Extraction, purification and analysis of histones

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Histone proteins are the major protein components of chromatin, the physiologically relevant form of the genome (or epigenome) in all eukaryotic cells. Chromatin is the substrate of many biological processes, such as gene regulation and transcription, replication, mitosis and apoptosis. Since histones are extensively post-translationally modified, the identification of these covalent marks on canonical and variant histones is crucial for the understanding of their biological significance. Many different biochemical techniques have been developed to purify and separate histone proteins. Here, we present standard protocols for acid extraction and salt extraction of histones from chromatin; separation of extracted histones by reversed-phase HPLC; analysis of histones and their specific post-translational modification profiles by acid urea (AU) gel electrophoresis and the additional separation of non-canonical histone variants by triton AU(TAU) and 2D TAU electrophoresis; and immunoblotting of isolated histone proteins with modification-specific antibodies.

INTRODUCTION

Chromatin, a macromolecular complex composed of DNA and protein, is the heritable material of eukaryotic cells. The repeating unit structure of chromatin is the nucleosome, in which DNA is wrapped around a core octamer unit of dimers of the four histone proteins (H2A, H2B, H3 and H4 and/or their variant isoforms) (**Fig. 1**) and further assembled into the larger chromatin fiber including the linker histone H1. The canonical histone proteins and the less abundant histone variants are subject to an extensive array of post-translational modifications (PTMs), including Lys and Arg methylation; Lys acetylation, ubiquitylation, SUMOylation, and ADP-ribosylation; Ser, Thr, and Tyr phosphorylation; and citrullination of methyl-Arg residues[1–4]. Accumulating evidence suggests that the combination of modifications on nucleosomes and the presence or absence of various histone variants contribute to the encoding of epigenetic information, the ultimate regulation of gene expression[5]. Therefore, the study of histone proteins, and the PTMs that they carry, has become increasingly important as more investigations are conducted into the 'epigenetic signatures' of these important chromosomal proteins.

Histones were among the first proteins studied, in part because of their relative ease of isolation. Albrecht Kossel coined the term 'histon' in 1884 when he isolated acid-soluble proteins from bird erythrocyte nuclei (reviewed in ref. 6). This had followed Friedrich Miescher's pioneering work in 1874 demonstrating the existence of the alkali-labile and acid-insoluble material he termed 'nuclein', later identified as DNA[7]. In retrospect, these early investigations, utilizing simple acid and base extractions, demonstrated that the two discrete components of the genetic material were nucleic acid and protein and suggested the use of acid extraction for histone preparation.

After Avery's demonstration of DNA as the genetic material[8] and Watson and Crick's structure of DNA[9], the study of histones and chromatin moved slowly as more attention was paid to the nucleic acid component of the genetic material. Some work on histones through the 1950s utilized high-salt extraction of chromatin to isolate the histones. Simple fractionation of the histone population using a variety of dilute acids was attempted by a number of research groups. More extensive characterization ensued and histone extraction from various cell types suggested different populations of histones existed in different tissues[10], foreshadowing the significant role histones and their variants have in epigenetic regulation. In 1967 Johns[11] introduced the concept of separating

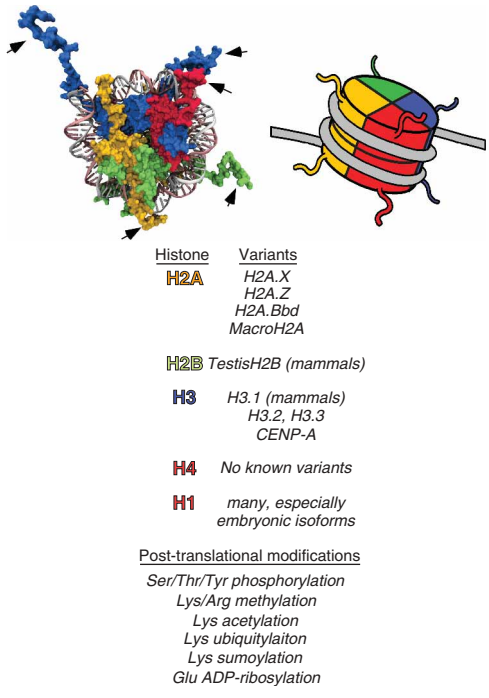

Figure 1 | Depiction of histones in the nucleosome. Core histone proteins H2A (orange), H2B (green), H3 (blue) and H4 (red) and a 147-bp DNA strand (silver and red) are shown in the 1.9-Å nucleosome core particle structure[31] (as rendered by the software package VMD[32]) and in a cartoon diagram to clearly illustrate the positions of the core histone proteins. The N-terminal tails of the histone proteins are noted with arrows; these tails are the location of the majority of the post-translational modifications (PTMs) of histones. The histone proteins, the major known histone variants[33] and the range of PTMs found on these histone proteins are noted below. The functions of histone modifications have been recently reviewed[34].

histone proteins for analysis on acidic polyacrylamide gels, and Panyim and Chalkley[12] developed the acid urea (AU) gel, which cleanly separated histones, histone variants and differently modified histone isoforms (such as acetylated and phosphorylated histones) on the basis of differences in their charge. Meanwhile, Allfrey and coworkers'[13] discovery of acetylated and methylated histones in 1964 led them presciently to hypothesize that histone modifications, notably acetylation, methylation and phosphorylation, may regulate gene expression. Finally, the discoveries in 1996 that a histone acetyltransferase[14] and a histone deacetylase[15] were known transcriptional regulators prefaced an explosion in histone and chromatin research in the past decade[16]. These advances all depend on a reliable and effective isolation of histone proteins.

To study the function of histones, histone variants and particularly histone modifications, many laboratories, including our own, routinely begin by acid extraction of histones from cells or nuclei, further purifying them by reversed-phase HPLC (RP-HPLC), a method that was used first for the analysis of histones in 1983, (ref. 17). Both the extracted and HPLC-purified histones are easily analyzed by a variety of techniques: AU gel analysis; immunoblotting with modification-specific antibodies after separation by Tris–Gly or Tris–tricine SDS–polyacrylamide gel electrophoresis (SDS-PAGE); and mass spectroscopy (MS) to determine the identity and abundance of variants and PTMs and also to search for novel modifications[18]. The still-relevant utility of AU gel analysis was recently demonstrated by the identification of 'long-distance' linkage between acetylation and methylation[19]. In these experiments, di- and tri-methylation on histone H3K4 were specifically found on slower-migrating H3 bands on an AU gel, indicating that H3K4 methylation was linked to H3 acetylation.

Model systems and cellular sources of histones

The following protocols include our standardized approach to histone extraction, purification and analysis. The techniques focus on isolation from mammalian cell culture, but the procedures following nuclei extraction are identical for yeast, *Tetrahymena thermophila*, *Xenopus laevis* egg extracts and other model systems in which nuclei are extractable. Please see the referenced methods for isolation of yeast nuclei[20], *Tetrahymena* nuclei[21], and *Xenopus* egg extract[22] and *Xenopus* nuclear isolation[23]. Histones can also be extracted from animal tissue or plant cells following isolation of their nuclei, although those procedures may differ from those presented here, so be sure to follow an established nuclear isolation procedure for that particular cell type. After successful nuclear or chromatin isolation, follow our protocols for purification and analysis of histone proteins. Alternatively, a cruder histone preparation may be obtained simply by placing the entire cell or tissue in dilute acid; in that case, follow our protocols disregarding the chromatin isolation steps. As histones are among the most highly conserved eukaryotic proteins, their biochemical behavior is remarkably similar across many species and model organisms, so the majority of these protocols are of general utility.

