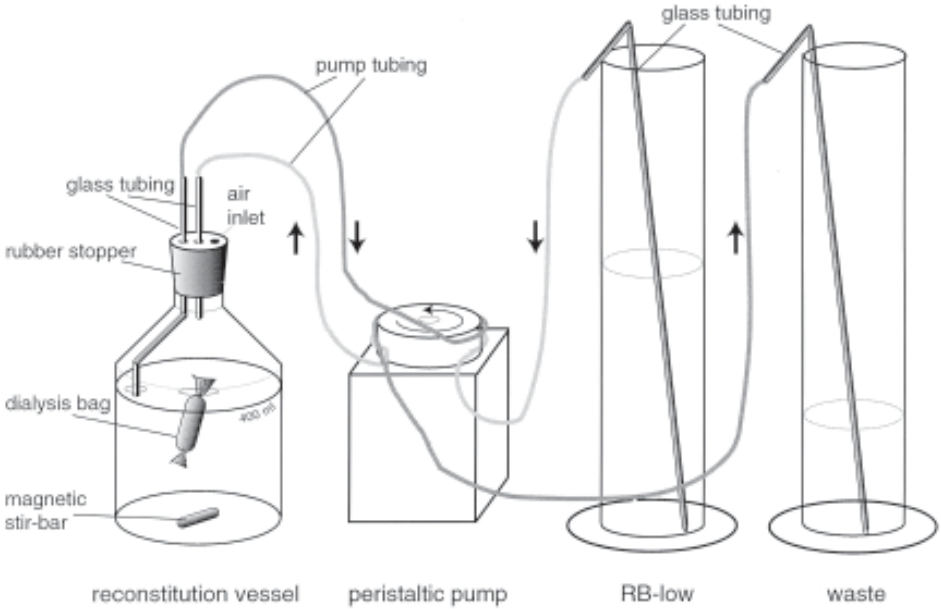

Fig. 1. Schematic drawing of the experimental apparatus for reconstitution. We use a 500-mL glass flask as a reconstitution vessel, and a peristaltic pump with a four-channel head. Standard glass tubes are bent in the appropriate manner and are connected by silicone tubing. The reconstitution vessel contains RB-high to start.

3. Acrylamide stock solution: 29.5% acrylamide, 0.5% *bis*-acrylamide in water. Deionized by stirring with Amberlite MB3, and stored at 4°C.
4. Dialysis membrane (molecular weight cutoff: 6–8 kDa), cut to a circle with a radius of 3 cm. Prepare according to the supplier and rinse thoroughly with distilled water before use.
5. Concentration device as specified in **Subheading 2.3.5.**
6. Storage buffers: TCS buffer:
   20 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 1 m*M* DTT
   CCS buffer: 20 m*M* K-Cacodylate, pH 6.0, 1 m*M* EDTA.

3. Methods

3.1. Histone Expression

Expression plasmids for the individual histone proteins and their N-terminally truncated versions, based on the T7-expression system ***(9)***, have been described previously ***(2)***. High level expression of the *Xenopus laevis* histone genes for H2A, H2B, and H3 does not necessitate adaptation of the codon usage, despite the presence of several codons with low usage in *Escherichia coli*. These proteins can be expressed with similar efficiencies as N-terminal

fusion proteins or with the coding region fused directly to the promoter. In contrast, the *Xenopus laevis* gene for histone H4 could only be expressed after redesigning the coding region of H4 to optimize for codon usage in *E. coli* ***(2)***.

Expression levels of histones H2A and H2B appear to be insensitive to the sequence variant that we have expressed, but histone H3 expression levels do vary between different sequence variants or mutated genes. H4 expression is sensitive to amino acid substitutions and can drop to an undetectable level for certain point mutations. Typical yields for H2A, H2B, and H3 are 50–80 mg of pure protein per liter of cell culture, while yields for H4 are 4–5 times lower (*see* **Note 1**).

