# Chapter 2

# Characterization of Posttranslational Modifications on Histone Variants

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## Abstract

The study of histone variants and histone posttranslational modifications (PTMs) is a trending topic in different fields including developmental biology, neurobiology, and immunology; as well as in the understanding of molecular mechanisms leading to diverse diseases, such as cancer. Since the establishment of histone PTMs starts immediately after their synthesis and it continues once they are assembled into chromatin, here we describe a classic protocol of subcellular fractionation aiming to study histones at different stages of maturation. This includes newly synthesized histones enriched in cytosolic fractions; a pool of newly synthesized, evicted, and stored histones enriched in nuclear soluble fractions; and chromatin-associated histones enriched in chromatin pellet. To study specific histone variants and the establishment of their PTMs, we describe a protocol for obtaining histone variants expressed in bacteria. In addition, we describe a Triton-Acetic acid-Urea (TAU) gel electrophoresis protocol adapted to work on mini-gels, which can be coupled to Western blot to analyze PTMs on histone variants. Finally, we describe a Chromatin immunoprecipitation (ChIP) assay for studying histone PTMs, or tagged histone variants, on specific DNA sequences.

**Key words** Histones, Histone variants, Recombinant histones, Histone purification, PTMs, TAU gel electrophoresis, Acid extraction, ChIP

## 1 Introduction

Histones are proteins that, together with DNA, structure the chromatin inside the nucleus of eukaryotic cells. They have low molecular weights, between 10 and 16 kDa, high proportion of lysines and arginines in their amino acid sequences and isoelectric points of around pH 10 [1, 2]. Consequently, histones have a positive net charge under physiological conditions, which makes them highly affine to DNA. Although the main function of his-

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tones is to compact the DNA, they are far from being just DNA packaging proteins. A growing variety of histone Posttranslational Modifications (PTMs) have been described, which are capable of directly impacting into chromatin structure by altering histone-DNA interactions or by acting as an assembly point for different protein machineries. Thus, histone PTMs influence all the functions where DNA is involved, including gene expression, some being correlated with gene activation and others with silencing [3, 4]. Histone variants add another level of complexity to the chromatin structure. They are defined as histones whose expression is not linked to the cell cycle progression, unlike canonical histones H3, H4, H2A, and H2B, which are synthesized coupled to DNA replication [5–7]. Histone variants have diverse roles; for instance, H3.3 regulates gene transcription and silencing [8]; phosphorylated H2A.X participates in the response to DNA double-strand break [9].

Here, we describe a variety of techniques for the study of the different pools of histones that are found inside the cell. These pools include histones bound to chromatin, called nucleosomal histones, and diverse non-nucleosomal histones, called soluble histones. Among the soluble pool, nuclear histones are composed of evicted, stored, and newly synthesized histones, while the cytosolic population is enriched on newly synthesized histones [10, 11]. We describe a subcellular fractionation method, based in Dignam et al. [12] that isolates cytosolic extract, nuclear extract, and nuclear pellet from HeLa cells. In addition, this fractionation method can be coupled to acid extraction to obtain histone-enriched samples by exploiting their basic character [1]. These isolation methodologies allow recovering histone pools for the analyses of PTMs and histone variants in different cellular reservoirs by classic and simple assays, like Western blot, taking advantage of the extensive variety of commercially available antibodies.

Furthermore, we describe a method to obtain high amounts of recombinant histones by using bacterial expression vectors [13]. This gives the possibility of expressing the desired histone variant without PTMs, and therefore, being suitable for different assays such as the evaluation of enzymatic activities and specificities related to the establishment of PTMs. Moreover, we describe a technique to separate histone variants based on the changes in the amino acidic composition between variants and canonical histones by using TAU (Triton X-100, Acetic acid, Urea) gel electrophoresis [14]. When this protocol is coupled to Western blot assays, PTM analyses on histones variants are greatly facilitated. Finally, we describe a ChIP (Chromatin Immunoprecipitation) methodology to study PTMs and histone variants on specific DNA sequences.