In sum, the following techniques (outlined in **Fig. 2**) are standard approaches for the experimental study of histone proteins. The extraction techniques are robust, and at least for routine cell culture of HeLa cells, yeast or *Tetrahymena*, the source material is cheap, so optimization of the described techniques is recommended to suit particular needs. We do not specifically address routine SDS-PAGE analysis, staining and SDS-PAGE gel western blotting, as these are standard techniques, although our experience dictates that adjustments are sometimes needed (e.g., electrophoretic transfer of proteins is routine from SDS-PAGE and AU gels, but is diminished from triton AU (TAU) gels, often requiring increased transfer times or currents). A wealth of modification-specific histone antibodies for immunoblotting are now available from a number of suppliers, including Millipore (formerly Upstate), Abcam and LP Bio. Most of these antibodies work across a range of species, although investigators should check with the supplier to confirm that it is true for a particular antibody of interest. As epitopes can be masked by neighboring PTMs, investigators are encouraged to use independent techniques (MS, analytical gel electrophoresis, etc) to verify positive or negative antibody results.

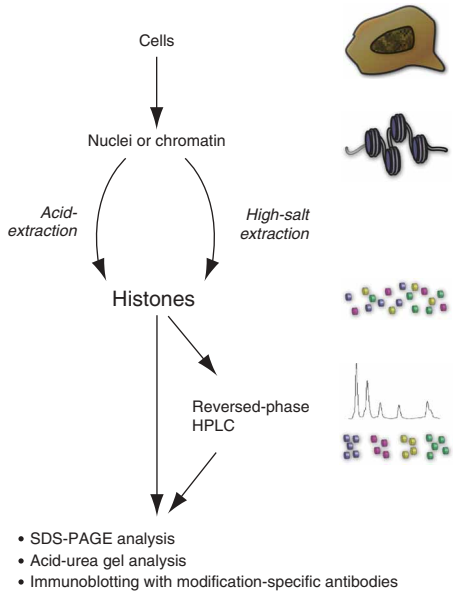

Figure 2 | Schematic flowchart of histone isolation and purification procedures. Cells are grown and collected under the specific experimental conditions required. The cells are lysed under hypotonic conditions and the intact nuclei are collected; alternatively, nuclei or chromatin can be obtained by other methods[35]. The nuclei are extracted in acidic or high-salt conditions to selectively remove histones, which can subsequently be further purified by reversed-phase chromatography or used immediately for other analysis, including immunoblotting and mass spectroscopy.

Experimental design and considerations

Most of the histone extraction work has been based on solubility of histones in acids, either dilute HCl or H_2SO_4, conditions under which most other nuclear proteins and nucleic acids will precipitate[24]. Note that the acid extraction method, when applied to plant cells, slime molds and some fungi (including the yeast *Saccharomyces cerevisiae*), may produce a histone preparation that contains large amounts of carbohydrates and polyphenols that readily cross-link and oxidize the histone proteins.

For the potential discovery of novel PTMs, it would be wise to consider infrequently utilized histone extraction techniques, such as high-salt extraction, in place of the typical acid extraction, as some histone modifications may be quite acid-labile[25] (such as His phosphorylation)[26]. Furthermore, acid and high salt may differentially extract histones from various chromatin environments, such as heterochromatin and euchromatin. Utilization of

both approaches may lead to a more complete analysis of the histones in a particular sample. Therefore we also present a less commonly used histone isolation strategy using high-salt extraction[27].

High-salt extraction is a useful alternative to acid extraction for a few reasons. First of all, a neutral pH is maintained and any acid-labile histone modifications should therefore remain. Second, acid extraction and subsequent TCA-precipitation occasionally produce insoluble material that may contain histones, reducing the yield of the preparation. Free histones, which are not incorporated into chromatin and might therefore represent a pool of either newly synthesized histones or histones ejected from chromatin, can be isolated and analyzed for pre- or post-incorporation specific modifications. Finally, salt extraction can differentially extract H2A and H2B (at greater than 1 M NaCl) and H3 and H4 (at greater than 1.5 M NaCl)[27]. For downstream applications in which maintenance of neutral pH is crucial, such as determination of acid-labile modifications, ensure that those methods are performed at a neutral pH. In these cases, histones should be chromatographically separated by gel filtration or ion-exchange chromatography, instead of RP-HPLC. Alternatively, perform RP-HPLC in the absence of trifluoroacetic acid (TFA), but expect some peak broadening.

RP-HPLC, which separates molecules on the basis of hydrophobicity, is a high-resolution method ideally suited for histone proteins. Acid-extracted or salt-extracted histones can be purified to near-homogeneity in one run on a standard C8 or C18 column, using an acetonitrile gradient. The method presented is optimized for separation of total histones. If further separation between individual histone proteins is required, the fractions can be re-loaded onto the column and separated using an RP-HPLC method with a shallower gradient, typically improving the separation.

SDS-PAGE (using standard Tris–Gly or alternatively using Tris–tricine (see http://www.natureprotocols.com/2006/06/23/tricinesdspage.php) is very useful for separation of the different histones. However, it does not allow for the separation of histone isoforms with different acetylation levels or phosphorylation status. This is accomplished by AU gel electrophoresis, a method that separates histones according to the individual charges introduced by acetylation and phosphorylation. TAU and 2D TAU gel electrophoresis further separates certain histone variants, such as some H2A variants and the H3 variants H3.1, H3.2 and H3.3, by the binding of the detergent Triton X-100 to hydrophobic regions of the proteins.

In AU gel electrophoresis, proteins are denatured by a high concentration of urea. Since urea denatures without affecting the charge of proteins (as opposed to the ionic detergent SDS), the charge of migrating proteins is determined solely by the number of protonated groups under the acidic running conditions of the protocol. Acetylation reduces the positive charge of a Lys, while phosphorylation introduces an additional negative charge. Therefore, these modifications greatly affect the migration of histones in AU gel electrophoresis, allowing for separation of the different modified isoforms.

For optimal resolution, we run long AU gels (40 cm). Alternatively, for fast separation we run short AU gels, using a standard minigel system (such as the BioRad Protean III system). Here, we present detailed protocols for preparing and running long and short AU gels as well as short TAU gels. Long AU gels consist of a separating gel section and a stacking gel section, which are poured without gel-loading wells. Only after the gel has been pre-run overnight are the wells for sample loading cast. Short AU and TAU gels, on the other hand, have no stacking gel. They are cast in one step, and no pre-running step is required.

MATERIALS

REAGENTS

- 100× Phosphatase inhibitor cocktails I and II (Calbiochem, cat. nos. 524624 and 524625)
- 10× Dulbecco's PBS (acid extraction) (Invitrogen, cat. no. 14200-075)
- NuPAGE 10–20% SDS gel (Invitrogen, cat. no. EC6136)
- Histones from calf thymus (Roche, cat. no. 223565)
- Recombinant histone H4 produced in *Escherichia coli* (Millipore, cat. no. 14-697)
- 0.2 M (0.4 N) sulfuric acid (H_2SO_4) (acid extraction) **! CAUTION** Has corrosive properties. Avoid skin contact and swallowing of H_2SO_4. H_2SO_4 should be used with appropriate safety measures, such as protective gloves, glasses and clothing and adequate ventilation.
- 100% TCA (acid extraction) 500 g, add 227 ml ddH_2O for 100% (wt/vol), keep at 4 °C indefinitely (Fisher Scientific, cat. no. A322) **! CAUTION** Corrosive and causes severe burns. Inhalation may cause lung damage.
- 100% ice-cold acetone (acid extraction) **! CAUTION** Acetone can damage the mucosa of the mouth and can irritate and damage skin. Acetone should be used with appropriate safety measures, such as protective gloves, glasses and clothing, and adequate ventilation. Store acetone in explosion- or flame-proof freezer.
- NP40 substitute (acid extraction) (Fluka, cat. no. 74385)
- Sodium butyrate (acid extraction) (Sigma, cat. no. B5887)
- 2-Mercaptoethanol (acid extraction and RP-HPLC) (EMD Chemicals, cat. no. 6010)
- Acetonitrile, synthesis grade or better (RP-HPLC) (Fisher, cat. no. BA170-4)
- TFA (RP-HPLC) (Sigma, cat. no. 302031) **! CAUTION** Highly corrosive. It is extremely destructive to the upper respiratory tract, eyes, and skin, and should not be disposed of into sewage systems as it is highly toxic to aquatic organisms. TFA is also volatile; dispense in a hood.
- Urea (AU/TAU gels) (Sigma, cat. no. U0631)
- *N,N,N′,N′*-tetramethylethylenediamine (TEMED) (AU gels)
- 10% (wt/vol) ammonium persulfate (APS) in ddH_2O (AU/TAU gels)
- Glacial acetic acid (AU/TAU gels)
- Triton X-100 (TAU gels) (Sigma, cat. no. T8787)
- 0.2% (wt/vol) Pyronin Y dye (AU/TAU gels) (Sigma, cat. no. P9172) in ddH_2O
- Methanol (AU/TAU gels and SDS-PAGE and AU/TAU western blotting)
- Short TAU gel lane run with histones (2D TAU gels) (prepared as described in Steps 58–59)
- 0.125 M Tris–Cl, pH 8.8 (2D TAU gels)
- 0.7% Acetic acid (AcOH) (AU/TAU western blotting)
- Phenylmethanesulfonylfluoride (PMSF)
- Sucrose
- DTT
- KCl
- HEPES
- Pierce SilverSNAP II (Pierce)
- Magnesium chloride ($MgCl_2$)
- Glycerol
- Brilliant Blue G-250 (Serva, cat. no. 17524)

