Isolation of Histones and Nucleosome Cores from Mammalian Cells

In vitro analysis of DNA in chromatin is often important for understanding mechanisms of regulation of transcription and other processes that occur on DNA. The basic unit of chromatin is the nucleosome core, containing two copies each of the core histones H2A, H2B, H3, and H4 to form a histone octamer that wraps ~145 base pairs of DNA in a left-handed superhelix. In vivo, chromatin is associated with linker histones (such as H1), which facilitate the ordered packing of nucleosomes. This linker histone–containing particle is properly termed the nucleosome (or chromatosome), while the linker histone–free particle is the nucleosome core.

Long arrays of nucleosome cores (poly- or oligonucleosome cores) as well as pure populations of mono- and dinucleosome cores can be purified. These purified nucleosomes are useful for a variety of applications; for example, they can act as histone donors for the assembly of nucleosomes on defined-sequence DNA. The core histones can be purified away from chromosomal DNA and from linker histones, as well. These are useful in a variety of procedures for in vitro chromatin assembly. The method here does not denature the histones (as does the acid extraction protocol in *UNIT 21.2*), and may yield histones that are more active for in vitro assemblies.

Pure polynucleosome cores and histones are useful in the in vitro assembly of chromatin on defined-sequence DNA. These can be readily isolated from mammalian tissue culture cells. In Basic Protocol 1, nuclei are isolated and purified. In Basic Protocol 2, polynucleosomes lacking linker histones are isolated from these nuclei. In Basic Protocol 3, pure populations of mono- and dinucleosome cores are isolated from oligonucleosome fractions from Basic Protocol 2. In Basic Protocol 4, core histones are isolated from purified nuclei.

The large genome size of mammals makes mammalian tissue culture cells abundant sources for chromatin and histones. The core histones are highly evolutionarily conserved, and human histones are frequently used in assembly systems derived from *Drosophila* and for in vitro transcription systems from eukaryotes as simple as yeast. The assumption is that, at the level of simple hypo-acetylated nucleosome cores, histone source matters little. Thus far, this assumption appears to be warranted. However, when histone modifications, linker histones, and other nonhistone chromosomal proteins begin to be considered, the organismal, tissue, and cell-type source become increasingly important.

NOTE: Unless otherwise indicated, keep all solutions and materials on ice or at 4°C.

PREPARATION OF A WASHED NUCLEAR PELLET

BASIC PROTOCOL 1

The isolation of nucleosomes and free histones requires clean nuclei as starting material. In this protocol, nuclei are released from cultured cells by physical shear forces (homogenization) and nonionic detergent (NP-40; Côté et al., 1995). Several washes with a buffer containing detergent are then used to remove membranes and yield a pellet containing relatively clean nuclei. Subsequent extraction with 0.3 M KCl removes most loosely bound proteins from chromatin. This yields a nuclear pellet and what is, effectively, a nuclear extract (from which several transcription factors can be isolated). However, the NP-40 washes may make the extract less active than nuclear extracts prepared by other means. If both nuclear extract and chromatin are desired, the nuclear extract should be

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prepared as in *UNIT 12.1*, and the "spent" nuclear pellet from this procedure can be used to prepare chromatin (Schnitzler et al., 1998).

Materials

Mammalian tissue cell culture (e.g., HeLa cells)
PBS (*APPENDIX 2*)
Lysis buffer (see recipe)
Buffer B (see recipe)
2 M NaCl
Buffer B/0.6 M KCl/10% (v/v) glycerol

Dounce homogenizer with type B pestle
Light microscope

Additional reagents and equipment for mammalian cell culture (*APPENDIX 3F*) and quantitation of DNA (*APPENDIX 3D*)

1. Grow and harvest 3 liters of mammalian tissue culture cells (e.g., HeLa) at 1×10^6 cells/ml and wash twice in 1 liter PBS.

   See APPENDIX 3F for counting cells.

2. Resuspend pellet in 20 pellet vol (~40 ml) lysis buffer, transfer to a Dounce homogenizer, and lyse cells with ~15 strokes of a type B pestle. Monitor lysis by light microscopy.

   The cells should be clearly lysed when viewed by light microscopy. The NP-40 in the buffer solubilizes membranes, resulting in a clean (white or light tan) pellet.