## 2 Materials

Prepare all solutions using distilled water and analytical grade reagents. Prepare and store all stock solutions at room temperature, unless otherwise indicated, up to a month. When handling irritant or volatile compounds, like glacial Acetic acid, work under fume hood. Prepare all working solutions the day before use and keep them at 4 °C, unless otherwise indicated. Add PMSF and DTT to the working solutions at the moment of use (*see* **Note 1**). DTT can be replaced by β-Mercaptoethanol (*see* **Note 2**). Cell culture solutions must be handled in sterility conditions and stored at 4 °C.

### 2.1 Stock Solutions

1. 2.5 M KCl. Dissolve 186.38 g of Potassium chloride in distilled water to a final volume of 1000 mL.
2. 1 M TRIS pH 7.9. Dissolve 121.14 g of Tris(hydroxymethyl) aminomethane (TRIS) in distilled water to a final volume of 1000 mL, adjusting pH to 7.9 with concentrated HCl.
3. 5 M NaCl. Dissolve 292.20 g of Sodium chloride in distilled water to a final volume of 1000 mL.
4. 1 M $MgCl_2$. Dissolve 4.76 g of Magnesium chloride in distilled water to a final volume of 50 mL.
5. 0.5 M EDTA pH 8.0. Dissolve 73.06 g of Ethylenediaminetetraacetic acid (EDTA) in distilled water to a final volume of 500 mL, adjusting pH to 8.0 with NaOH pellets (*see* **Note 3**).
6. 0.1 M PMSF. Dissolve 0.87 g of Phenylmethanesulfonyl fluoride (PMSF) in isopropanol to a final volume of 50 mL (*see* **Note 4**).
7. 1 M DTT. Dissolve 1.54 g of Dithiothreitol (DTT) in distilled water to a final volume of 10 mL. Store at −20 °C.
8. Antibiotic 1000× (*see* **Note 5**).
9. 1 M IPTG 1000×. Dissolve 2.38 g of Isopropyl β-D-1-thiogalactopyranoside (IPTG) in distilled water to a final volume of 10 mL.
10. Running Buffer 10×: 250 mM TRIS, 2 M Glycine, 1% Sodium Dodecyl Sulfate (SDS). Dissolve 30.3 g of TRIS, 150.1 g of Glycine and 10 g of SDS in distilled water, to a final volume of 1000 mL.
11. T-TBS 10×: 200 mM TRIS pH 7.9, 1.5 M NaCl, 1% Tween 20. Mix 200 mL of 1 M TRIS pH 7.9, 300 mL of 5 M NaCl and 10 mL of 100% Tween 20, adding distilled water to a final volume of 1000 mL.
12. 30% Acrylamide-0.8% Bis-acrylamide. Dissolve 150 g of Acrylamide and 4 g of N, N′-Methylenebis(acrylamide) (Bis-

acrylamide) in distilled water, to a final volume of 500 mL. Keep at 4 °C, protected from light (*see* **Note 6**).