EQUIPMENT

- 50-ml Falcon tubes (acid extraction and AU/TAU gels)
- 15-ml Falcon tubes (AU/TAU gels)
- 1.5-ml tubes (acid extraction, RP-HPLC and AU/TAU gels)
- Dialysis tubing with low molecular weight cut-off (e.g., 3,250 MWCO, Spectra/Por 133 110) and plastic clamps (acid extraction/high-salt extraction)
- Lyophilizer or SpeedVac (Savant) with vacuum pump (RP-HPLC and AU/TAU gels)

- 1-ml glass Hamilton syringe with metal needle (#1001) (RP-HPLC)
- Glass plates (AU gels); for long gels we use 40 × 20 cm^2 plates; for small gels a regular minigel system such as the Hoefer Mighty Small (Hoefer) or the BioRad Protean III system (BioRad) and spacers (0.75 mm)
- Plastic combs (AU/TAU gels) (see EQUIPMENT SETUP)
- Gel electrophoresis apparatus (AU/TAU gels)
- Rocking platform (AU/TAU gels, SDS-PAGE and AU/TAU western blotting)
- Stir plate (AU/TAU gels)
- 0.45-µm filters (Millipore)
- Gel chamber (BioRad or NuPAGE) (2D TAU gels)
- Pre-cast 2D-well gels (2D TAU gels) (e.g., Invitrogen NuPAGE Tris–Gly 10–20% 2D well gels, cat. no. EC6136)
- Small plastic containers (AU/TAU gels and SDS-PAGE and AU/TAU western blotting)
- Polyvinylidene difluoride (PVDF) membrane (various pore sizes) (western blotting) or nitrocellulose membrane
- Whatman 3 MM paper (SDS-PAGE and AU/TAU western blotting)
- Wet-transfer chamber (western blotting)
- Power supply (500 V/500 mA) (SDS-PAGE and AU/TAU gels and SDS-PAGE and AU/TAU western blotting)
- HPLC system with pumps, UV detector and fraction collector, and 5-ml sample loop, such as Beckman System Gold (Beckman)
- 4.6-mm-diameter C8 column (Perkin-Elmer Aquapore, cat. nos. RP-300, 0711-0059)

REAGENT SETUP

Appropriate cell culture or nuclei source For mammalian cell culture, 10^6 cells produce an adequate quantity of histones for routine analysis of total histones by western blotting; 10^7 would be preferable for MS analysis. ▲ CRITICAL Wash cells well with PBS to remove serum proteins and aspirate completely so medium does not interfere with hypotonic lysis.

Hypotonic lysis buffer (acid and salt extraction) Make a 10-ml solution containing 10 mM Tris–Cl pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$ and 1 mM DTT and chill on ice. Add protease and phosphatase inhibitors described below to a 1× concentration just before use in lysis of cells.

25× solution of complete protease inhibitor cocktail One tablet (Roche, cat. no. 1697 498) dissolved in 2 ml of ddH_2O (can be stored for up to 12 weeks at −20 °C). ▲ CRITICAL Add DTT, PMSF (final concentration of 1 mM) and protease and phosphatase inhibitors just before use.

Extraction buffer (high-salt extraction) Prepare 10 ml of a solution containing 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol. Add NP40 or NP40 substitute to 0.2% for cell lysis. Add protease and phosphatase inhibitors to 1× from stock solutions. Add sodium butyrate to 10 mM to inhibit histone deacetylase activity.

No-salt buffer (high-salt extraction) Prepare 10 ml of a solution containing 3 mM EDTA, 0.2 mM EGTA.

High-salt solubilization buffer (high-salt extraction) Prepare 10 ml of a solution containing 50 mM Tris–Cl pH 8.0, 2.5 M NaCl, 0.05% NP40.

Dialysis solution (high-salt extraction) Prepare 2 l of 10 mM Tris–Cl pH 8.0, chilled to 4 °C.

Solvent A (RP-HPLC) (5% acetonitrile, 0.1% TFA) Prepare 1 l by addition of 50 ml acetonitrile to 950 ml of ddH_2O. Add 1 ml TFA using a syringe in a fume hood.

Solvent B (RP-HPLC) (90% acetonitrile, 0.1% TFA) Prepare 1 l by addition of 100 ml of ddH_2O to 900 ml of acetonitrile. Add 1 ml TFA using a syringe in a fume hood.

Solvent C (RP-HPLC) (90% acetonitrile, no TFA) Prepare 1 l by addition of 100 ml of ddH_2O to 900 ml of acetonitrile.

Note: Solvents A to C for RP-HPLC should be filtered using 0.45-µm filters.

60%:0.4% acrylamide:bisacrylamide solution (AU/TAU gels) Consists of 250 g acrylamide in 417 ml ddH_2O. Stir overnight and add 1.67 g bisacrylamide. Filter the solution and store in dark container protected from light. ! CAUTION Acrylamide and bisacrylamide are potent neurotoxins. Use appropriate safety measures, such as protective gloves and safety goggles, and handle under adequate ventilation.

Running buffer (AU/TAU gels) 5% AcOH.

Protamine sulfate solution (AU/TAU gels) (Sigma, cat. no. P4505, grade III from Herring) Should be prepared fresh at 25 mg ml^{-1} in ddH_2O and stored in 500-µl aliquots at −80 °C.

Amido black solution (SDS-PAGE and AU/TAU western blotting) Prepare a solution containing 0.5% amido black dye (JT Baker, cat. no. A586-03) in 10% isopropanol and 10% AcOH.

Coomassie brilliant blue solution (AU/TAU gels) 0.1% (wt/vol) Brilliant Blue G-250 (serva), 50% methanol, 10% AcOH. Solubilize Brilliant Blue G-250 powder in methanol, then add AcOH and finally ddH_2O. Filter solution and store at room temperature (20–25 °C).

Silver staining (AU/TAU gels) We use the Pierce SilverSNAP II kit for silver staining. Alternatively, researchers may use homemade silver stains[28].

Transfer buffer (AU/TAU western blotting) 0.7% AcOH

EQUIPMENT SETUP

Plastic combs Make a 'one-finger' comb by cutting a piece comprising one 'finger' from a plastic comb as is commonly used for pouring SDS-PAGE gels (**Fig. 3**). Save for future use (2D TAU gels).

PROCEDURE

Acid extraction of histones ● TIMING 2 d

1| *Preparation of nuclear extracts* ● TIMING Day 1, 1–1.5 h. Collect tissue culture cells (5 × 10^6 cells ml^{-1}) in 50-ml tubes and pellet (10 min, 300*g*).