   Alternatively, the nuclear pellets remaining after nuclear extract preparation (UNIT 12.1) can be resuspended by homogenization in the same buffer. If this is done, wash as described in steps 2 to 4, and then proceed to purification of H1-depleted oligonucleosomes (see Basic Protocol 2).

3. Centrifuge 15 min at $3000 \times g$, 4°C.

4. Repeat resuspension and pelleting twice with lysis buffer and once with buffer B. Just before the final pelleting, estimate the yield of DNA in the nuclei by diluting 10- to 30-fold into 2 M NaCl and measuring A_{260} (*APPENDIX 3D*).

   A few strokes with the type B pestle can be used to help resuspend the pellet.

5. Resuspend nuclei in 2 pellet vol buffer B and measure the total volume of the suspension.

6. While gently stirring, add 1 total vol of buffer B/0.6 M KCl/10% glycerol in a dropwise fashion. Continue gentle stirring for 10 min at 4°C. Homogenize gently, if necessary.

7. Pellet nuclei 30 min at $17{,}500 \times g$, 4°C.

   Nuclear pellets can be frozen in liquid nitrogen or dry ice and kept for more than a year at –80°C.

SOLUBILIZATION AND PURIFICATION OF HISTONE H1–DEPLETED OLIGONUCLEOSOMES

BASIC PROTOCOL 2

Oligonucleosome cores lacking histone H1 are commonly used to assemble labeled, defined-sequence mononucleosomes by the method of octamer transfer at high salt, amongst other applications. The NP-40-washed nuclear pellet is washed at 0.4 M NaCl to remove nonhistone proteins. The pellet is then solubilized in 0.65 M NaCl. At this salt concentration, the linker histones are released from the nuclei, resulting in chromatin decompaction. The resultant nuclei are much less resistant to shear forces, and vigorous homogenization fragments the chromatin to a size that allows their separation from other nuclear fragments. After dialysis to reduce the salt concentration, digestion with the endonuclease micrococcal nuclease releases even smaller fragments. These fragments are size-separated by either gradient centrifugation or gel filtration, and different size distributions are pooled and stored frozen. Size separation is done at salt concentrations (0.6 M NaCl) at which linker histones do not bind, allowing their efficient separation from oligonucleosome cores (Côté et al., 1995; Schnitzler et al., 1998).

Materials

Washed nuclear pellet (see Basic Protocol 1)
MSB (see recipe)
HSB (see recipe)
2 M NaCl
LSB (see recipe)
0.1 M $CaCl_2$
50 U/µl micrococcal nuclease (see recipe)
0.5 M EGTA, pH 8.0
HSB without sucrose
HSB/glycerol: HSB containing 10% and 40% (v/v) glycerol instead of sucrose
0.5% (w/v) SDS
0.5 mg/ml proteinase K
Dialysis buffer (see recipe)

Dounce homogenizer with type B pestle
6 to 8 kDa MWCO dialysis membrane
~1.6 × 58–cm Sepharose CL-6B column (Amersham Pharmacia Biotech; for gel filtration) and accessories
Refrigerated ultracentrafuge with appropriate rotor and tubes, e.g.,
  Beckman SW55 rotor with polyallomer or ultraclear tubes (for gel filtration)
  Beckman SW28 rotor with 1 × 3.5–in. (2.5 × 8.9–cm) polyallomer tubes (for centrifugation)
Gradient maker (for centrifugation)
21-G needle (for centrifugation)
Tubing (for centrifugation): e.g., 21-G needle infusion set with 12-in. (30.5-cm) tubing (Abbot Laboratories)
50°C water bath
6 to 8 kDa MWCO dialysis bag

Additional reagents and equipment for quantitation of DNA (*APPENDIX 3D*), pouring gradients (*UNIT 10.10*), agarose gel electrophoresis (*UNIT 2.5A*), and SDS-PAGE (*UNIT 10.2A*)

Purify oligonucleosome cores

1. Resuspend ~2 ml nuclear pellet with 40 ml MSB. Centrifuge 10 min at 10,000 × *g*, 4°C (e.g., 9200 rpm in Beckman JA-20 rotor).

   Dounce homogenization (a few strokes with type B pestle) can be used to resuspend nuclei.

2. Resuspend nuclei in 4 pellet vol of HSB and homogenize in a Dounce homogenizer with 40 to 50 strokes of a type B pestle to release oligonucleosome fragments.

