Expression, Purification and Reconstitution of Recombinant Yeast Histones

This protocol must be used as a supplement for Luger et al., Methods Enzymol. 304, p3-19 (1999).

This protocol was optimized for purification of histones from a 2L culture.

1. Expression

- Express each histone in the suggested strains: BL21 pLysS for H2A, H2B, and H3 and BL21 for H4.
- Grow cells in 2xTY-AC media as described, first performing a small-scale test expression to determine the culture with best expression.
- For large scale expression, use 2 precultures as described in the original protocol and grow the final 4x500mL cultures to an OD600=0.4 before inducing with 0.2mM IPTG. Grow all cultures at 37°C.
- Following induction, shake cultures 4 hrs (for H2A and H2B) and 3hrs (for H3 and H4).
- Harvest cells at room temperature; resuspend each liter of cells in 16.7mL wash buffer, and flash-freeze in liquid nitrogen as described.

[protocol from Ali Hamish]
Bl21-Codon plus (RIL) from Stratagene is used as the host, and cells are routinely grown at 37°C on LB medium + 0.1 % glucose + selective pressure (Kan 20 µg/ml and Chloramphenicol 40 µg/ml). Expression is induced at an A600 of O.6-0.7 by addition of IPTG to a final concentration of 1 mM and the culture is incubated for 2h at 37°C. 150 µg/ml of rifampicin is then added and the culture is incubated for another 2 hours (in total 4 hours).

2. Inclusion Body Purification

- Thaw cells in a 30°C water bath with constant gentle stirring. H2A, H2B, H3 suspensions all become quite viscous as cells lyse, but cells expressing H4 don't lyse well without pLysS.
- Transfer cell suspensions to disposable polyallomer Beckman centrifuge tubes (25x89mm), adjusting the volume to 25mL/L cells. Shear DNA by sonication with 6 x10-15s pulses (or until viscosity is reduced), keeping the suspension on ice at all times.
- Pellet inclusion bodies by centrifugation for 20 min at 14K in a JA-17 rotor. Resuspend cells in TW buffer (25mL/L original culture) using 10mL plastic pipette. Pellets never completely resuspend, but should be broken up as much as possible. (Cells expressing H4 don't lyse well until resuspension in TW buffer so suspension should be resonicated at this point or only sonicated once at this step.) Spin at 14K (JA-17 rotor) at 4°C.
- As in the original protocol, wash pellet a total of 2 times with TW buffer and 2 times with wash buffer.
- Following the washes, store pellet at -80°C.

3. Purification by Ion Exchange Chromatography (SP Sepharose)

(Note: All manipulations in this section were performed on material from 2L starting culture)

Column Set-Up: Pack one HR10/10 column with Q Sepharose and one with SP Sepharose. Set up columns in tandem so the sample first passes over the Q column and then over the SP Sepharose. (All of our chromatography was done on the low pressure Gradifrac system from Pharmacia.) Before starting the salt

gradient, remove the Q Sepharose column. Equilibrate the columns with U Buffer-10%NaCl to load H2A and H2B and U Buffer-20%NaCl to load H3 and H4.

- Soak pellets in 350 µL DMSO for 30 min at room temp and combine into 1 tube. Mince with spatula. Add 13.3mL unfolding buffer and rotate gently for 1hr.
- Remove cell debris by 20 min spin at 14K (JA-17) at room temp.
- Save supernatant. Reextract pellet with 3.3mL unfolding buffer and respin.
- Combine supernatants and dialzye against urea dialysis buffer (1 or 2 buffer changes over 3hrs, checking conductivity periodically).
- Remove any insoluble material by 20min spin at 14K (JA-17).
- Equilibrate the columns (Q and SP) with 10 or 20%B at a flow rate of 2mL/min.
- Inject samples and allow flow through to come off.
- Remove Q Sepharose column.
- Begin salt gradient (see table 1 below).
- Analyze fractions by SDS-PAGE. Pool pure histone fractions and dialyze against three changes of 2L deionized water (+5mM 2-mercaptoethanol and 0.2mM PMSF) at 4°C (Spectra/Por 6 MWCO=3500 Da, The Spectrum Companies). The second dialysis step should be done overnight.
- Lyophilize histone fractions and dissolve in a small volume of water. Measure the OD276 and determine the approximate concentration of the yeast histones using the molar extinction coefficients in the original protocol. Check by SDS-PAGE.
- Lyophilize in aliquots appropriate for octamer reconstitution.