Note: For the alternative method of high-salt extraction see **Box 1**.

2| Discard supernatant, wash cell pellet with 10 ml PBS and spin again (10 min, 300*g*). Again, discard supernatant.

■ PAUSE POINT Cell pellet can be flash-frozen in liquid nitrogen and stored at −80 °C indefinitely.

3| Re-suspend cell pellet in 1 ml hypotonic lysis buffer and transfer to 1.5-ml tube.

▲ CRITICAL STEP Steps 4–15 are all performed at 4 °C. This procedure can be scaled up to accommodate larger cell culture volumes, but more tubes may be necessary.

4| Incubate for 30 min on rotator at 4 °C to promote hypotonic swelling of cells and lysis by mechanical shearing during rotation.

5| Pellet the intact nuclei by spinning in cooled tabletop centrifuge: 10,000*g*, 10 min, 4 °C.

6| Entirely discard supernatant with pipette and re-suspend nuclei in 400 µl 0.4 N H_2SO_4.

▲ CRITICAL STEP Nuclei have to be re-suspended very well, with no clumps left in solution. If necessary, vortex the solution until clumps are dissolved.

7| Incubate on rotator for at least 30 min or overnight.

8| Spin samples in cooled tabletop centrifuge to remove nuclear debris: 16,000*g*, 10 min.

9| Transfer the supernatant containing histones into a fresh 1.5-ml tube.
Note: Proceed either by TCA-precipitation of histones (Steps 10–17) or by dialysis and lyophilization (Steps 18–22).

10| *TCA-precipitation of histones* ● **TIMING** Day 2, 1.5 h. Add 132 µl TCA drop by drop to histone solution and invert the tube several times to mix the solutions (final concentration of TCA is 33%).
▲ **CRITICAL STEP** The histones precipitate in TCA. The solution will appear milky over time.

11| Incubate on ice for 30 min.
■ **PAUSE POINT** This step can be extended to an overnight incubation.

12| Pellet histones by spinning in cooled tabletop centrifuge: 16,000*g*, 10 min at 4 °C.

13| Carefully remove supernatant with pipette and wash histone pellet with ice-cold acetone without disturbing it. Acetone is used to remove acid from the solution without dissolving the protein pellet.

14| Spin in microcentrifuge 16,000*g*, 5 min at 4 °C.

15| Repeat Steps 13 and 14.

16| Carefully remove all of the supernatant with pipette and air-dry histone pellet for 20 min at room temperature.

17| Dissolve histone pellet in appropriate volume of ddH_2O (typically 100 µl, scale with quantity of cellular source) and transfer into fresh 1.5-ml tube. Go to Step 22.
▲ **CRITICAL STEP** Histones will be visible as a 'smear' on the tube wall. To dissolve the histones, pipette up and down the tube wall. Occasionally an insoluble pellet remains, which can be removed by spinning for 10 min at 16,000*g* at 4 °C. As this insoluble pellet may contain histones, an alternative to TCA precipitation follows.

18| *Dialysis and lyophilization* ● **TIMING** Typically 2 d. (Do not continue from Step 17, only from Step 9.) Dialyze supernatant from acid extraction in dialysis tubing with low molecular weight cut-off against ddH_2O for 2 h to overnight. 2-Mercaptoethanol (e.g., 10 µl of 100% in 1 l ddH_2O) may be added to prevent oxidation. Instead of ddH_2O, 2.5% AcOH may be used for dialysis to ensure histone solubility.
▲ **CRITICAL STEP** Ensure that a low molecular weight (approximately 3,000) cut-off dialysis membrane is used, as histones are small proteins and may be lost in higher weight cut-off membranes.
■ **PAUSE POINT** Dialysis can continue overnight.

19| Carefully remove dialyzed solution from membrane and freeze at −80 °C in a 15-ml Falcon tube or in multiple 1.5-ml centrifuge tubes.

20| Load frozen histone solution into a SpeedVac (1.5-ml tubes with opened cap) or a lyophilizer (15-ml tubes with a hole punched through the cover) and apply a vacuum.
▲ **CRITICAL STEP** Do not apply heat.

21| After complete solvent removal (anywhere from 3 h to overnight), remove tubes from lyophilizer and either store the powder at −20 °C or −80 °C or dissolve in appropriate volume of ddH_2O (approximately 100 µl).
■ **PAUSE POINT** Histones can be stored frozen at −20 °C or −80 °C indefinitely.

22| Separate 1, 3 and 5 µl of the histone solution on 15% SDS/PAGE gel and stain with Coomassie Brilliant Blue solution to characterize concentration of the histones before running SDS-PAGE gels (**Fig. 3**) or performing SDS-PAGE western

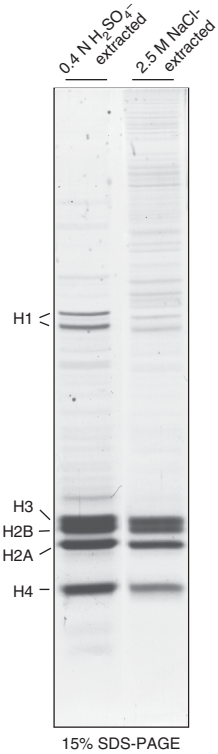

Figure 3 | Coomassie Blue–stained SDS gel with acid- and salt-extracted histones. Two microliters of acid-extracted and 2 µl of salt-extracted histones from HeLa cells were run on a 15% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel and stained with Coomassie Blue dye. The locations of the linker histone protein H1 and the core histone proteins H3, H2B, H2A and H4 are noted. A similar banding pattern is observed for most mammalian species' histone proteins.

blotting, RP-HPLC (see Step 23, **Fig. 4**), AU, TAU (see Step 30, **Fig. 5**) or 2D TAU (see Step 53, **Fig. 6**) gel electrophoresis. As a positive control, commercially available histone preparations (e.g., histones from calf thymus from Roche, cat. no. 223565, 10 mg lyophilized) can be used as a concentration standard. For the analysis of post-translational histone modifications by western blotting, recombinant histones can be used as negative controls, as these do not contain any modifications (e.g., recombinant histone H4 produced in *Escherichia coli* from Millipore, cat. no. 14-697, 1 mg lyophilized), As a control for good transfer of histones out of the gel onto the membrane, the membrane can be stained with amido black solution.
? TROUBLESHOOTING

RP-HPLC purification of histones ● TIMING 5–6 h, including column washing and storage

23| Dissolve histones (either acid- or salt-extracted from 10^7 cells) in 200 µl ddH_2O.

24| Load histone sample into sample loop with a glass Hamilton syringe and inject sample onto column to initiate the programmed run (see **Box 2**, **Table 1** and **Fig. 4**).
▲ CRITICAL STEP Do not use a plastic syringe, as contaminants from the plastic may interfere with subsequent MS.

25| Switch sample loop back from 'inject' position to 'load' position before the 5-min mark to prevent a delay in the gradient.

26| Watch chromatogram during run and check to ensure that fraction collector is working properly.

27| After the run finishes, the fractions can be frozen at −80 °C and lyophilized in a SpeedVac for 4 h or overnight. Lyophilized fractions can be stored at −80 °C dried or they can be re-dissolved in 100 µl ddH_2O. The addition of a trace amount of 2-mercaptoethanol (e.g., 5 µl of 0.1 M 2-mercaptoethanol) to the fractions prevents the oxidative damage of histones during and after lyophilization.