13. Upper Buffer: 0.5 M TRIS pH 6.8, 0.4% SDS. Dissolve 30.3 g of TRIS and 2 g of SDS in distilled water to a final volume of 500 mL, adjusting pH with concentrated HCl. Keep at 4 °C.
14. Lower Buffer: 1.5 M TRIS pH 8.8, 0.4% SDS. Dissolve 90.9 g of TRIS and 2 g of SDS in distilled water to a final volume of 500 mL, adjusting pH with concentrated HCl. Keep at 4 °C.
15. 10% APS. Dissolve 1 g of Ammonium persulfate (APS) with distilled water to a final volume of 10 mL. Make 250 μL aliquots and keep at −20 °C until use. After thawing an aliquot, keep it at 4 °C up to a week.
16. Laemmli Buffer 5×: 312.5 mM TRIS pH 7.9, 10% SDS, 25% glycerol, 5% β-mercaptoethanol, 0.005% Bromophenol Blue [15] . Mix 3.125 mL of 1 M TRIS pH 7.9, 1 g of SDS, 2.5 mL of 100% Glycerol and 0.5 mg of Bromophenol Blue. Dissolve in distilled water to a final volume of 10 mL. Store this buffer at −20 °C in 1.8 mL aliquots. Before use, thaw one aliquot and add 200 μL of 100% β-mercaptoethanol. When β-mercaptoethanol is added, store this buffer at room temperature.
17. 40% Acrylamide-0.67% Bis-acrylamide. Dissolve 100 g of Acrylamide and 1.675 g of Bis-acrylamide in distilled water to a final volume of 250 mL. Keep at 4 °C, protected from light (*see* **Note 6**).
18. 1% Methyl green. Dissolve 0.1 g of Methyl green in distilled water to a final volume of 10 mL. Make 1 mL aliquots and keep at −20 °C.
19. 25 mg/mL Protamine sulfate. Dissolve 0.5 g of Protamine sulfate in distilled water to a final volume of 20 mL. Make 1 mL aliquots and keep at −20 °C.
20. TAU sample Buffer 2×: 6 M Urea, 0.02% Methyl green, 5% Acetic acid, 12.5 mg/mL, 12.5 mg/mL Protamine Sulfate, 10% glycerol. Mix 0.36 g of Urea, 20 μL of 1% Methyl green, 50 μL of glacial Acetic acid, 500 μL of 25 mg/mL Protamine sulfate, 100 μL of 100% glycerol. Add distilled water to a final volume of 1 mL. Keep at −20 °C.
21. 2.5 M LiCl. Dissolve 5.3 g of Lithium chloride in distilled water to a final volume of 50 mL.
22. 2.5 M Glycine. Dissolve 9.4 g of Glycine in distilled water to a final volume of 50 mL.
23. 1 M $NaHCO_3$. Dissolve 4.2 g of Sodium bicarbonate in distilled water to a final volume of 50 mL.

24. 10% Triton X-100. Mix 1 mL of Triton X-100 and 9 mL of distilled water.
25. 10% SDS. Dissolve 1 g of SDS in distilled water to a final volume of 10 mL.
26. 10% Sodium deoxycholate. Dissolve 1 g of Sodium deoxycholate in distilled water to a final volume of 10 mL.
27. 10% NP40. Mix 1 mL of NP40 and 9 mL of distilled water.
28. 0.5 M HEPES pH 7.9. Dissolve 59.6 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in distilled water to a final volume of 500 mL, adjusting pH to 7.9 with concentrated HCl.

### 2.2 Working Solutions and Buffers

1. Buffer A. Mix 5 mL of 1 M TRIS pH 7.9, 750 μL of 1 M $MgCl_2$ and 2 mL of 2.5 M KCl. Add distilled water to a final volume of 500 mL and keep at 4 °C. At the moment of use, prepare an aliquot of 10 mL and add 5 μL of 1 M DTT and 20 μL of 0.1 M PMSF.
2. Buffer B 10×. Mix 30 mL of 1 M TRIS pH 7.9, 56 mL of 2.5 M KCl and 3 mL of 1 M $MgCl_2$. Add distilled water to a final volume of 100 mL. Keep at 4 °C.
3. Buffer C. Mix 10 mL of 1 M TRIS pH 7.9, 750 μL of 1 M $MgCl_2$, 42 mL of 5 M NaCl, 200 μL of 0.5 M EDTA and 125 mL of 100% glycerol. Add distilled water to a final volume of 500 mL. At the moment of use, prepare an aliquot of 10 mL and add 5 μL of 1 M DTT and 50 μL of 0.1 M PMSF. Keep at 4 °C.
4. BC50 Buffer. Mix 20 mL of 1 M TRIS pH 7.9, 20 mL of 2.5 M KCl, 400 μL of 0.5 M EDTA and 200 mL of 100% glycerol. Add distilled water to a final volume of 1000 mL. At the moment of use, add 500 μL of 1 M DTT (or 700 μL of 100% β-mercaptoethanol) and 2 mL of 0.1 M PMSF. Keep at 4 °C.
5. Buffer E. Mix 2.5 mL of 1 M TRIS pH 7.9, 250 μL of 1 M $MgCl_2$, 50 μL of 0.5 M EDTA and 12.5 mL of 100% glycerol. Add distilled water to a final volume of 50 mL. At the moment of use, add 25 μL of 1 M DTT and 100 μL of 0.1 M PMSF. Keep at 4 °C.
6. LB (Luria-Bertani) Medium. Mix 10 g of NaCl, 5 g of yeast extract and 10 g of Tryptone. Add distilled water to a final volume of 1000 mL and sterilize by autoclaving. Keep at 4 °C.
7. 0.5 M HCl-10% Glycerol. Mix 420 μL of 37% Hydrochloric acid and 1 mL of 100% glycerol. Add distilled water to a final volume of 10 mL.

