HISTONES (from Yeast cells)

The protocol below is given for the histone isolation from undegraded crude nuclear preparations and from whole cells, as developed from standard procedures in 1999 [see Waterborg (2000) J. Biol. Chem 275(17), 13007].

At 12 Mbp in the yeast genome, it is *assumed* that 0.012 pg/haploid genome yields, between G1 and G2 cells in log cultures, approx. 0.02 pg DNA (and thus histones) per nucleus or cell.

Histone purity is less than expected after isolation of nuclei, as judged by HPLC, SDS and AUT analysis. The level of H4 acetylation observed (Y1 preparation) is at least as high or up to 20% higher than the highest values reported. The best prep method reported is in Davie, Saunders, Walsh and Weber (1981) Nucleic Acids Res. **9**: 3205-3216. It requires use of 100 mM butyrate during spheroplasting and nuclear isolation in all buffers for standard wild-type cells or at least 30 mM butyrate in low-protease (≤5%) strains. H4 Ac 0 to 4 = 14, 27, 31, 17 and 11 % (1.84 AcK/H4).

The observation of methionine oxidation of alfalfa H2B (see Binder 63) has prompted the inclusion of 2-mercaptoethanol in all buffers from the time that the cells are disrupted in the 40% GuCl homogenization step.

While it is possible to isolate intact histones from whole cells (see Y1-Y5 preps, 1999, binders 144 and 147), non-histone proteins that elute across a wide range of acetonitrile concentrations (30 to >50%) cause problems of contamination between samples, regenerating the column and bands in AU gels that obscure histone bands. Preparation Y5 established that, while HCl back-extraction of a whole cell extract is feasible and produces a fair yield (see details below), homogenization of cells by beading in a buffer selected to maximize nuclear and chromatin insolubility rather than nuclear purity, and minimize changes due to deacetylation or proteolysis, is effective. Acetylation levels were unchanged; no trace of proteolysis of histones. This method is described below as the Preferred method.

REQUIRED:

<u>Nuclear Isolation Buffer</u> (NIB)

The composition of this buffer has not been 'optimized' experimentally. It was based on the premise to create an isotonic, sucrose-based buffer that would stabilize nuclei and prevent solubilization of chromatin.

NIB composition: 0.25 M sucrose, 10 mM $MgCl_2$, 2.5 mM spermidine, 0.5 mM spermine, 20 mM HEPES, 100 mM butyrate, 0.1 % (w/v) Triton X-100, 5 mM 2-mercaptoethanol, pH 7.

NIB can be prepared 1 day before use and stored at 4°C until use.

PMSF to 1 mM is added just prior to use.

Required approx. 50(-100) ml per sample of up to 2 x 10^{10} cells; 500 ml for 8 samples (10^{10} cells)

Prepare: _____ *ml NIB [100 ml] for* _____ *samples*

_____ g sucrose [8.56 g]

_____ ml 1 M $MgCl_2$ stock [1 ml]

_____ µl spermidine stock [363 µl] (100 mg/ml in water, at MW=145.2 → 689 mM)

_____ µl spermine stock [101 µl] (100 mg/ml in water, at MW=202.3 → 494 mM)

_____ ml 1 M HEPES stock [2 ml]

_____ ml butyric acid [0.92 ml] (10.87 M solution)

_____ ml 25% Triton X-100 stock [0.40 ml]

_____ µl 2-mercaptoethanol (14.3 M) [35 µl]

Adjust pH to 7 by adding approx. 2.0-2.2 ml 5 N KOH per 100 ml.

Adjust with water to final volume. Storage (4°C): _____

Use of butyrate is based on Davie experience (NAR 9,3205,1981) despite the observation that butyrate added to live yeast cells does not affect acetylation level.

<u>PMSF stock</u>: freshly prepare 50 mM PMSF in isopropanol:

For 10 ml, weigh 87 mg PMSF, and add isopropanol to 10 ml volume. Dissolve completely before use. Stable for up to a few days in isopropanol; rapidly decomposes in water.
Add freshly, just prior to use, 2 ml per 100 ml NIB.