3. Pellet nuclei 20 min at 10,000 × *g*, 4°C, and collect the supernatant. Dilute a sample of this supernatant into 2 M NaCl and measure A_{260} (APPENDIX 3D) to calculate the percent of chromosomal DNA that has been solubilized from the nuclei.

Typically ~3 to 4 mg of oligonucleosome cores can be expected at this point (~50% of the DNA in the nuclei).

4. Dialyze supernatant overnight at 4°C against 4 liters LSB with 6 to 8 kDa MWCO dialysis membrane. When collecting the dialysate, mix the sample (pressing on the sides of the dialysis bag) in order to collect those oligonucleosomes that precipitated during dialysis.

5. Add 0.1 M $CaCl_2$ to 3 mM (final) and warm the sample for 5 min at 37°C.

6. Add 50 U/µl micrococcal nuclease to 10 U/ml (final) and incubate 5 min at 37°C.

The solution should become clearer, because digestion will resolubilize precipitated oligonucleosomes.

7. Stop digestion with 0.1 vol of 0.5 M EGTA and chill on ice.

8. While gently vortexing, add 2 M NaCl dropwise to a final concentration of 0.6 M.

For separation by gel filtration:

9a. Pour an ~1.6 × 58–cm Sepharose CL-6B column and equilibrate with several column volumes of HSB without sucrose at 12 to 24 ml/hr.

10a. Centrifuge polynucleosome fragments 30 min at 150,000 × *g*, 4°C, (e.g., 40,000 rpm in a Beckman SW55 rotor) to remove insoluble material.

11a. Load supernatant onto the column, run the column at 12 ml/hr, and collect 2-ml fractions.

If the load exceeds 10% of the column volume, rerun the column with additional material or use a larger column with proportional dimensions.

For separation by centrifugation:

9b. Pour two (or more) 34-ml linear gradients (UNIT 10.10) in 1 × 3.5–in. polyallomer centrifuge tubes using HSB/glycerol, with 10% at the top to 40% at the bottom.

For this procedure, perform all steps and maintain all buffers at 4°C.

10b. Carefully layer 2 ml quenched digestion reaction on top of each gradient and centrifuge 16 hr at 100,000 × *g*, 4°C (e.g., 27,500 rpm in a Beckman SW28 rotor).

A 4-ml load onto a 32-ml gradient also gives good resolution with higher final concentration.

11b. Harvest the gradients by piercing the bottom edge (where it bends upward) with a 21-G needle attached to outlet tubing. Hold the needle at an ~30° angle, with the beveled edge up, to pierce the tube, and then lodge the needle tip near the very bottom of the tube (without puncturing the opposite end of the tube). To start the flow, cover the top with Parafilm and apply gentle pressure. Collect 1- to 1.5-ml fractions.

Analyze and store oligonucleosome cores

12. Determine DNA concentration of fractions by diluting an aliquot 10- to 40-fold into 2 M NaCl and measuring absorbance at 260 nm.

13. Measure DNA lengths of oligonucleosomes by treating aliquots of fractions (≥0.5 µg) with 0.5% SDS and 0.5 mg/ml proteinase K for 1 hr at 50°C, and separating by native 1.5% agarose TBE gel electrophoresis (*UNIT 2.5A*). Stain with 0.1 µg/ml ethidium bromide.

    Ethidium bromide staining should show a smear of overdigested DNA, a band of DNA at ~150 bp (mononucleosomes), and larger bands equal to ~150 bp plus multiples of ~200 bp (di-, tri-, tetranucleosomes and so on). Higher multiples will be nearer the column void or the bottom of the gradient.

    CAUTION: *Ethidium bromide is a mutagen and should be handled appropriately.*

14. Analyze 5 to 20 µl of selected fractions for the presence of all four core histones (H2A, H2B, H3, and H4) and for the absence of H1 on a 15% SDS-PAGE gel (*UNIT 10.2A*). Stain the gel with Coomassie brilliant blue.

    Molecular weights of core histones are 14.1 kDa (H2A), 13.8 kDa (H2B), 15.3 kDa (H3), and 11.3 kDa (H4). H1 (21.5 kDa) should separate from most of the DNA and run at the top of the gradient or at the end of the included volume on the column.