1. Transform BL21 (DE3) pLysS cells with 0.1–1 µg of the pET-histone expression plasmid and plate on AC agar plates. Incubate the plates at 37°C overnight.
2. First, perform a test expression by incubating five 5-mL aliquots of 2X TY-AC, each inoculated with one single colony from the agar plate. Shake at 37°C for approx 4 h, or until the OD_{600} is between 0.3 and 0.6. Transfer 0.5 mL of the culture into a sterile Eppendorf tube, add 0.2 mL of sterile glycerol, mix well and store at –80°C (this will serve as the glycerol stock for large-scale expressions). Induce all but one culture by addition of IPTG to a final concentration of 0.2 m*M*. Leave one sample uninduced as a negative control. Incubate for another 2–3 h at 37°C, harvest by centrifugation, and boil the cell pellets in 100 µL of protein gel loading buffer. Load 20 µL per sample on an 18% SDS-polyacrylamide gel (SDS-PAGE), and determine the culture with the maximum expression.
3. The evening before performing the large scale expression, restreak the glycerol stock for this culture on an AC agar plate, and incubate at 37°C over night (*see* **Note 1**).
4. The next morning, inoculate five aliquots of 4 mL 2X TY-AC media with one colony from this plate and shake at 37°C for approx 4 h (OD_{600} ~ 0.3). Use the combined precultures to inoculate 100 mL of 2X TY-AC media. Shake at 37°C for about 2 h, or until the culture is slightly turbid (OD_{600} ~ 0.4) (*see* **Note 2**).
5. Inoculate 12 2-L Erlenmeyer flasks containing 500 mL 2X TY-AC media with 8 mL of the 100-mL starter culture. Shake at 200 rpm and 37°C until the OD_{600} has reached 0.6 (this takes about 3 h, *see* **Note 2**). Induce by addition of 0.2 m*M* IPTG (final concentration) and shake for another 2 h (H3 and H4) or 3 h (H2A and H2B).
6. Harvest the cells by centrifugation at room temperature. Resuspend homogeneously in 100 mL wash buffer and flash-freeze in liquid nitrogen (*see* **Note 3**).

3.2. Histone Purification

The purification protocol involves three steps: preparation of inclusion bodies, gel filtration under denaturing conditions, and HPLC-ion exchange chromatography under denaturing conditions.

Analysis of the pure proteins by gel electrophoresis (SDS- and triton-urea-acid PAGE), mass spectroscopy, amino acid analysis, and by sequencing the

N-terminus using Edman degradation shows that all preparations were highly homogenous and free of modifications. Whereas the terminally truncated histone proteins retain the N-terminal methionine residue, the full-length histones are completely free of this methionine residue and begin with the native sequence ***(2)***.

3.2.1. Inclusion Body Preparation

1. Start the equilibration of the Sephacryl S-200 gel filtration column in the morning: prepare 2 L of 9*M* urea in water. Heat to dissolve and deionize with Amberlite MB3 (*see* **Note 4**). Prepare 2 L of SAU-1000 buffer. Equilibrate the column with 2 L of filtered and degassed SAU-1000 buffer at a flow rate of 3 mL/min.
2. To avoid overloading of the gel filtration column, no more than the equivalent of 6 L of cell culture should be processed at one time. For histones with moderate expression (e.g., all H4-variants), up to 12 L of culture can be used. Thaw the cell suspension in a warm water bath. The cell suspension will become extremely viscous as lysis occurs. Stir occasionally until completely thawed (20–30 min). Transfer into a wide, short measuring cylinder and adjust the volume with wash buffer to 150 mL. Reduce viscosity by shearing with a Turrax stirrer. Check whether the mixture is still viscous by using a pasteur pipet, and if necessary, repeat shearing step. Centrifuge immediately for 20 min at 4°C and 23,000*g*. The pellet contains inclusion bodies of the corresponding histone protein.
3. Resuspend the pellet completely in 150 mL TW buffer using a 10-mL plastic pipet. Spin for 15 min at 4°C at 12,000 rpm. Repeat this step twice with TW buffer and twice with wash buffer (*see* **Note 5**). After the last wash, the drained pellet can be stored at –20°C until further processing.
4. With a spatula, transfer the pellet to a 50-mL centrifuge tube. Add 1 mL of DMSO and soak the pellet for 30 min at room temperature. Mince the pellet with a spatula. Slowly add 40 mL of unfolding buffer and stir gently for 1 h at room temperature. The pellet should eventually almost completely dissolve (*see* **Note 4**).
5. Remove cell debris by centrifugation at 20°C and 23,000*g*. The supernatant contains the unfolded proteins. Reextract the pellet with 10 mL of unfolding buffer, and combine the supernatants.