BOX 1 | HIGH-SALT EXTRACTION OF HISTONES ● TIMING 4–6 H

1. Re-suspend 10^7 cells in 1 ml extraction buffer with 0.2% NP40. Incubate for 10 min on ice, occasionally rotating.
▲ CRITICAL STEP Steps 1–16 are all performed at 4 °C. The main downside to salt extraction is that cellular modifying enzymes and proteases can remain active for longer than in the acid-extraction protocol. It is therefore crucial that protease inhibitors, phosphatase inhibitors and histone deacetylase inhibitors are included in the extraction buffers if analysis of modifications is desired.
2. Spin in 4 °C cooled microcentrifuge: 6,500*g*, 5 min.
3. Remove supernatant (the cytoplasm) completely and carefully with a 1-ml pipette.
4. Wash pellet (the nuclei) in 1 ml extraction buffer (without NP40) by re-suspending the pellet and incubating on ice for 1 min.
5. Spin in 4 °C cooled microcentrifuge: 6,500*g*, 5 min.
6. Completely remove supernatant.
7. Lyse the nuclei by re-suspending the nuclei pellet in 1 ml no-salt buffer and vortexing intermittently for 1 min (10 s on, 10 s off).
8. Incubate on a rotator at 4 °C for 30 min.
9. Spin and pellet chromatin and nuclear debris in microcentrifuge: 6,500*g*, 5 min.
10. Remove supernatant containing the nucleoplasm and save if analysis of unincorporated histones is desired.
Note: The chromatin pellet containing the DNA and histones should appear somewhat glassy.
11. Re-suspend the chromatin pellet in 400 µl of High-Salt Solubilization buffer and vortex for 2 min.
▲ CRITICAL STEP Chromatin should be re-suspended very well, with no clumps left. The solublization buffer volume can be increased to improve the final yield; however, the resulting solution of histones will be more dilute.
12. Incubate on rotator at 4 °C for 30 min.
13. Spin and pellet DNA and nuclear debris in microcentrifuge: 16,000*g*, 10 min.
14. Cut 5 cm of 3,500 MWCO dialysis tubing, pre-wet and close on one end with clamp. Transfer supernatant containing extracted histones into dialysis tubing.
▲ CRITICAL STEP Ensure that there is adequate empty space in the tubing for a two- to threefold increase in volume of the solution after dialysis. Clamp off the top of the tubing, such that there are a few centimeters' slack of tubing past the clamp for ease of removal later, and put into a 1-l beaker with dialysis solution in cold room with stir bar. Stir slowly, with modest movement of the tubing, for 1 h.
15. *Change dialysis solution*: Remove tubing with gloved hand on clamp, discard solution and add remaining 1 l of fresh dialysis buffer. Stir for 1 h.
▲ CRITICAL STEP 2 h of dialysis should bring the histone solution to 100–200 mM NaCl. If lower salt concentrations are required, continue dialysis for longer time with additional buffer changes.
? TROUBLESHOOTING Some protein may precipitate after rapid dialysis against no salt. Histones remain in solution, but it is possible that some histone protein will be lost. If precipitation occurs, a step-dialysis against 1, 0.5 and 0.2 M NaCl may be performed to limit protein precipitation owing to rapid change in ionic strength.
16. Remove solution from dialysis tubing carefully: First, put a 15-ml conical tube on ice with the cap off. Remove dialysis tubing from flask and carefully open one clamp. Insert top end of tubing into conical tube and squeeze out entire solution with gloved hands.
■ PAUSE POINT Histone solutions can be stored frozen at −20 °C or −80 °C.
17. See Step 22 of the acid-extraction protocol for SDS–polyacrylamide gel electrophoresis analysis of recovered histones.

BOX 2 | REVERSED-PHASE HPLC (RP-HPLC)

- Prepare solvents as described in the REAGENT SETUP. Prime lines and pumps of HPLC according to the manufacturer's instructions. Set the UV detector to 214 nm.
- Equilibrate the column in Buffer A for 30 min at 0.8 ml min^{-1}.
- Wash the column with a short programmed gradient, from 0 to 100% B over 20 min and then re-equilibrate in 0% B for 10 min. Watch the chromatogram: if any peaks of higher than 0.05 mAU appear, continue wash runs until column is cleaned.
- Set up a method with the buffer conditions in **Table 1**, at a flow rate of 0.8 ml min^{-1} (see **Fig. 4** for a plot of the gradient program).
- Program fraction collector to collect 1-min fractions (0.8 ml), either from time 0 min (including H1 histones) or from time 40 min (just the core histone fractions). Fraction collection can be stopped at 80 min.
- If it is necessary to increase the separation between histone peaks, then increase the duration of that section of the gradient to make it shallower. For instance, the 'core histone separation' section of the program can be increased from 75 to 150 min, with each later section of the time program correspondingly changed.
- At the end of the chromatographic run wash the column using the wash program as above twice, or until the baseline absorbance at 214 nm does not have noticeable contamination (i.e., no peaks above 0.05–0.1 AU).
- Continue with another run or switch and prime the solvent lines and pumps to Solvent C for storage.
- Equilibrate the column in Solvent C for 15 min at 0.8 ml min^{-1} and shut the system down according to the manufacturer's instructions.

TABLE 1 | Buffer conditions for reversed-phase HPLC (RP-HPLC) separation of histones.

Time (min)	Solvent B (%)	Step
0–5	0	Injection
5–15	0 (to 35)	Ramp-up
15–25	35	Non-histones and H1s
25–100	35 (to 65)	Core histone separation
100–120	65 (to 100)	Typically no proteins (wash)
120–130	100	Wash
130–135	100 (to 0)	Wash
135–145	0	Wash

■ **PAUSE POINT** Histone fractions can be stored frozen at −80 °C.

28| Run 5 µl of each re-dissolved fraction corresponding to peaks on the chromatogram on a 15% SDS-PAGE gel and Coomassie stain to determine the abundance of individual histones (**Fig. 4**).
? TROUBLESHOOTING

29| Continue with MS or other analysis.

AU/TAU gel electrophoresis ● TIMING
Long AU gel: 2 d; short AU/TAU gel: 2 h
30| *Pour separating gel* ● **TIMING** 2–3 h (long AU gel) or 1 h (short AU/TAU gel). Set up gel plates in gel-pouring apparatus.

31| Mix components of separating gel except for TEMED and 10% APS according to **Table 2**. Stir until urea is completely dissolved. Do not apply heat to dissolve urea. For long AU gels, degas the solution under vacuum to prevent the formation of air bubbles during polymerization. This step is not necessary for small gels.

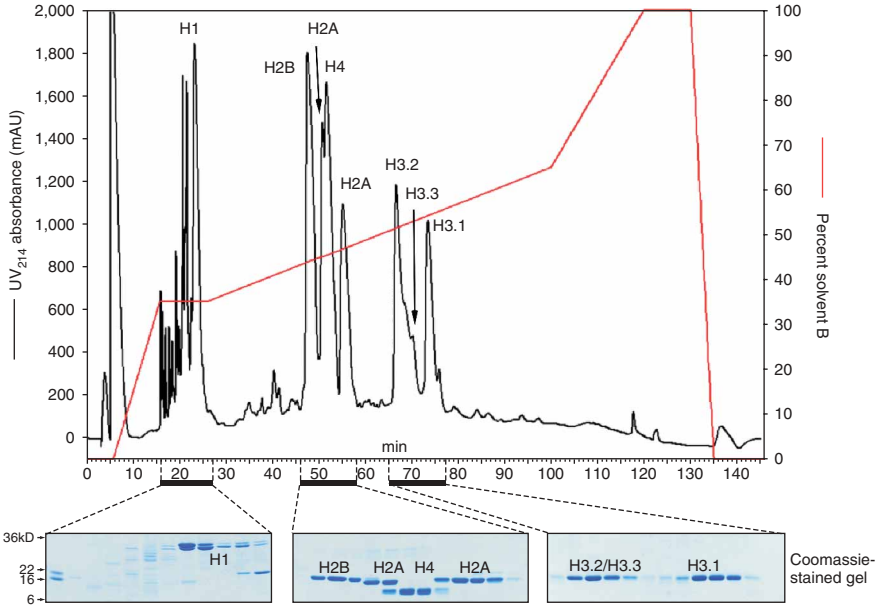

Figure 4 | Chromatogram and Coomassie-stained gels from reversed-phase HPLC (RP-HPLC) separation of histones. Histones from HEK293 cells were separated on a C8 reversed-phase column in a ddH_2O/acetonitrile gradient. The chromatogram shows the retention of proteins (black line) on the column over the course of the acetonitrile gradient program (red line). Peak fractions were lyophilized and a small sample of each re-dissolved fraction was run on an SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel and Coomassie stained. The Coomassie-stained gels correspond to the eluted fraction range as depicted in the Figure. The locations of the various histone proteins as identified by mass spectroscopy are noted on the chromatogram and the gel.