4. Octamer Reconstitution

We followed the original protocol very closely at this step. We tried to use the protein concentrations suggested in the original protocol. However, due to inaccuracies in quantitation, we most likely used a lower protein concentration than written.
(Information from Cindy White and Karolyn Lugar-8/01)

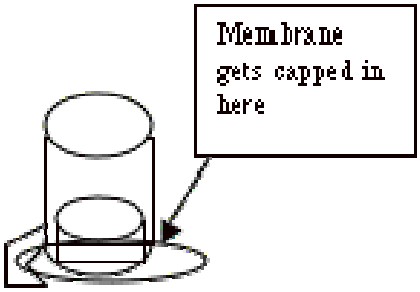
yH2A - ExCo=4350 MW=13858g/mol
yH2B - ExCo=7250 MW=14106g/mol
yH3 - ExCo=2900 MW=15225g/mol
yH4 - ExCo=5800 MW=11237g/mol

- Dissolve each histone aliquot to a concentration of 2mg/mL in unfolding buffer and allow unfolding to proceed for 2-3 hours. (We have performed reconstitutions starting from approximately 2 and 3mg of each histone.)
- Mixed the four histones in equimolar ratios and adjust to a final concentration of 1mg/mL with unfolding buffer.
- Dialyze against four changes of 2L refolding buffer. The second and fourth dialysis steps should be performed overnight at 4°C. (Spectra/Por 6 MWCO=3500Da, The Spectrum Companies)
- Centrifuge reaction at 14K (JA-17) for 20 minutes at at 4°C.
- Concentrate to 1mL using Centricon 10 and filter concentrated mix.
- Equilibrate Superdex-200 column with refolding buffer at a flow rate of 1mL/min.
- Inject sample onto column. High molecular aggregates elute at 45ml, histone octamer at 57mL, tetramer at 65mL, and dimer at 74mL.
- Analyze fractions by SDS-PAGE. Pool pure peak fractions.
- Concentrate on Centricon 10 and dilute to 2M NaCl, 50% glycerol. Store at -20°C.
- Approximate concentration by spec (1mg/ml =0.45 OD276: by K. Lugar) and SDS-PAGE analysis.

5. Reconstitution of Nucleosome Core Particle by Salt Dialysis

For this method of reconstitution, octamer is added to DNA in approximately a 1:1 ratio. We do our reaction in 150mL (10 ng / µl DNA. DNA:histone =1:~1.05: need to be titrated), using the top of an Eppendorf tube as a dialysis vessel (see Diagram 1).

Diagram 1: Vessel for Salt Dialysis



To make the vessel, use a razor blade to cut a siliconized Eppendorf tube (PGC #505-201) around the 1.25mL mark. Discard the bottom of the tube. Cut off the hinge which joins the cap to the tube. To use as a dialysis vessel, remove the cap and pipette your solution (150mL maximum) into the cap. Overlay the cap with a square of dialysis tubing and reattach the top of the Eppendorf tube, lining up the remnants of the hinge. Occasionally, tubes will burst open so before loading the sample, we test the vessel. If it does not pop open in a few seconds, it tends to hold up during dialysis.