3.2.2. Gel Filtration

1. Load a maximum of 60 mL of the sample on the equilibrated S-200 column, at a flow rate of 3 mL/min. Record the elution profile at a wavelength of 280 nm, and collect fractions of an appropriate size.
2. Analyze peak fractions by 18% SDS-PAGE. The first peak will contain DNA and larger proteins but can be merged with the histone peak. Because of the high dilution factor and the small molar absorption of histones, the histone peak is often small and unobtrusive. Pool fractions containing histone proteins (*see* **Note 6**).
3. The protein is dialyzed thoroughly against at least three changes of distilled water containing 2 m*M* 2-mercaptoethanol, at 4°C. Dialysis bags with a cutoff of 6–8 kDa are sufficient even for the globular domain of H4, but leave enough room for

Table 1
Molecular Weights and Molar Extinction Coefficients (ε) for Full-Length and Trypsin Resistant Globular Domains of Histone Proteins[a]

Full-length protein			Globular domains		
Histone	Mol. wt	ε (cm/*M*), 276 nm	Amino acid	Mol. wt	ε (cm/*M*), 276 nm
H2A	13,960	4050	19–118	11,862	4050
H2B	13,774	6070	27–122	11,288	6070
H3	15,273	4040	27–135	12,653	4040
H4	11,236	5400	20–102	9,521	5040

[a]ε was calculated according to Gill and von Hippel ***(14)***. The molecular weights were determined by a summation of amino acids and were confirmed by mass spectrometry ***(2)***.

the volume increase. Determine the concentration of the dialyzed sample, using the molecular extinction coefficients listed in **Table 1**. Lyophilize, and store at –20°C.

3.2.3. Purification by Ion Exchange Chromatography (SP-5PW)

1. Dissolve lyophilized histone protein in SAU-200 and remove insoluble matter by centrifugation. Equilibrate the preparative SP-5PW HPLC column with SAU-200 buffer. Inject a maximum of 15 mg of protein for each run. Using a flow rate of 4 mL/min, elute proteins with the gradients given in **Table 2** (buffer A = SAU–200, buffer B = SAU–600).
2. Analyze the peak-fractions by SDS-PAGE. Pool the fractions containing pure histone protein, dialyze against water as described under **Subheading 3.2.2.3.**, and lyophilize.
3. Dissolve in a small volume of water (determine the concentration using the values given in **Table 1**) and lyophilize in Nunc vials in aliquots suitable for subsequent octamer refolding reactions (*see* **Subheading 3.3.1.**). For example: 4.5 mg H3, 3.5 mg H4, 4.0 mg H2A, 4.0 mg H2B). The purified histones can be stored at –20°C for an unlimited length of time.

3.3. Refolding of the Histone Octamer

The protocol below is valid for the refolding of histone octamers from lyophilized recombinant histone proteins. (All combinations of recombinant *Xenopus laevis* full-length and globular domain histone proteins have been refolded to functional histone octamers.) The method works best for 6–15 mg of total protein. For smaller amounts of protein, scale down the gel filtration column (*see* **Note 7**).

1. Dissolve each histone aliquot to a concentration of approximately 2 mg/mL in unfolding buffer. Use a Pasteur pipet to ensure protein sticking to the sides of the tube is dissolved. Do not vortex. Unfolding should be allowed to proceed for at least 30 min and for no more than 3 h (*see* **Note 4**).

Table 2
Salt Gradients for Elution of Histone Proteins from an SP-Column[a]

H2A, H2B		H3		H4	
t (min)	B (%)	t (min)	B (%)	t (min)	B (%)
0	0	0	0	0	0
10	0	3	30	5	50
11	40	8	30	10	59
46	100	40	100	40	100
60	100	50	100	50	100
61	0	51	0	51	0

[a]Salt gradients for elution of histone proteins from an SP-5PW HPLC column (2.15 × 15.0 cm): the flow rate is 4 mL/min, buffer A is SAUDE 200, buffer B is SAUDE 600.