TABLE 2 | Components of separating gel (15% 60%:0.4% acryl:bisacrylamide/6 M urea/5% acetic acid).

Component	Long AU gel (70 ml)	Short AU gel (10 ml)	Short TAU gel (10 ml)
Urea (g)	25.2	3.6	3.6
60%:0.4% acrylamide:bisacrylamide (ml)	17.5	2.5	2.5
Glacial acetic acid	3.5 ml	500 µl	500 µl
dH_2O (ml)	28.7	4.1	3.73
10% Triton X-100	—	—	370 µl
N,N,N′,N′-tetramethylethylenediamine (µl)	420	60	60
10% APS (µl)	980	140	140

▲ **CRITICAL** All components of AU/TAU gels that contain urea (gel solutions and sample buffer) have to be prepared freshly each time, because the rapid degradation of urea at any pH above 8 leads to rapid carbamylation modification of histones.

32| Add TEMED and 10% APS and mix gently to start polymerization.

33| Pour separating gel. For long AU gels pour to 3 cm below top of plate. Overlay with ddH_2O and allow 1 h to polymerize. For short AU/TAU gels pour gel to the top, insert comb and allow to polymerize for 20–30 min.
Note: For preparation of long AU gels continue with Step 34. For short AU/TAU gels continue at Step 45.

34| *Pour stacking gel (long AU gels only)* ● **TIMING** 2–3 h. Pour off ddH_2O from the top of the polymerized gel. Residual ddH_2O may be removed by absorbing it with a Whatman 3 MM paper.

35| Mix components of stacking gel except for TEMED and 10% APS according to the table below. Stir until urea is completely dissolved.

Component	Long AU gel (5 ml)
Urea	1.8 g
60%:0.4% acrylamide:bisacrylamide	0.5 ml
Glacial acetic acid	250 µl
dH_2O	2.8 ml
N,N,N′,N′-tetramethylethylenediamine	30 µl
10% ammonium persulfate	70 µl

36| Add TEMED and 10% APS to start polymerization. Pour stacking gel to 1.5 cm below the edge of the plate, overlay with ddH_2O and allow to polymerize for at least 2 h.

37| *Pre-run gel* ● **TIMING** 8–10 h. Pre-running the gel with AU sample buffer removes residual radicals from the polymerization process, which might otherwise react with proteins of the sample and alter their migration characteristics. Set up gel in running chamber (running buffer: 5% AcOH). Use a syringe to rinse the top of the gel and wash away residual unpolymerized acrylamide.

38| Load 500 µl of AU sample buffer (prepared as shown below) on top of the stacking gel.

Component	
Urea	0.36 g
0.2% Pyronin Y	100 µl
Glacial acetic acid	50 µl
Protamine sulfate[a] (25 mg ml^{-1})	500 µl

[a]Protamine, a small, highly cationic protein, serves as a scavenger for radicals during pre-running and as a blocking agent to reduce non-specific association of proteins with the gel matrix during the actual run of the sample.

39| Pre-run the gel at 300 V overnight (6–12 h).
▲ **CRITICAL STEP** Histones are positively charged under the acidic running conditions of AU gel electrophoresis and thus, unlike in SDS-PAGE gels, run toward the *negative* end. It is therefore crucial to switch electrode leads.
? TROUBLESHOOTING

40| Remove gel from running chamber.

41| *Pour loading wells* ● **TIMING** 1–2 h. Mix components of gel for loading wells (according to the table below) except for TEMED and 10% APS. Vortex until urea is dissolved.

Component	Long AU gel (5 ml)
Urea	1.8 g
60%:0.4% acrylamide:bisacrylamide	1.25 ml
Glacial acetic acid	250 μl
dH_2O	2.05 ml
N,N,N′,N′-tetramethylethylenediamine	60 μl
10% ammonium persulfate	140 μl

42| Add TEMED and 10% APS to initiate polymerization.

43| Pour acrylamide solution up close to the edge of the plate. Insert comb.
▲ **CRITICAL STEP** For long AU gels, the comb must be inserted deep enough that the teeth of the comb are pushed approximately 1 mm into the stacking gel.
? TROUBLESHOOTING

44| Allow gel to polymerize for 1 h.

Preparation of samples and running the gel

45| *Sample preparation* ● **TIMING** 0.5 h. Dry samples down to protein pellet in SpeedVac.
Note: Since the differently charged isoforms of histones are separated, it is necessary to load more sample per lane than for SDS-PAGE.

46| Prepare AU sample buffer by mixing the components given shown below and vortexing until urea is dissolved.

Component	
Urea	0.36 g
0.2% Pyronin Y	100 μl
Glacial acetic acid	50 μl
Protamine sulfate[a] (25 mg ml^{-1})	500 μl

[a]Protamine, a small, highly cationic protein, serves as a scavenger for radicals during pre-running and as a blocking agent to reduce non-specific association of proteins with the gel matrix during the actual run of the sample.

47| Dissolve samples in 10 μl (long AU gels) or 5 μl (short AU/TAU gels) AU sample buffer.
▲ **CRITICAL STEP** Do not boil the samples, as this will cause the urea to react covalently with the histone amines (carbamylation), and thereby change the migration behavior of the proteins.

48| *Run the gel* ● **TIMING** Long AU gels: 0.5 h handling, 30 h running. Short AU or TAU gels: 0.5 h handling, 0.5–1.5 h running. Transfer gel into running chamber (running buffer: 5% AcOH).

49| Remove comb.
▲ **CRITICAL STEP** If the 'fingers' between the wells are displaced from the stacking gel during comb removal, reposition them (we use a thin spatula for this); otherwise the sample will leak between loading wells.

50| Right before sample loading, flush wells with a syringe to remove excess urea and unpolymerized acrylamide.

51| Load samples.
▲ **CRITICAL STEP** Put sample buffer in lanes without sample. For 2D TAU gels leave one lane empty (i.e., add no sample buffer) between samples in order to later identify sample lane.

52| For long AU gels, run samples into stacking gel at 200 V for 1–2 h, then run gel at 400 V for another 30–32 h (at this point, mammalian histone H4 will be close to the bottom of the gel, see **Fig. 6**). For short AU/TAU gels, run samples for 0.5–1.5 h at 200 V.
▲ **CRITICAL STEP** Histones are positively charged under the acidic running conditions of AU/TAU gels. Therefore, unlike SDS-PAGE gels, the proteins run toward the negative end. Remember to switch electrode leads.
? TROUBLESHOOTING

53| Disassemble gel after the run and mark gel for orientation. AU/TAU gels can be stained with Coomassie Brilliant Blue solution or by silver staining. Alternatively, the gel can be transferred to a nitrocellulose or PVDF membrane for western blotting (see protocol in **Box 3**). The gel can also be run in a second dimension to further differentiate the histone isoforms (2D TAU, proceed with Steps 54–66).

BOX 3 | WESTERN BLOTTING OF AU/TAU GELS

Preparation of acid urea/triton acid urea gel for transfer ● TIMING Day 1, 20–30 min.