8. Inclusion Bodies Wash Buffer. Mix 35 mL of 1 M TRIS pH 7.9, 10 mL of 5 M NaCl and 1 mL of 0.5 M EDTA. Add distilled water to a final volume of 500 mL.
9. Unfolding Buffer. Mix 66.8 g of Guanidium chloride, 2 mL of 1 M TRIS pH 7.9 and 1 mL of 1 M DTT. Add distilled water to a final volume of 100 mL. Keep at 4 °C.
10. T-TBS 1×. Mix 100 mL of T-TBS 10× and 900 mL of distilled water.
11. Running Buffer 1×. Mix 100 mL of Running Buffer 10× and 900 mL of distilled water.
12. Transfer Buffer. Mix 200 mL of 100% methanol, 100 mL of Running Buffer 10× and 700 mL of distilled water.
13. Red Ponceau staining solution: 0.1% Ponceau salt, 5% Acetic acid. Mix 0.5 g of Ponceau salt and 25 mL of glacial Acetic acid. Add distilled water to a final volume of 500 mL.
14. Blocking Buffers. To prepare T-TBS 5% Milk, dissolve 2.5 g of skim milk in T-TBS 1× to a final volume of 50 mL. To prepare T-TBS 1% BSA, dissolve 0.5 g of lyophilized Bovine Serum Albumin in T-TBS 1× to a final volume of 50 mL (*see* **Note 7**).
15. Laemmli Buffer 1×. Mix 200 µL of Laemmli Buffer 5× and 800 µL of distilled water.
16. TAU Running Buffer: 5% Acetic acid. Dilute 50 mL of glacial Acetic acid in distilled water to a final volume of 1000 mL.
17. TAU gel Wash Buffer 1: 0.5% SDS, 50 mM Acetic acid. Dissolve 2.5 g of SDS in 1.45 mL of glacial Acetic acid and add distilled water to a final volume of 250 mL.
18. TAU gel Wash Buffer 2: 62.5 mM TRIS pH 6.8, 2% SDS, 5% β-mercaptoethanol. Dissolve 1.9 g of TRIS and 5 g of SDS in distilled water adding 12.5 mL of β-mercaptoethanol, and adjusting pH to 6.8 with concentrated HCl, to a final volume of 250 mL.
19. Cell Lysis Buffer. Mix 500 µL of 1 M TRIS pH 7.9, 100 µL of 5 M NaCl and 1 mL of 10% NP40. Add distilled water to a final volume of 50 mL. Filter with a 0.2 µm filter. Add protease inhibitors cocktail 10× to final concentration of 1× at the moment of use (*see* **Note 8**). Keep at 4 °C.
20. Sonication Buffer. Mix 5 mL of 0.5 M HEPES pH 7.9, 1.4 mL of 5 M NaCl, 100 µL of 0.5 M EDTA, 5 mL of 10% Triton X-100, 500 µL of 10% Sodium deoxycholate and 500 µL of 10% SDS. Add distilled water to a final volume of 50 mL. Filter with a 0.2 µm filter.
21. Elution Buffer. Mix 500 µL of 1 M $NaHCO_3$ and 1 mL of 10% SDS. Add distilled water to a final volume of 10 mL. Filter

with a 0.2 μm filter. This solution must be prepared at the moment of use.