2. Determine the concentration of the unfolded histone proteins by measuring OD_{276} (**Table 1**) of the undiluted solution against unfolding buffer (remove solid matter by centrifugation, if necessary).
3. Mix the four histone proteins to equimolar ratios and adjust to a total final protein concentration of 1 mg/mL using unfolding buffer.
4. Dialyze at 4°C against at least three changes of 2 L of refolding buffer (for 15 mg setup, use dialysis bags with a flat width of 2.5 cm). The second or third dialysis step should be performed overnight. Octamer should always be kept at 0–4°C.
5. Remove precipitated protein by centrifugation. There should be almost no precipitate in the "ideal" refolding reaction. Concentrate to a final volume of 1 mL.
6. Gel filtration is performed at 4°C at a flow rate of 1 mL/min. Load a maximum of 1.5 mL or 15 mg of the concentrated histone octamer on the Superdex-200 gel filtration column previously equilibrated with refolding buffer. High-molecular-weight aggregates will elute after about 45 mL, histone octamer at 65–68 mL, and histone (H2A-H2B) dimer at 84 mL (*see* **Note 7**).
7. Check the purity and stoichiometry of the fractions on an 18% SDS-PAGE. Dilute 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. Pool fractions that contain equimolar amounts of the histone proteins.
8. Determine the concentration (A_{276} = 0.45 for a solution of 1 mg/mL). Use for nucleosome core particle reconstitution with DNA immediately or concentrate to 3–15 mg/mL, adjust to 50% (v/v) glycerol, and store at –20°C.

3.4. Reconstitution of Nucleosome Core Particles

Specific DNA fragments of the desired length or sequence can be obtained by a number of methods (e.g., ***11,12***). Reconstitution of histone octamer with DNA is accomplished using a modification of the salt gradient method

described by Thomas and Butler ***(13)***. Briefly, octamer and DNA is mixed at 2 *M* KCl, and the salt concentration is reduced by dialysis to 0.25 *M* KCl over a period of 36 h ***(12)***. The procedure works equally well for large (up to 10 mg) and small (0.1 mg) amounts of nucleosome core particles. Multiple setups can be dialyzed in one vessel. If smaller amounts need to be reconstituted, use dialysis buttons (e.g., Hampton Research, Laguna Hills, CA), or the apparatus described in Chapter 4.

1. Histone octamer is added to the DNA to a 0.9 molar ratio of octamer to DNA, with a final DNA concentration of 6 µ*M*. Before adding the histone octamer (*see* **Note 8**), adjust the salt concentration of the DNA solution to 2 *M* using 4 *M* (or solid) KCl, and add DTT to a final concentration of 10 m*M*. Incubate at 4°C for 30 min.
2. Prepare 400 mL RB-high and 1600 mL RB-low buffer and chill these to 4°C. Set up the dialysis apparatus as shown in **Fig. 1**. Calibrate the pump to a flow rate of 0.7–0.8 mL/min.
3. Transfer the sample to a dialysis bag and start dialysis against 400 mL RB-high at 4°C under constant stirring. Using the peristaltic pump, continually remove buffer from the dialysis vessel and replace with RB-low. Over a period of 36 h, an exponential gradient is generated (*see* **Note 9**). After the gradient has finished, dialyze for at least 3 h against RB-low. If the samples are not further processed within the next 24 h, dialyze against CCS buffer. If the core particle will be purified by preparative gel electrophoresis (*see* **Subheading 3.5.2.**), dialyze against TCS buffer.