- Miracloth (Calbiochem #475855, 22-25 micron pore size).
- vortex, funnel, Sorvall centrifuge, sonicator.

Other solutions:

0.1M KPi : 100 mM Potassium Phosphate buffer (pH 6.8) made by adding equal volumes of 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4.

5% GuCl (Guanidine Hydrochloride) : 5 % (w/v) Guanidine hydrochloride in 0.1M KPi, made pH 6.8 by addition of 5 N KOH (or by dilution of 40% GuCl with 0.1M KPi).
The Refractive Index of 5% GuCl : n=1.3465 (~7.4 degrees Brix, (% sucrose equivalents).

40% GuCl : 40 % (w/v) Guanidine hydrochloride in 0.1M KPi, made pH 6.8 by addition of 5 N KOH.
The Refractive Index of 40% GuCl : (» 32 degrees Brix).

BioRex-70 (BioRad ion exchange resin: a weakly carboxylic cation exchanger, R-COO^-, good stability, Selectivity H>>Ag>K>Na>Li and H>>Fe>Ba>Sr>Ca>Mg). Use mesh size 200-400 (or, alternatively, 100-200 mesh).
Prepare at least 1 day in advance or store prepared at room temperature in 5% GuCl.

2-Mercaptoethanol : 14.26 M (commercial stock solution).

Preparation of BioRex-70

1. Weigh 25 g BioRex-70 into a 250 ml cylinder or capped (graduated) tube (.... g).
2. Add 250 ml 5% GuCl and resuspend the resin.
   Preferably, mix on a rotation-mixing platform: __:___ - __:___ hours.
   __:___ - __:___ hours.
   __:___ - __:___ hours.
   If equilibration is for several hours each time, 3 washes will suffice for complete equilibration.
3. Let the resin settle.
4. Decant the supernatant.
5. Measure the Refractive Index of the supernatant and compare with RI of 5% GuCl
   RI= _____ RI= _____ RI= _____ RI= _____ RI= _____
6. Measure the pH of the supernatant and compare with pH 6.8.
   pH= _____ pH= _____ pH= _____ pH= _____ pH= _____
7. Repeat washing steps 2 - 6 with 100 ml 5% GuCl several times until the supernatant RI and pH values are identical to those of 5% GuCl.
8. Store the resin in 5% GuCl in a closed, graduated tube at room temperature until use.

HISTONE EXTRACTION Date: ___, ___-___-___

1. Typical preparation amount for RP-HPLC fractionation and analysis of separated histone pools on AU gel lanes: 5 x 10^9 cells (100 µg total histones @ 0.02 pg/cell) in 100 ml i.e. use mid-log 5 x 10^7 cells/ml culture directly or concentrate 2(-4) fold to ≤ 50 ml for cultures that will be inhibited for growth, e.g. by cycloheximide.
   - Note that for incubations with growth (e.g. without cycloheximide), cells should NOT be concentrated but used at (or even below) 5 x 10^7 cells/ml, e.g. for continued 1-2x doubling in 2-3 hours before a steady-state density of 10-20 x 10^7 cells/ml is reached (Y6-E/G samples reached 25 x 10^7 and showed a reduction in H4 acetylation !).
   - Note that histones, isolated in Y6 from approx. 1.5 x 10^{10} cells, using the nuclear preparation method, gave overloaded H2B and H3 lanes, with heavy H4. Continued growth for 120 min, likely giving 2.5 x 10^{10} cells in 100 ml, even gave some H4 overload and severe overload of H2B, H3 and H2A. Thus, the histone recovery using the nuclear method is so high that 5 x 10^9 cells will likely suffice for any analysis; The Y7 experiment, using this

recommendation and cycloheximide, suggests that 10^{10} cells i.e. 200 ml of 5 x 10^7 cells/ml is required for H4 AUT gel patterns, with H3 then of all or 2/3 of H3 pool and with _ of H2B pool per AUT gel lane (long, large gels).