15. Pool fractions that contain mono- and dinucleosomes, short oligonucleosomes (3 to 6 units), and long oligonucleosomes (>6 units) total three pooled fractions). Be careful to avoid fractions where the DNA is <150 bp (overdigested) or that contain H1.

16. If DNA concentration is low (<0.5 mg/ml), concentrate by putting sample in a 6 to 8 kDa MWCO dialysis bag and surrounding with solid sucrose. Once the volume has decreased 2 to 4 fold, rinse off sucrose with water and reclip the dialysis bag to the new volume.

    Adjusting the clip prevents samples from swelling back to the original volume in the next step.

17. If step 16 was bypassed, place samples in a 6 to 8 kDa MWCO dialysis bag and dialyze into 100 vol dialysis buffer at 4°C for >4 hr.

    Alternatively, or in addition, samples can be concentrated up to 1 mg/ml using Centriprep-10 concentrators (Amicon).

18. Divide into aliquots, freeze on dry ice or in liquid nitrogen, and store for up to 2 years at –80°C.

    The nucleosomes are also stable for many weeks at 4°C.

    NOTE: *When polynucleosome stocks are thawed, some precipitation may be observed. This will not interfere with the use of the polynucleosomes in salt-dilution assembly procedures, as long as they are well mixed before use to ensure proper concentration. Also, material precipitated during freezing appears to come back into solution when the stock is diluted. The addition of 20% (v/v) glycerol and rapid freezing in liquid nitrogen may help to reduce this effect.*

BASIC PROTOCOL 3

PURIFICATION OF MONO- AND DINUCLEOSOMES

The previous protocol rarely yields pure mono- and dinucleosomes, either because fractions don't separate well or because H1 may contaminate these fractions. By redigesting pure oligonucleosomes, however, mono- and dinucleosomes with little cross-contamination can be recovered (Schnitzler et al., 1998).

Materials

Oligonucleosomes: medium or large polynucleosome fractions (see Basic Protocol 2)
100 mM $CaCl_2$
1 M $MgCl_2$
50 U/µl micrococcal nuclease (see recipe)
0.5 M EDTA
10% and 30% (v/v) glycerol gradient buffer (see recipe)

30°C water bath
Ultracentrifuge with rotor (e.g., Beckman SW55) and 0.5 × 2.5–in. (1.3 × 6.4–cm) polyallomer tubes
Gradient maker

Additional reagents and equipment for harvesting, analyzing, and concentrating fractions (see Basic Protocol 2) and for nondenaturing acrylamide electrophoresis (*UNIT 10.2B*)

1. Thaw ~1 mg oligonucleosomes (ideally ~1 to 2 mg/ml) and warm to 30°C.
2. Add 100 mM $CaCl_2$ to 1.5 mM final and 1 M $MgCl_2$ to 3.5 mM final.
3. Add 50 U/µl micrococcal nuclease to 0.1 U/µg polynucleosomes and allow digestion to proceed for 10 min at 30°C.
4. Stop the reaction by adding 0.5 M EDTA to 15 mM.
5. Pellet insoluble material by microcentrifuging 30 sec at ~10,000 × g, 4°C.
6. Pour a 4.7-ml linear gradient in a 0.5 × 2.5–in. polyallomer ultracentrifuge tube using 10% glycerol gradient buffer at the top and 30% at the bottom.
7. Layer 0.5 ml digestion reaction onto the gradient and centrifuge 18 hr at 100,000 × *g*, 4°C (e.g., 35,000 rpm in SW55 rotor).
8. Harvest the gradient as described (see Basic Protocol 2, step 11b), taking about twenty four 5-drop fractions.
9. Analyze fractions by agarose gel electrophoresis as described (see Basic Protocol 2, step 13).
10. Pool clean mono- and dinucleosome peaks and concentrate, if desired (see Basic Protocol 2, steps 16 and 17, but perform final dialysis in 10% glycerol gradient buffer; see recipe). Store at 4°C (>1 month) or freeze in liquid nitrogen or on dry ice and store up to 2 years at −80°C.

    The integrity of mono- and dinucleosome cores can be checked by nondenaturing electrophoresis on either 5% acrylamide (UNIT 10.2B) or 1.2% agarose gels run at 4°C in 0.5-fold TBE electrophoresis buffer. Intact mononucleosomes will run much slower than the DNA they contain (~700 bp rather than 150 bp for samples treated with SDS and proteinase K).