3.5. Purification of the Nucleosome Core Particle

Two methods are described for the purification of nucleosome core particles from free octamer and/or free DNA. Both methods have been optimized for NCP with 146 bp DNA, but can easily be adjusted for nucleosomes with different length DNA. The first method uses HPLC DEAE-ion exchange chromatography ***(12)***. Free DNA elutes from the column at a higher salt concentration than NCP and thus can be easily separated from the complex. The second method uses gel electrophoresis under nondenaturing conditions as a purification principle ***(2)***. **Table 3** compares the advantages and disadvantages of the two methods. Both methods alone give rise to highly pure NCP preparations; the choice depends on available equipment and on the problem at hand. Ion exchange chromatography is suitable for large-scale preparations on a routine basis. However, certain modification of histone proteins (such as covalently bound heavy atoms) might completely alter the binding and elution properties of the NCP. If a significant amount of material appears as a high-molecular-weight band in a gel shift assay, or if the particle is prone to salt-dependent dissociation, the second method might be more suitable. The two methods can also be used in combination.

Table 3
Purification of Nucleosome Core Particles: Comparison of Two Methods

	DEAE-ion exchange	Preparative gel electrophoresis
Capacity	Up tp 10 mg NCP per run for a preparative column (2.15 × 15.0 cm)	Maximum of 3 mg NCP per run
Time	Fast, reliable	Slow, labor-intensive
Purification	No purification from higher order aggregates	Purification from both DNA and higher order complexes
Elution	Salt elution might cause dissociation	Free choice of elution buffer

Reconstitution usually leads to a heterogeneous population of NCP with respect to the position of the DNA on the histone octamer. A simple heating step (37–55°C for 20–180 min.) usually results in a uniquely positioned NCP preparation for DNA 145 to 147 bp in length which is suitable for biochemical studies and crystallization (*see* **Fig. 3**, and **Note 10**).

3.5.1. Purification by Ion Exchange Chromatography

1. Equilibrate the DEAE-5PW column with TES-250 at 4°C. Centrifuge the reconstitution mixture in Eppendorf tubes at 4°C and inject the supernatant on the column (a maximum of 10 mg core particle). Samples can either be in RB-low, TCS buffer, or CCS buffer. Using a flow rate of 4 mL/min, develop the column with the gradient given in **Table 4** (buffer A = TES-250, buffer B = TES-600) while monitoring the eluent at 260 nm. Adjust gradient for core particles containing longer DNA fragments or different temperatures and salts (NaCl instead of KCl). A typical chromatogram is shown in **Fig. 2A** (*see* **Note 10**).
2. Analyze the peak-fractions by non-denaturing gel electrophoresis, if necessary (*see* **Subheading 3.6.**, and **Note 10**).
3. Pool the peak fractions and immediately dialyze against three changes of TCS buffer at 4°C. Concentrate and store on ice until use. For prolonged storage, dialyze against CCS buffer, or add 5 m*M* potassium cacodylate at an appropriate pH to prevent microbial growth. Determine the concentration of NCP preparations by measuring the absorbency at 260 nm of a 200- to 500-fold dilution. The yield of the above reconstitution and purification method may vary between 20 and 80% of the DNA added to the reconstitution mixture.

*3.5.2. Purification by Preparative Gel Electrophoresis (*see *also* ***Subheading 3.6.****)*

1. Prepare 20 mL of a 5% polyacrylamide gel (ratio of acrylamide to *bis*-acrylamide 60:1), containing 0.2X TBE, and pour a cylindrical gel with an outer radius of 28 mm,

Table 4
Salt Gradient for Elution of NCP from a DEAE -5PW HPLC Column[a]

NCP146	
t (min)	B (%)
0	0
10	0
11	40
46	100
60	100
61	0

[a]The flow rate is 4 mL/min, buffer A is TES-250, buffer B is TES-600.

an inner radius of 19 mm and a height of 50 mm. Polymerize and assemble according to instructions given in the manual for the Model 491 Prep Cell. Prerun under constant recirculariza tion of the buffer for 90 min in 0.25X TBE at 4°C and 10 W. Record a base line at 260 nm using TCS as elution buffer (*see* **Notes 11** and **12**).