1. Disassemble acid urea/triton acid urea (AU/TAU) gel and mark for orientation.
2. Transfer gel into plastic container filled with 0.7% AcOH (transfer buffer) and incubate, slowly rocking, for 15 min (note that the gel will expand in this buffer).
3. Prepare Polyvinylidene difluoride (PVDF) membrane by cutting it to the size of the expanded gel and soak it for 30 s in 100% methanol. Then wash membrane for 1 min with ddH_2O and incubate it for 15 min in transfer buffer.
4. Cut four Whatman papers to size of the gel and the PVDF membrane.
5. Assemble gel, PVDF membrane and Whatman paper to a 'transfer sandwich' as follows: soak transfer sponge in transfer buffer and place on transfer screen. Then soak two pieces of Whatman paper in transfer buffer and place on sponge, followed by the PVDF membrane and the gel. Avoid the incorporation of air between the different layers. Then place two more Whatman papers and one sponge, soaked in transfer buffer, on the gel and close the transfer screen. Insert transfer screen in transfer chamber and fill up with transfer buffer.

Transfer of histones from AU or TAU gel onto PVDF membrane ● TIMING Day 1, 60 min.

6. Transfer the histone proteins from the AU or TAU gel onto the PVDF membrane at 500 mA for 20 min.

▲ CRITICAL STEP Histones are positively charged in the acidic buffer used to transfer AU/TAU gels. Therefore, unlike in transfers of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels, they migrate towards the negative pole. Remember to switch electrode leads! Note that increased transfer times (or current) may be required to efficiently transfer proteins from TAU versus AU gels. In addition, various pore sizes of PVDF membranes are available commercially, which can dramatically influence the retention of small (e.g., histones) versus larger polypeptides (we typically use PVDF membranes with 0.45-μm pore size).

? TROUBLESHOOTING If problems are encountered while transferring AU/TAU gels, one recommendation is to blot to two (or more) sheets of membrane, followed by staining, to determine whether retention is a significant problem. Staining of the gel, following transfer, enables one to judge whether a reasonable amount of protein has been transferred from the gel to the membrane. The Triton detergent in TAU gels frequently reduces the migration of proteins out of the gel, so increased transfer times may be required.

7. Disassemble 'transfer sandwich' and place PVDF membrane in plastic container with Ponceau-S solution or stain with Amido black to control for proper transfer of proteins and proceed to blocking and staining of the membrane with antibodies of choice as for a typical immunoblot.

2D TAU/SDS gel ● TIMING Approximately 3 h

54| 2D TAU gels can be run with a homemade SDS-PAGE gel as second dimension (**Fig. 5**) or with a pre-cast commercial gel with a '2D' well, such as the NuPAGE 10–20% SDS gel (Invitrogen, cat. no. EC6136). If you are using the pre-cast second-dimension gel, skip to Step 58.

55| *Preparation of 15% SDS-PAGE gels* ● TIMING 1 h. Clean glass plates and assemble using standard recipes.

▲ CRITICAL STEP Use wider spacers to obtain a thicker SDS-PAGE gel than the first-dimension TAU gel, which makes transfer of the TAU gel slice on top of it easier.

56| Carefully mix all components for the separation gel, avoiding bubble formation, and pour acrylamide to a level of 4 cm below the top of the short plate, overlay with ddH_2O or ethanol and let polymerize for 20 min.

57| Discard ddH_2O and carefully mix all components for the stacking gel. Pour acrylamide to a level of 2 cm below the top of the short plate, overlay with ddH_2O and let polymerize for 20 min.

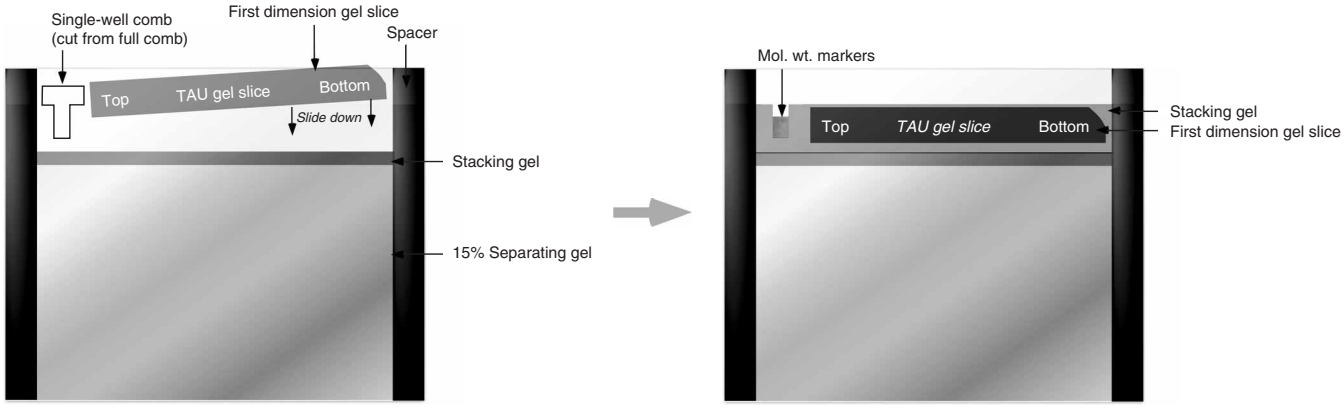

Figure 5 | Assembly of a 2D triton acid urea (TAU) gel. A 15% SDS-separating gel is poured in a standard minigel apparatus; alternatively, a commercial pre-cast 2D gel can be used. After soaking it in a neutralization buffer, the first-dimension TAU gel slice is placed on its side and inserted into the top of the SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel. A notch should be placed on one corner of the TAU gel slice to ensure correct orientation of the top and bottom of the first-dimension gel. Stacking gel can be polymerized around the gel slice, or alternatively the slice can be overlaid with 1× Laemmli (SDS) loading buffer. Then the gel is run under standard SDS/Tris–Gly conditions.

58| *Preparation of TAU gel lanes for 2D separation* ● **TIMING** Day 1, 1 h. After the TAU separation of the histone samples is complete (see Step 53), cut out the lanes where the sample was run.
▲ **CRITICAL STEP** Sample lanes can be identified by the faint red color of the loading buffer. Use fresh razor blades to cut out the sample lane. Also, cut out a small corner from the bottom of the TAU gel slice to identify top and bottom of gel slice before assembly.

59| Transfer TAU sample lanes carefully to small plastic containers (a pipette-tip box lid works well) filled with 0.125 M Tris–Cl, pH 8.8, and rock slowly for 5 min on rocking platform. Replace three times with fresh 0.125 M Tris–Cl, pH 8.8 solution.
▲ **CRITICAL STEP** By equilibrating the gel slices in Tris–Cl buffer, the acid in the gel will be neutralized.