- Chill 3L Lo buffer and 600mL Hi buffer in advance.
- Set up reactions in Siliconized tubes on ice. Add components in the following order: Hi Buffer (to final 150mL), then BSA (0.5mg/mL final conc.), then DNA, and finally histone octamer. Titrate to find the optimum histone: DNA ratio.
- For dialysis, we use tubing with a 14kDa MW cutoff. After testing the dialysis vessels, load the sample into the vessel and add to a 1L beaker containing 600mL prechilled Hi Buffer.
- Stir rapidly on a stir plate at 4°C so you can see the dialysis vessels moving up and down for at least 45 minutes.
- During this time, equilibrate two pumps at approximately 3mL/min.
- To begin salt gradient, pump Lo buffer into the beaker containing Hi buffer and pump solution from this beaker out into the sink.
- Allow to dialyze for approximately 20 hours or until most of Lo Buffer is gone.
- Recover samples by gently dabbing outside of tubing with a Kimwipe. Break dialysis tubing with a pipette tip and pipette off dialysate. Store at 0°C for approximately 1 week.
- We usually spike our reconstitution reaction with hot DNA for analysis by gel shift. We run 20x20 cm 5% polyacrylamide gels in 0.25xTBE at 100v, first prerunning the gels for 1 hour at 50v. Change running buffer following the prerun. To avoid disruption of DNA-protein interactions, we omit bromophenol blue from our loading buffer. We always see multiple bands following salt dialysis. Following overnight incubation at 55°C, we see a single nucleosome band shift using a 146bp 5S fragment from Xenopus borealis. Centrifuge and filter to remove aggregate.

6. Reconstitution of Nucleosome Core Particle Using NAP1

Sample assembly reaction:
ExB 5/50 + 0.1mg/mL BSA
0.75mg yNAP1
0.3mg yeast histone octamer
DNA

DNA:histone ratio should be approximately 1:1 by mass, but titrate to find optimal ratio. Add reagents **in the order** listed above, tapping gently after each addition.

- Incubate reaction for 4 hours at 30°C.
- Can be analyzed by gel shift as above. This method of assembly gives a single band using the 146bp 5S fragment, but it may not be possible to assemble the entire DNA into nucleosomes.

7. Reagents

(Note: I have only included reagents which have been changed from the original protocol. For example, we did not change unfolding and refolding buffers. For all reagents containing urea, we prepared 8M urea stock fresh each time, incubating it for 1 hour with AG-501 X8 anion/cation exchange resin from Biorad prior to use.)

- (1, 2) Wash Buffer: 50mM Tris-HCl pH7.6, 100mM NaCl, 1mM EDTA. Shortly before use, add 5mM 2-mercaptoethanol and 0.2mM PMSF
- (2) TW Buffer: Wash Buffer + 1% (v/v) Triton X-100
- (3) Urea Dialysis Buffer: 7M urea, 1mM EDTA, 10mM Tris pH 8.0, 0.1M NaCl. Shortly before use, add 5mM 2-mercaptoethanol and 0.2mM PMSF
- (3) Urea Buffer 0M NaCl (A): 7M urea, 10mM Tris-HCl pH8.0, 1mM EDTA. Shortly before use, add 1mM DTT and 0.2mM PMSF.
- (3) Urea Buffer 1M NaCl (B): 7M urea, 10mM Tris-HCl pH8.0, 1mM EDTA, 1M NaCl. Shortly before use, add 1mM DTT and 0.2mM PMSF.
- (6) Hi Buffer: 10mM Tris-HCl pH7.6, 1mM EDTA, 2M NaCl, 0.05% NP40. Shortly before use, add 5mM 2-mercaptoethanol.
- (6) Lo Buffer: 10mM Tris-HCl pH7.6, 1mM EDTA, 50mM NaCl, 0.05% NP40. Shortly before use, add 5mM 2-mercaptoethanol.
- (7) ExB5/50: 10mM Hepes pH7.6, 50mM KCl, 5mM MgCl2, 0.5mM EGTA, 0.1 mM EDTA, 10% glycerol.

Table 1: Salt gradients for histone elution off SP Sepharose

H2A, H2B		**H3**		**H4**	
Vol (mL)	%B	vol (mL)	%B	vol (mL)	%B
0	10	0	20	0	20
10	10	10	20	10	20
135	35	20	25	135	45
140	40	145	50	155	60
140.1	100	155	160	155.1	100
190	100	155.1	100	205	100
		205	100		

Salt gradients for elution of histones from SP Sepharose column (HR10/10 from Pharmacia): the flow rate is 2mL/min, buffer A is urea buffer 0M NaCl, buffer B is urea buffer 1M NaCl.