2. After reconstitution, dialyze NCP against TCS buffer and concentrate. A maximum of 600 µL or 3 mg is mixed with sucrose to a final concentration of 5% (v/v) and loaded on the preparative gel. Electrophorese at 10 W, and elute the complex at a flow rate of 0.7 mL/min with TCS buffer. Recirculate buffer. Record elution at an OD of 260 nm, and collect fractions of appropriate size (usually 0.7–1.0 mL). Free DNA will appear first, followed by pure NCP, and finally, by higher molecular weight aggregates (**Fig. 2B**, *see* **Note 11**).

3.6. High-Resolution Gel Shift Assay for Nucleosome Core Particles

The protocol given next routinely allows for the separation of NCP with different translational setting of the DNA by only 10 bp (**Fig. 3**; *see* **refs.** ***[2,5]***). We have observed that the ratio between acrylamide and bis-acrylamide can completely alter the relative mobilities of different NCP species with respect to the DNA size marker ***(2)***. The choice of gel buffer also has minor effects on the resolution of the different NCP species.

1. Prepare a native gel with the dimensions $20 \times 20 \times 0.1$ cm, using a 10- to 16-well comb. The gel material is 5% acrylamide with 60:1 acrylamide to *bis*-acrylamide, with 0.2X TBE.
2. Prerun gels for at least 3 h at 4°C and 200 V, while constantly recycling the running buffer (*see* **Note 12**).

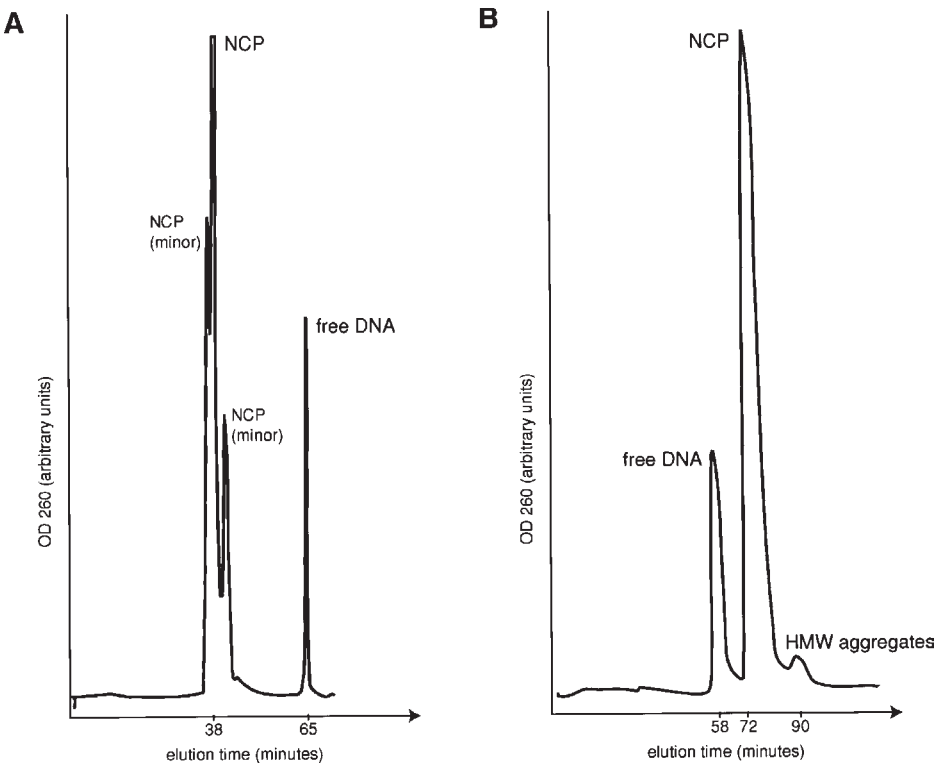

Fig. 2. Purification of reconstituted nucleosome core particle by **(A)** ion exchange chromatography or **(B)** preparative gel electrophoresis. Elution is monitored by the absorbance at 260 nm. Separation conditions are as given in the text. The approximate elution times are shown for each peak, but they are dependent on the flow rate and the geometry of the set-up. **(A)** Purification by HPLC ion exchange chromatography. NCPs with different rotational positions elute at different times from the column. The relative ratio between the main and minor peaks can vary. Usually, major and minor peaks are combined; additional peaks that usually exhibit baseline separation (not shown) are not included. In some cases, free octamer can be observed to elute in the beginning of the gradient (not shown). **(B)** Purification by preparative nondenaturing gel electrophoresis. Note that the three bands observed in **Fig. 3** cannot be discerned by this method. Faster elution will improve the separation of the peaks but will also yield a more dilute sample.