Cells in YPD (typically **YPD-S**) medium are used directly or concentrated from mid-log cultures (e.g. 5 x 10^7 cells/ml) by centrifugation e.g. 5 min 800 x ***g*** (2000 rpm) at Room temperature. Use 250 ml conical Corning tubes, spin, decant and retain (or re-add) (used) YPD(-S) medium to (maximally) 10 x 10^7 cells/ml.

Notes on details of samples and/or incubations are below, or on separate pages.

- Y6 (5/99): 1.25 x 10^{10} cells, incubated in Erlenmeijer flask, 30°C 150 rpm in incubator, with label in 90-100 ml YPD-S (pH approx 4) medium, grown for up to 2 hrs, thus likely up to 2.5 x 10^{10} cells, were collected as 2 plts in 50 ml tubes: plt volumes ≤ 1ml each, combined in NIB for beading.

2. Following incubation (or growth), collect cells from 50-(100 ml) samples into 1(or 2) marked 50 ml low-speed polypropylene tubes by centrifugation for 5 min at 800 x *g* (2000 rpm).
   Alternatively, collect 200-250 ml into 1 Corning tube (250 ml), spin, resuspend in water and transfer to 50 ml tube for spin, decant, freeze and store at –80°C.
   Decant and discard supernatant (or collect in radioactive waste container).

ID												
ml plt												

3. **Immediately** freeze the cell pellet (approx. 1 ml pellet from 10^{10} cells) in methanol-dry ice, and store (for at least 1 hour) at –80°C until isolation of histones.

   Date: ___, ___-___-___, __:__ → ___, ___-___-___, __:__

<u>Isolation of (crude) nuclei</u> (the "better" procedure to isolate histones)

4. Place 50 ml tubes with frozen cells on ice.
5. Use 3 ml **cold** Nuclear Isolation Buffer (NIB) to combine the cell pellets into one 50 ml tube with total sample volume of 4(-5) ml.
6. Add 500 micron glass beads (e.g. Sigma G9268, 425-600 μm, 30-40 U.S. sieve) until **just** no free liquid remains. This means that to 4 ml, glass is added to a total volume of approximately 10 ml.
   Homogenization on a fierce vortex of 10 ml in a 50 ml Corning tube is effective; a final volume of (15-)20 ml is TOO large and requires distribution across 2 tubes.
   After the homogenate is collected (see below), the glass beads are extensively washed with water, rinsed on a Buchner filter, dried in an oven and collected for re-use.
7. Vortex for 1(-2) min vigorously the initially almost dry ball of glass beads which will start to liquify with vigorous semi-liquid eruptions of the surface.
8. Cool on ice.
9. To eliminate free standing liquid, add glass beads until no free liquid remains. The final volume may increase to approx. 13 ml.
10. Vortex for 1(-2) min vigorously. If initial vortex homogenization was effective, little or no increase in liquid state is observed
11. Add NIB to the 30 ml mark, mix and let the glass beads settle.
12. Decant the supernatant through 2 layers of Miracloth (in a funnel) into a Sorvall high-speed centrifugation tube (35 ml effective sample volume) **in ice**.
13. Add 10 ml NIB to the glass beads in the tube, mix and pour all into the funnel.
    The glass beads are extensively washed with water, rinsed on a Buchner filter, dried in an oven and collected for re-use.

14. Sorvall centrifugation for 10 min at 16,000 rpm (30,000 *x g*), 4°C, with brake.
15. Decant the supernatant (into radioactive waste when cells were labeled !)

16. Resuspend the pellet in 10-20 ml NIB, combining equivalent samples from separate tubes into a single Sorvall tube.
    Use a pipet and vortexing for effective and complete resuspension.
17. Re-spin for 10 min at 16,000 rpm (30,000 *x g*), 4°C, with brake.
18. Decant the supernatant.