PURIFICATION OF CORE HISTONES BY HYDROXYLAPATITE CHROMATOGRAPHY

BASIC PROTOCOL 4

A second common method for assembling mononucleosomes or polynucleosomes from defined-sequence, labeled DNA relies on the direct assembly of nucleosomes from free histones and DNA as the salt concentration is decreased from 1 or 2 M. For this and other applications, the four core histones can be readily purified by binding chromatin fragments to hydroxylapatite resin (to which DNA binds very strongly), washing away histone H1 with 0.6 M NaCl, and eluting the histones from the resin-bound DNA at 2.5 M NaCl (Côté et al., 1995; Workman et al., 1991).

Materials

Nuclear pellet (see Basic Protocol 1)
HAP buffer (see recipe), with and without 2.5 M NaCl
BioGel HTP powder (Bio-Rad), with adsorption capacity 0.6 mg DNA per g dry powder
Bio-Rad Protein Assay (optional)
2 × 15–cm column and accessories
Centriprep-10 concentrators (Amicon; optional)

Additional reagents and equipment to determining protein concentration (*UNIT 10.1A*) and SDS-PAGE (*UNIT 10.2A*)

1. Resuspend ~2 ml nuclear pellet containing ~6 mg DNA in 25 ml HAP buffer and stir gently for 10 min at 4°C.

   A few strokes with a dounce homogenizer and a type B pestle can be used to aid resuspension. To avoid proteolysis, 1 µM pepstatin A and 1 µM leupeptin can be added to HAP buffer.

2. While stirring, add 10 g dry BioGel HTP powder.

3. Add just enough HAP buffer to make a slurry. Pour this into a 2 × 15–cm column and collect the eluant.

4. Wash resin with 10 vol HAP buffer (~300 ml at 30 ml/hr).

   The eluant from step 3 and the first ⅔ column volume of wash contain H1, which can be further purified as described (Côté et al., 1995) in order to reconstitute chromatosomes.

5. Elute the core histones with HAP buffer containing 2.5 M NaCl, collecting 8-ml fractions.

6. Determine protein concentration of fractions by A_{230} or A_{280} readings or by protein assay, and pool the peak fractions.

   Because histones are small positively charged proteins, many methods for determining protein concentration give differing results. Since nucleosome assembly protocols require careful determination of the molar ratio of histones to DNA, it is essential to use the same method for determining histone concentration as used in the assembly protocol. One reasonably good method is to measure absorbance at 230 nm. The extinction coefficient (calculated for chicken erythrocyte histones, but likely to be similar for mammalian histones) is 4.3 A_{230} units/mg histones/ml. Alternatively, a Bio-Rad or other protein assay (UNIT 10.1A) can be performed with BSA as a standard. The Bio-Rad assay tends to yield concentration estimates that are ~1.6-fold higher than the A_{230} method.

7. Measure the salt concentration of pooled fractions by conductivity readings.

   The salt concentration should be ~2 M.

8. Measure the purity of the core histones by SDS-PAGE as described (see Basic Protocol 2, step 14).

9. If necessary, concentrate the core histones to between 2 and 10 mg/ml using Centriprep-10 concentrators.

10. Divide into aliquots, freeze on dry ice, and store up to 4 years at –80°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2;*** *for suppliers, see* ***APPENDIX 4.***

Buffer B

20 mM HEPES, pH 7.5
3 mM $MgCl_2$
0.2 mM EGTA
Store up to several weeks at 4°C
Immediately before use add:
3 mM 2-mercaptoethanol
0.4 mM PMSF (see recipe)
1 µM pepstatin A (see recipe)
1 µM leupeptin (see recipe)

Dialysis buffer

20 mM HEPES, pH 7.5
1 mM EDTA
Store up to several weeks at 4°C
Immediately before use add:
1 mM 2-mercaptoethanol
0.5 mM PMSF (see recipe)

Glycerol gradient buffer, 10% and 30% (v/v)

20 mM HEPES, pH 7.9
1 mM EDTA
30 mM KCl
0.1% Nonidet P-40 (NP-40)
10% or 30% (v/v) glycerol
Store up to several weeks at 4°C

This buffer can be adjusted as desired. Virtually any solution with >0.2 mM EDTA, no divalent cations, and salt from 0 to 600 mM will work well as the basis for a glycerol gradient buffer.