3. Rinse slots well with 0.20X TBE shortly before loading samples. Load 1–2 pmol of core particle solution, containing 5% (v/v) sucrose, in no more than 10 µL. Samples can be supplemented with bromophenol blue for easier handling.
4. Run the gel for a suitable length of time or until bromophenol blue has reached the bottom of the gel. Recycle the running buffer at all times (*see* **Note 12**).

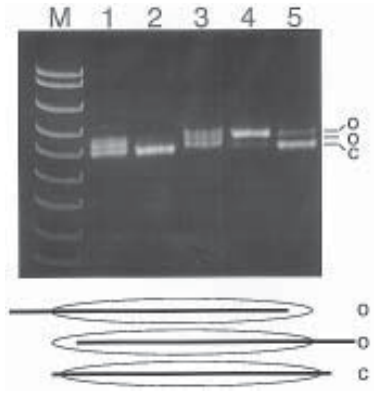

Fig. 3. Nondenaturing gel electrophoresis of NCP reveals multiple positions of the histone octamer on the DNA. Lanes 1–3: purified NCP reconstituted from recombinant full-length histone proteins and a 146-bp fragment derived from the 5S RNA gene of *Lytechinus variegatus*. Before (lanes 1 and 3) and after (lane 2) heating for 1 h at 37°C. Lanes 4 and 5: purified NCP prepared from recombinant full-length histone proteins and a 146-bp palindromic DNA fragment derived from human alpha satellite DNA; before (lane 4) and after (lane 5) heating for 2 h at 37°C. Note that NCP containing the asymmetric 5S RNA DNA fragment reconstitutes in two off-centered and one centered rotational position (marked with o and c, respectively), ***(5)***, whereas NCP containing a palindromic sequence reconstitutes mainly to the off-centered positions. Nucleosomes in the two off-centered positions cannot be distinguished on the gel, because the sequence symmetry results in an identical exit angle of the ends of the DNA from the histone octamer. The relative positions of the DNA on the histone octamer in the three bands is shown schematically (*ovals*: histone octamer, *bold line*: DNA).

5. Stain with ethidium bromide. Note that free DNA is stained significantly better by ethidium bromide than DNA bound to the histone octamer. Subsequent staining with Coomasssie brilliant blue is also possible.

4. Notes

1. Glycerol stocks of transformed BL21(DE3)pLys-S can be kept at –80°C for at least 2 mo. If the glycerol stock has not been used for some time, restreak and perform the small-scale expression test again. For some difficult cases (i.e., histone variants), expression of histone proteins in cells lacking the pLysS plasmid proved to be more successful (T. J. Rechsteiner, unpublished results).
2. Values obtained by measurements of the optical density at 600 nm depend on the geometry of the spectrophotometer. We use a Pharmacia Novaspec™ spectrophotometer (Pharmacia). Trial experiments should be performed for each individual histone variant to determine the optimal optical density for induction. We have observed that some point mutants exhibit quite different optima of cell density for induction. The starter culture should never be grown to densities

higher than OD_{600} ~ 0.6, since the cells loose the ability to be induced even after dilution.