19. ___ µl 2-mercaptoethanol is freshly added to _____ ml 40% GuCl [0.1 µl/ml] to a final concentration of 0.1% (=approx.12.5 mM).
    Add 1 volume (or 1 ml, whatever is less) of 40% GuCl (with 2-mercaptoethanol) to the pellet.
    Typically: 3 ml + 1 ml per sample.
20. Transfer by pipet tip the likely viscous sample to a 15 ml conical polypropylene tube (for samples of less than 2 ml) or to a 50 ml tube for large samples.
    If necessary, the sample can now be frozen at –80°C or the histone extraction procedure can continue.

    cms note: This is super viscous so I add 1mL 40%GuHCl and scrape the goop up with a blue pipet tip and transfer it to a 6mL snap-top falcon (2063) [which seems to be the best tube for the sonication that follows]. I then add another ~1 mL 40% GuHCl, vortex and scrape to get as much of the remaining goop that I can. Sonication is best if the volume is less than 3 mL/tube and there is no more than 1L-worth of cells per tube.

21. Homogenize the cells by sonication ON ICE within the 50 ml (or 15 ml) tubes, using a micro-probe sonicator, for 2 x for 30 seconds with 60 sec cooling break at 60(-80) Watts.
    Microtip on Branson Sonifier 400: setting #2, 15% output; Medium probe: setting #7, 20% output.
    Avoid foaming during sonication.
    The viscous solution (viscous only at high DNA concentrations) will have become thin.

    cms note: Using a Fisher Scientific 60 sonicator, I sonicate 2X 30 sec at setting 10.

22. Transfer the sonicate by pipet to a capped 5 ml Sorvall tube (plastic, for SS34 rotor).
23. Clarify the homogenate by centrifugation for 10 min at 30,000 x *g* (Sorvall SS34: 16,000 rpm) 4°C.
24. Carefully decant the tan-colored supernatant from the pellet of nuclear debris into a new capped 5 ml Sorvall tube.

    Color supernatant: _____ Color pellet: _____

    cms note: From Yeast, sup is clear/yellowish and pellet is white and dark around edges.

Estimate the volume of the supernatant by comparison with tubes with known volumes.
If desired, add 5% GuCl to get equal volumes in all tubes.

ID												
vol												

25. Add slowly with mixing: _____ µl concentrated HCl (37%=12.1N) to a final concentration of 0.25 N.
    The solution will likely turn slightly turbid. Observations:

26. Place the tubes in melting ice for (at least) 15 min to allow full precipitation of DNA and acidic, non-histone proteins: ___:___ - ___:___ hours.
    Resulting color: _____
27. Clarify the solution by centrifugation for 30 min at 30,000(-40,000) x *g* (Sorvall SS34: 16,000 (to 18,000) rpm), 4°C and NO BRAKE.

    cms note: Remember to put brake back on ☺

28. Carefully collect by decanting the clear supernatant from the small, white pellet.
    (color: _____ size: _____)
    If part of the precipitate, as pellet or floating on top of the solution, should end up in the supernatant, spin again to remove it before continuing to the next step.
29. Note the volume of the supernatant:

ID												
vol												

30. Add 4 (-6) volumes of 0.1M KPi to decrease the GuCl concentration from (nominally) 40% to 5%.

ml KPi												

31. Measure the Refractive Index :

n=												

    Add more 0.1M KPi (or 40% GuCl) to make the RI identical to that of 5% GuCl (~7.4 Brix).

KPi												
n=												
KPi												
n=												
KPi												
n=												

32. Adjust the pH of the solution with 5N KOH to pH 6.8.

pH												
KOH												

33. **Add mercaptoethanol** (!!) to 5 mM to this solution by adding 0.4 µl/ml.

Vol												
µl 2-me												

34. Skip this step if histones are extracted from nuclei.
    In case of whole cell extraction, the solution has now become cloudy, especially using early to late stationary yeast cultures.
    Clarify by centrifugation for 20 min at 5000 rpm at 4°C in Sorvall RC3 HG-4L or IEC PR6000 in conical 50 or 250 ml or large 1000 ml tubes.
    Pellet observed: _____

35. Add BioRex-70 resin at **200 µl settled resin per 10^{10} cells** (nominally 200 µg DNA):
    _____ ml resin in storage tube.
    _____ ml total volume in storage tube: _____ ml suspension ≈ 1 ml resin

ID												
ml												

36. Place the capped tubes on a nutator, a rotator for head-over-head mixing or horizontally on a rotary shaker-incubator, overnight at room temperature.
    Mixing: Date: ___, ___-___-___ ___:___ hours → ___, ___-___-___ ___:___ hours.