High-salt buffer (HSB)

20 mM HEPES, pH 7.5
0.65 M NaCl
1 mM EDTA
0.34 M sucrose
Store up to several weeks at 4°C
Immediately before use add:
1 mM 2-mercaptoethanol
0.5 mM PMSF (see recipe)

Hydroxylapatite (HAP) buffer

50 mM sodium phosphate, pH 6.8
0.6 M NaCl
Store up to several weeks at 4°C
Immediately before use add:
1 mM 2-mercaptoethanol
0.5 mM PMSF (see recipe)

Leupeptin

Prepare a 1 mM aqueous stock solution and store up to 1 year at −20°C.

Low-salt buffer (LSB)

20 mM HEPES, pH 7.5
0.1 M NaCl
1 mM EDTA
Store up to several weeks at 4°C
Immediately before use add:
1 mM 2-mercaptoethanol
0.5 mM PMSF (see recipe)

Lysis buffer

20 mM HEPES, pH 7.5
0.25 M sucrose
3 mM $MgCl_2$
0.5% (v/v) Nonidet P-40 (NP-40)
Store up to several weeks at 4°C
Immediately before use add:
3 mM 2-mercaptoethanol
0.4 mM PMSF (see recipe)
1 µM pepstatin A (see recipe)
1 µM leupeptin (see recipe)

Medium-salt buffer (MSB)

20 mM HEPES, pH 7.5
0.4 M NaCl
1 mM EDTA
5% (v/v) glycerol
Store up to several weeks at 4°C
Immediately before use add:
1 mM 2-mercaptoethanol
0.5 mM PMSF (see recipe)
1 µM pepstatin A (see recipe)
1 µM leupeptin (see recipe)

Micrococcal nuclease, 50 U/µl

50 U/µl micrococcal nuclease (from solid; Sigma)
50% (v/v) glycerol
50 mM Tris·Cl, pH 8 (*APPENDIX 2*)
0.05 mM $CaCl_2$
Store at −20°C (can be stable for >1 year)

Note that different suppliers define units of micrococcal nuclease differently. If Sigma enzyme is not used, be sure that the unit definition is the same, or determine the proper conversion ratio to Sigma units.

Pepstatin A

Prepare a 1 mM stock solution in methanol and store up to 2 years at −20°C.

Phenylmethylsulfonyl fluoride (PMSF)

Prepare a 100 mM stock solution in isopropanol and store up to 4 years at room temperature.

COMMENTARY

Background Information

Use of polynucleosomes and histones in chromatin assembly

A principle use of oligonucleosome cores and core histones is the in vitro assembly of nucleosome cores on defined-sequence DNAs. Several templates exist that allow the formation of radiolabeled mononucleosomes. Many of these contain "rotational phasing sequences," which are essentially DNA sequences that cause the DNA to bend, promoting wrapping around the histone core with one face outwards (a single rotational phase). Frequently used mononucleosome templates are those based on 5S rDNA sequences and those with artificial phasing sequences (e.g., Kwon et al., 1994; Hayes and Lee, 1997; Luger et al., 1997; Schnitzler et al., 1998). Mononucleosomes can be formed by incubating the template DNA with excess polynucleosomes (octamer transfer method; Côté et al., 1995) or with roughly equimolar free histones at 1 or 2 M NaCl (salt assembly; Imbalzano et al., 1996). At this high salt concentration the H2A/H2B dimers dissociate and the H3/H4 tetramers readily transfer from donor polynucleosomes, or begin to assemble de novo onto the template DNA. As the salt concentration is reduced, either by dilution or dialysis, the H2A/H2B dimers are added and the complete nucleosome is formed. Polynucleosomes can be formed by salt assembly on any linear DNA template, although the resulting nucleosomes assume somewhat random positions (Stein, 1989). Templates that are made up of a repeated array of 5S rDNA sequences, however, result in arrays of reasonably well-positioned nucleosomes (Carruthers et al., 1999).