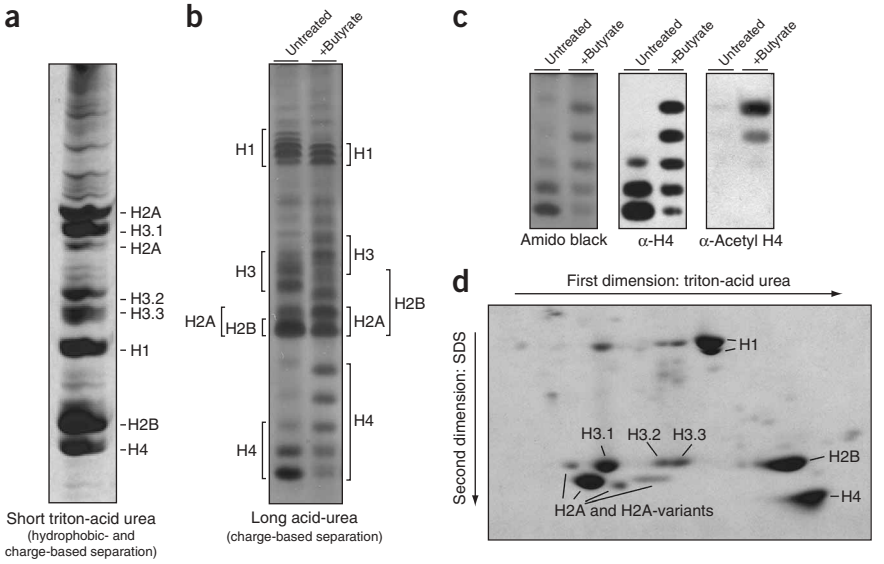

Figure 6 | Examples of triton acid urea (TAU), AU and 2D TAU gels. (**a**) Acid-extracted mammalian histones were separated on a short TAU gel and silver stained. The locations of the major forms of the core histones are noted. (**b**) Acid-extracted histones from untreated (lane 1) and sodium butyrate (deacetylase inhibitor)–treated (lane 2) HeLa cells were separated on a long AU gel, transferred to a polyvinylidene difluoride (PVDF) membrane and stained with Amido black. The regions where core histone isoforms with different charge migrate are noted. (**c**) A long AU gel run with histones from untreated (lane 1) and sodium butyrate–treated (lane 2) HeLa cells was transferred to PVDF and stained with Amido black. The membrane was immunoblotted with antibodies against histone H4 and penta-acetylated H4. Note the correlation between the slower-migrating H4 isoforms and the specific recognition of acetylation by the acetyl-H4 antibody. (**d**) Acid-extracted mammalian histones were first run on a short TAU gel and then run in a second dimension on a standard SDS gel. The locations of histone isoforms are noted on the gel. **Figure 6d** reproduced from the *Journal of Biological Chemistry* with permission of the authors[18].

60| Carefully transfer the gel slice horizontally between the glass plates (see depiction in **Fig. 5**). Leave some room to the left and right of the glass plate and to the separating gel.
▲ **CRITICAL STEP** It is tricky to place the gel slice in between the glass plates without breaking it. Therefore, ensure that the second-dimension gel is thicker than the first-dimension gel (i.e., 1-mm-thick SDS gel for a 0.75-mm-thick TAU gel). Try to transfer it on the top of the glass plate and then gently push the gel slice with both thumbs or a metal spatula down between the two glass plates by applying soft pressure to different parts of the gel slice. Start pushing on one end of the gel slice and move on to the other end of the gel slice. Repeat these steps until the gel slice is pushed in between the glass plates. Prepare 2 ml of fresh stacking gel and pour it very carefully between the glass plates, thereby covering the TAU gel slice. To avoid bubbles, hold the glass plates at an angle and pour in more acrylamide solution to flush out bubbles. The TAU gel should be completely covered in stacking gel.
? TROUBLESHOOTING

61| (Only for 'homemade' gels; skip to Step 65 if using a pre-cast gel). Insert 'one-finger' comb into one side of the gel before it polymerizes. This well will be used to load a protein marker in order to later identify the molecular weight of the proteins and to determine the appropriate protein running distance (cross-reference with histone SDS gel in **Fig. 3**).

62| Let the gel polymerize for 30 min.

63| Fit assembled 2D TAU gel into chamber and fill carefully with Tris–Gly SDS running buffer without disturbing the TAU gel slice.

64| Remove comb finger carefully and flush out marker well with Tris–Gly SDS running buffer to remove bubbles and non-polymerized acrylamide.

65| Load protein molecular weight marker in marker well.

66| Run the gel as usual using Tris–Gly SDS running buffer.
Note: Disassemble glass plates, remove TAU gel slice and stacking gel, and place separating gel carefully in plastic container filled with Coomassie Brilliant Blue solution. Alternatively silver-stain gel or proceed to transfer (regular SDS-PAGE transfer protocols) to perform western blotting techniques.

● TIMING

Coomassie staining and destaining: 4 h,
Silver staining: 1 h, and
Transfer: 1 h

? TROUBLESHOOTING

Step 22 (acid extraction)

If histones are contaminated with a large background of other cellular proteins visible on a Coomassie-stained gel, it is possible that the hypotonic lysis was not completely effective. In that case, we recommend that the lysis buffer volume be increased and a Dounce homogenizer be used after initial hypotonic swelling. The cells should also be freeze-thawed before lysis.

Step 28 (RP-HPLC)

If poor separation is encountered between histone proteins, first ensure that TFA was added to the solvents, as low pH ensures sharp peaks. H2A and H4 frequently have somewhat overlapping retention on C8 columns, so those eluted fractions can be re-injected onto the C8 column and run with a shallower gradient ramp to increase the separation.

Step 39 (AU/TAU gels)

Note that the ability of Triton X-100 detergents to bind to hydrophobic regions of the proteins is strongly influenced by the oxidation state of the proteins. If separation of histones by TAU gel electrophoresis is compromised by high levels of histone oxidation (i.e., smearing and poor band resolution), additional pre-running steps with 2.5 M cysteamine can be applied to 'scavenge' oxidative free radicals from the gel[29]. For an additional, discontinuous AU/TAU gel system that systematically incorporates anti-oxidation practices, see ref. 30.

Step 43 (AU/TAU gels)

Use freshly prepared 10% APS to ensure good polymerization. Keep the 60% acrylamide stock in an amber bottle away from light to prevent degradation of the stock.

Step 52 (AU/TAU gels)

It can be difficult to determine the extent of migration of histone proteins through AU and TAU gels without prior experience. Five to ten micrograms of cytochrome *c* protein can be used as a marker, as it appears brownish while run. It typically migrates near histone H4. However, it tends to become diffuse and difficult to see as the gel is run, so hold a white paper behind the gel to visualize it.

Step 60 (2D TAU gels)

When using precise SDS gels for the second dimension, an alternative to casting the first-dimension gel slice in stacking gel and allowing it to polymerize is just to layer 1× SDS (Laemmli) loading buffer, as the pre-cast gels do not require a stack.

ANTICIPATED RESULTS

Acid- or high-salt extraction of histones from nuclei or chromatin typically results in a tremendous enrichment of histone proteins. Histones are very abundant proteins, usually present in cells in equal mass with the DNA[6]. Investigators following the protocols presented should be able to routinely isolate quantities of purified histones for many follow-up experiments, including immunoblotting and MS.

Figure 3 illustrates a Coomassie-stained SDS-PAGE gel showing what is expected from acid or salt extractions of human nuclei. Notice that most of the proteins present are core histone proteins, running in a banding pattern typical for histones (between 11 and 17 kDa). Many isoforms of histone H1 proteins run between 25 and 35 kDa. Also notice that in the high-salt extraction more high molecular weight proteins are apparent. These are likely chromatin-associated proteins that are acid-insoluble, so they only appear when extracted in neutral conditions. Note that histones from different species and different cell types may have somewhat different banding patterns, but there are usually three discrete bands of H2A, H2B and H3 with a lower H4 band.

Figure 6 demonstrates the relative benefits of the TAU gel and the long AU gel. Histones run on a short TAU or AU gel will not exhibit the same pronounced separation of charge-dependent isoforms that the long AU gel will. But the short gel can be turned on its side and run in a second dimension with an SDS-PAGE gel, separating the proteins on the basis of size, which allows explicit identification of protein variants. Furthermore, the non-ionic detergent Triton X-100 (and other such non-ionic detergents in this series) binds to hydrophobic residues and retards the mobility of certain histones (in particular, histones in the H2A and H3 families). Note that the ability of these detergents to bind to hydrophobic regions of the proteins is strongly influenced by the oxidation state of the proteins. Thus, efforts are needed to keep the histone samples reduced by incubation with charged and neutral reducing agents, including the addition of cysteamine to the AU/TAU running buffers

('scavenging' oxidative free radicals) (see TROUBLESHOOTING tip for Step 39 for more details). The long AU gel, while more time-consuming to run, allows for unambiguous separation of acetylated- or phosphorylated-histone proteins.

Finally, **Figure 4** shows a typical HPLC purification of acid-extracted histones. The chromatogram and stained gels show how much separation can be expected between each histone.

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