Date: ___, ___-___-___

37. **Let** the resin **settle** in the tubes.
    Do not spin to prevent carbohydrates to compact with the resin.
38. Remove by aspirator the histone-free supernatant and discard. The supernatant will likely to be very turbid when whole-cell extracts are prepared.
    Supernatant looks: _____

39. Mercaptoethanol to approx. 1.3 mM (0.1 µl/ml) is added to the 5% and 40% GuCl solutions to be used to prevent oxidation of histones:
    _____ ml 5% GuCl: add _____ µl 2-mercaptoethanol.
    _____ ml 40% GuCl: add _____ µl 2-mercaptoethanol.
40. Wash the resin with 5% GuCl until the supernatant is clear and free of color.

ID												
# wash												
ml 5% GuCl												

    Typically: _____ (≥ 3) washes with _____ ml (45 ml) 5% GuCl.

41. Add just enough 5% GuCl to resuspend the resin and transfer it by Pasteur pipet to an appropriately sized column.
    Typical column: BioRad 'disposable' 2(-10) ml polypropylene column
    Larger volume options: a Sephadex 10ml PD-10 column (re-used), or a disposable polypropylene syringe with a porous polyethylene disk (range: 3 - 60 ml).
    Flow-rate control with typical colum is only needed for 'slowed flow' during 40% GuCl elution. This is achieved by adding a 25 Gauge, 5/8 inch syringe needle. With these very small columns with resin volumes less than 5 ml, the resin does not dry even if no solvent is applied for 15 min after the flow stops.
    The same needle, or a larger size for larger columns, can be used during resin washing and elution, with a small rubber cork as 'stop-flow' to prevent causing dried resin. When using a pump: DO NOT let the resin run dry!
42. Wash the column with 8 column volumes 5% GuCl.
    The wash solvent is discarded (or collected for disposal if radioactive).

ID												
column vol												
ml 5% GuCl												

43. The short 25 Gauge needle gives a correct, slow flow rate for column elution in a small volume.

44. Place a **collecting tube** below the syringe.

45. Elute the column with 10 column volumes (... ml) 40% GuCl.

ID												
# aliquots												
ml 40% GuCl *vol eluate*												

46. Resin columns used with labeled nuclear preparations are discarded.
    Columns with resin-only (if >5 ml resin volume) can be re-cycled/re-equilibrated.

47. Prepare Spectrapor 3 (MW cut-off 3500) for use by soaking in water.
    18mm Spectrapor 3 holds 1 ml/cm; 45 mm Spectrapor 3 holds 6.4 ml/cm. Boiling in 1 mM EDTA for a few minutes is optional but not required and typically not done.
    If low MW-cutoff membrane is unavailable, use 'normal' (~10000 MW cutoff) boiled in 5 mM EDTA in water for at least 10 minutes.
    Cut pieces for 1 or 2 samples, bordered by knots (5 cm/knot), 1 knot between 2 samples.
48. Pipet or pour (using a funnel) the eluate into the dialysis bag.
    - Close each end by a double knot.
    - Do NOT exclude all air. It will assure a vertical position of all dialysis bags during dialysis, perpendicular to the stirred current.
    - Use simple rubber bands to tie each bag to a marked, identified (inverted) pipet.
      Multiple sets of small samples, up to 2 per tubing, separated by a single knot, with clearly marked order on the pipet, may be tied to a single holder.