Although salt assembly methods are the simplest assembly systems, containing only DNA and the core histones, other systems for chromatin assembly exist. Heat-treated extracts of *Xenopus* oocytes allow assembly of poorly positioned nucleosomes at lower salt concentrations (Workman et al., 1991). Many cellular factors that facilitate such random deposition are known and can be purified from a variety of sources (Ito et al., 1996, and references therein). Unheated *Xenopus* oocyte and *Drosophila* egg extracts contain ATP-dependent factors that result in uniform spacing of nucleosomes assembled onto templates (Sessa and Ruberti, 1990; Becker et al., 1994). The factors responsible for some of these activities have been identified, and defined in vitro reconstitution systems for spaced nucleosomes using pure proteins are now possible (Ito et al., 1997; Varga-Weisz et al., 1997).

Variations for histone and polynucleosome isolation

Polynucleosomes that contain linker histones can be isolated by a protocol that uses micrococcal nuclease to release chromatin fragments from nuclei (Kornberg et al., 1989). Partially purified linker histone H1 is a by-product of the chromatography step in Basic Protocol 4. This can be further purified and used to reconstitute chromatosomes (Côté et al., 1995). Treatment of cultured cells with the histone deacetylase inhibitor butyrate results in hyperacetylation of histone tails (Vettese-Dadey et al., 1995). The acetylation state of the histones can be assayed by Triton/acid/urea gels (*UNIT 21.2*). Nuclei from these cells can be subjected to the above protocols to yield hyperacetylated oligonucleosome cores or histones. A stepwise elution of histone from hydroxlapatite allows H2A and H2B to be separated from H3 and H4 (Simon and Felsenfeld, 1979). The N- and C-terminal tails of the histones in oligonucleosome cores can be cleaved off by treatment with trypsin. These proteolyzed arrays can be subjected to hydroxylapatite chromatography to yield tailless histones, and both the arrays and histones can be used in nucleosome assembly reactions (Guyon et al., 1999).

Critical Parameters

As with any protein isolation, everything should be kept on ice or at 4°C except where noted. Small amounts of endogenous nucleases

and residual micrococcal nuclease may be present in poly- and mononucleosome samples. To avoid slow hydrolysis during storage, samples should always be stored in buffers containing EDTA, which chelates the divalent cations required for endonuclease action.

Reducing agents (2-mercaptoethanol and DTT) and protease inhibitors (PMSF, pepstatin A, and leupeptin) used in these experiments should all be added to the buffers just before use. Other buffer components can be stored at 4°C for several weeks.

Troubleshooting

It is important to check the integrity and purity of histones in oligonucleosome and histone preparations. Each of the four core histones should stain roughly evenly using Coomassie stain. If one stains much more weakly (often H2A), protease inhibitors should be added at steps where it is an option.

Anticipated Results

The nuclear pellet from 3 liters of HeLa cells should yield 4 to 10 mg of DNA. Of this, ~1 to 2 mg of medium polynucleosomes (Basic Protocol 2) or ~3 to 8 mg of core histones (Basic Protocol 4) should be obtained. The yield of mononucleosomes (Basic Protocol 3) is generally ~20% to 30% of the mass of input polynucleosomes.

Time Considerations

Basic Protocol 1 should take ~3 hours (less if starting with spent nuclear pellet). Basic Protocol 2 will take ≥2 days to complete. The dialysis step can be reduced to ~2 to 3 hr by dialyzing into LSB with only 50 mM salt, taking samples from within the bag and monitoring salt concentration with a conductivity meter. Oligonucleosomes can be collected when the salt concentration is 100 mM. The column washes in the gel filtration method are often conveniently done overnight (washing more slowly or with a greater volume is acceptable). Depending on the experimental needs, the lengthy concentration steps may not be necessary. Be aware that nucleosomes below ~10 ng/µl may not be stable without 0.1% NP-40 or >50 µg/ml BSA as carriers.

Basic Protocol 3 takes a few hours to set up, plus an overnight centrifugation and an hour to collect fractions. Basic Protocol 4 calls for ~10 hr wash time at 0.6 M NaCl to remove all traces of H1. The flow rate during this wash can be increased (up to 60 ml/hr) without creating excessive H1 contamination.

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Key References

Côté, et al., 1995. See above.

Basic Protocols 1, 2, and 4 are modifications of protocols in this paper.

Wasserman, P.M. and Wolffe, A.P. (eds.) 1999. Chromatin. *Methods Enzymol.* Vol. 304.

This volume contains many other useful and current protocols for the assembly and analysis of chromatin.

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