3. Cells expressing histone proteins (especially H4) are prone to lysis and should be centrifuged at room temperature, and for the same reason, it is not recommended to wash the cell pellet. Resuspend cells well before freezing, as this will improve lysis upon thawing. The cells can be stored at –20°C.
4. Urea in solution is in a slow equilibrium with isocyanate which can irreversibly modify proteins. Do not use urea-containing solutions older than 24 h, and always deionize urea stock solutions before use. Storage of protein in buffers containing guanidinium HCl or urea for more than 24 h is not recommended.
5. If lysis by freezing and thawing is not complete, additional lysis will occur upon the addition of TW buffer and the cell suspension will become viscous again. In that case, repeat shearing. Two consecutive cycles of freezing and thawing of the cell suspension can improve lysis significantly.
6. Unusually early elution of histone proteins from the Sephacryl S-200 gel filtration column (within the large DNA peak), might result from the formation of unspecific complexes between cellular DNA and histones. The presence of 1 *M* NaCl usually inhibits complex formation between cellular DNA and denatured histones, but this can occasionally remain a problem depending on the shearing of the DNA. For this reason, or if the resolution of the gel filtration column is insufficient, the first fractions of the histone peak might be contaminated with DNA. Before pooling, check the first few histone-containing fractions by UV-spectroscopy for DNA contamination. Discard if the spectrum exhibits a ratio of $OD_{260} : OD_{280} > 1.0$, since DNA and histone will form a precipitate after removal of the salt. Minor precipitate can be removed by centrifugation.
7. Other gel filtration resins of a similar separation range, such as Superose 12 or Sephacryl S-300 (both from Pharmacia) can also be used, but give a lower resolution. Sephadex G-100 does not separate histone octamer from high-molecular-weight aggregates and is therefore not recommended. Separation between histone octamer and excess H2A-H2B dimer is better than from excess $(H3\text{-}H4)_2$ tetramer. Yields of pure histone octamer are usually between 50 and 75%. Significant amounts of octamer (or high-molecular-weight aggregates) can remain attached to the column. Clean the column with NaOH as recommended by the supplier.
8. If histone octamer from a glycerol stock is used, dialyze over night against refolding buffer and determine the concentration. The required accuracy in the ratio between histone and DNA ratio cannot be maintained if pipeted directly from the glycerol stock. Histone octamer should always be added last to the reconstitution mixture to avoid premature mixing octamer and DNA at <2*M* salt concentrations.
9. Ensure that the dialysis bag can circle freely and rapidly to allow constant mixing its contents. Take care to adjust the position of the inlet and outlet tubing as shown in **Fig. 1**. This is important for two reasons:
   a. Rotation of the dialysis bag is inhibited if it gets caught in the tubing.
   b. Uneven pump speed can result in either overflowing or running dry of the dialysis vessel.

Formation of large amounts of precipitate during dialysis, or unexpectedly low yields, may result for two reasons:

a. An excess of histone octamer (or histone protein) has been added. Any change in the given molar ratio between octamer and DNA will reduce yields significantly.
b. Stalled motion of the dialysis bag creates an uneven salt gradient within the bag itself. Sometimes, precipitate is formed at salt concentrations of about 400 m*M* but will dissolve again later.

10. NCPs reconstituted on small DNA fragments are usually heterogeneous with respect to the relative position of the DNA on the histone octamer. This is seen by the appearance of several bands on high-resolution gel shift assays (**Fig. 3**). As a consequence, NCP elutes from the DEAE-column as a major peak with several shoulders (**Fig. 2A**). As a rule, the major peak fractions, including the shoulders, are pooled without analysis by nondenaturing PAGE. The peaks are not distinguished by preparative electrophoresis (**Fig. 2B**). Depending on the sequence and the length of the DNA fragment, the octamer can be moved to one single position by incubation at elevated temperature after reconstitution and purification of NCP (**Fig. 3**). The temperatures and incubation times necessary for this transition have to be checked individually for each sequence 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°C for a complete shift, whereas other sequences might require as long as 2 h at 55°C. Shifting to a unique position occurs completely without dissociation of the DNA; an excess of competitor DNA does not exchange onto the histone octamer during the process.
11. Different conditions for preparative gel electrophoresis can be tested by small-scale native gel electrophoresis experiments, following the guidelines given in the instruction manual for the Model 491 Prep Cell. The ratio between acrylamide and bis-acrylamide, the length of the gel, and the elution speed can greatly alter the relative mobility and the separation of the components. We have also noticed that the choice of elution buffer and electrophoresis buffer greatly influence the relative mobility of the different species. Improved resolution between the different peaks is often a tradeoff with high dilution of the sample. Note that high dilution of NCP during purification might result in a partial dissociation of DNA and octamer.
12. Recycling of the buffer and temperature equilibration are essential for good resolution.

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