49. Initial dialysis: 1 hour against 100 volumes of 2.5% (v/v) acetic acid (with approx. 1 mM 2-mercaptoethanol: 0.1 µl/ml or 100 µl/liter) at 4°C, to remove the major amount of GuCl.
    The use of 0.5, 1.0, 2.0 or 5.0 liter polypropylene beakers with handle, with large magnetic flea, on magnetic stirrer, in a 4◦C cold-room works very well.
    _____ ml 2.5% HAc/2me. Date: ___, ___-___-___ ___:___ h. → ___, ___-___-___ ___:___ h.

50. Dialysis 2: 1 hour against 100 volumes.
    _____ ml 2.5% HAc/2me. Date: ___, ___-___-___ ___:___ h. → ___, ___-___-___ ___:___ h.

51. Dialysis 3 : overnight against 100 volumes.
    _____ ml 2.5% HAc/2me. Date: ___, ___-___-___ ___:___ h. → ___, ___-___-___ ___:___ h.

Ultra-filtration through Centricon-100 (2 ml sample volume) or CentriPlus-100 (15 ml sample volume) through Amicon YM-100 membrane (100,000 D MW cut-off) gives a filtrate with quantitative histone yield, removing “insolubles” and compounds that elute heterogeneously at high acetonitrile concentrations (30% - >60%) in reversed-phase ProteinPlus HPLC.
Incorporation of total label into dialsate is reduced to 25% (*Ac 2h), 3% (*Ac 2h +cycloheximide), 50-60% (*arg, lys 2h), 15-20% (*met 2hr ±cycloheximide), 10% (*AdoMet 2hr ±cycloheximide) (Y9 experiment), apparently retaining all label incorporated in yeast histones.

52. Transfer the dialysate(s) to CODED/labeled, capped polypropylene tube(s) (15, 50 or 250 ml).

ID												
total (ml)												
for LSC:												

53. Centricon-100 filtration of dialysates in aliquots of up to 2 ml at 4°C and 1000x*g* (2200 rpm) in rubber adapter-fitted IEC PR6000 (or RC-2C HG-4L rotor with adapters).
    - initial 2 ml spin 60 min: _____ - _____h. Result: _____
    - load more/rest of dialysate: _____ - _____h. Result: _____
    - further spin(s): _____ - _____h. Result: _____
    - ONE wash of the small residual volume with (up to) 2 ml 2.5% HAc and spin to "completion" (overnight). Date: ___, ___-___-___ ___:___ h. → ___, ___-___-___ ___:___ h.

54. Transfer the filtrate(s) to CODED/labeled, capped polypropylene tube(s) (15, 50 or 250 ml).

ID												
total (ml)												
for												
for												
for												
for												

Aliquots of these dialysates may be taken for lyophilization in separate tubes, e.g. in 1.5 or 0.5 ml Eppendorf tubes, for uses as sample in single HPLC or gel analyses.

55. Puncture the caps of the tubes.
56. Freeze the tubes in –70°C freezer (or with methanol-dry ice or liquid nitrogen.)
    Date: ___, ___-___-___ ___:___ hours. → ___, ___-___-___ ___:___ hours.
    Do NOT place tube in insulating (polystyrene) holders: this causes melting and prevent sublimation.
57. Lyophilize until dry in an oil-pump vacuum with methanol-filled cold-trap and chemical trap.
    Despite all traps, do NOT use the oil pump for more than 250 hours (after heavy use, especially drying gels, less !) without **completely** changing the oil (disassemble and wipe all accessible parts in the Savant oil pump). (Do NOT use the so-called 'cleaning solvent': it causes the pump to seize!)
    Lyophilization. Date: ___, ___-___-___ ___:___ hours. → ___, ___-___-___ ___:___ hours.
    Result: _____ Continue → ___, ___-___-___ ___:___ hours.
    Result: _____ Continue → ___, ___-___-___ ___:___ hours.

Observations:

Dried histone lyophilizates can be stored indefinitely at –20°C in closed (new caps!) tubes, especially under nitrogen atmosphere. Even storage at room temperature is acceptable.

NOTE : the residual mercaptoethanol in the lyophilizate will confer protection against oxidation if excessive air contact is avoided: store dry histones in tightly capped tubes !