22. IP Wash Buffer. Mix 10 mL of 2.5 M LiCl, 5 mL of 1 M TRIS pH 7.9, 5 mL of 10% NP40 and 500 μL of 10% Sodium deoxycholate. Add distilled water to a final volume of 50 mL. Filter with a 0.2 μm filter.
23. Nuclei Buffer. Mix 2.5 mL of 1 M TRIS pH 7.9, 1 mL of 0.5 M EDTA and 5 mL of 10% SDS. Add distilled water to a final volume of 50 mL. Filter with a 0.2 μm filter. Add protease inhibitors cocktail 10× to final concentration of 1× at the moment of use.
24. TE Buffer. Mix 2.5 mL of 1 M TRIS pH 7.9 and 200 μL of 0.5 M EDTA. Add distilled water to a final volume of 50 mL. Filter with a 0.2 μm filter.

### 2.3 Cell Culture

1. Culture plates.
2. HeLa cells.
3. PBS 1×: Dilute 1 volume of sterile PBS 10× to 1× by adding 9 volumes of sterile water.
4. Culture medium, 10% FBS: DMEM medium high-glucose supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin.
5. 0.01% Trypsin-EDTA: Dilute 1 volume of commercially available 0.05% Trypsin-EDTA by adding 4 volumes of sterile PBS 1×.

### 2.4 Other Reagents, Materials, and Equipment

1. Laboratory consumables/plastic material (centrifuge tubes, serological pipettes, etc.).
2. $CO_2$ incubator for cell culture.
3. Refrigerated microcentrifuge (e.g., Heraeus™ Fresco™ 17 Microcentrifuge from Thermo Fisher Scientific).
4. Dounce tissue grinder with a loose type pestle.
5. Probe type sonicator (E.g., Sonic Dismembrator, Model 120 from *Thermo Fisher Scientific*).
6. Cold acetone (−20 °C).
7. Trichloroacetic acid (TCA).
8. 37% Formaldehyde solution.
9. Bacterial strains for protein expression (E.g., BL21), previously transformed with a plasmid coding for histones (untagged Histone H3 sequence cloned on the vector pET3a [16]).
10. Mini-PROTEAN® Tetra Cell system from *Bio-Rad* or equivalent.
11. Tetra Blotting Module system from *Bio-Rad* or equivalent.

12. Nitrocellulose or PVDF membrane.
13. Electrophoresis power supply.
14. 100% methanol.
15. 100% Tetramethylethylenediamine (TEMED).
16. Lab filter paper, Whatman® type, grade 3 MM.
17. Prestained protein ladder (e.g., PageRuler™ Prestained Protein Ladder, 10–180 kDa from Thermo Fisher Scientific).
18. Primary antibodies against histones or histone PTMs.
19. Secondary antibodies conjugated to Horseradish peroxidase (HRP), directed against the primary antibodies Ig type.
20. Chemiluminescence substrate for the detection of HRP conjugated secondary antibodies (e.g., Pierce™ Enhanced Chemiluminescence (ECL) Western Blotting Substrate from *Thermo Fisher Scientific*).
21. Autoradiography cassettes and films.
22. Urea.
23. 50 μg/μL Cytochrome-C, diluted in distilled water.
24. Glacial Acetic acid.
25. Ethylene Glycol bis(succinimidyl succinate) (EGS).
26. Protease inhibitors (Cocktail).
27. Bovine Serum Albumin.
28. Orbital incubator shaker.
29. Microbiological incubator.
30. SOC medium for *E. coli*.
31. Dialysis tubing of 1 K MWCO (e.g., Spectra/Por®7 Dialysis Membrane, Thomas Scientific).
32. Bradford reagent (e.g., Coomassie Protein Assay Reagent, Thermo Scientific).

## 3 Methods

### *3.1 Isolation of Histones*

#### *3.1.1 Histones from Cytosolic, Nuclear and Chromatin Extracts Derived from HeLa Cells*

As mentioned previously, there are different populations of histones inside the cell and each population is at a different state of maturation. The cellular fractionation protocol described here is based on Dignam et al. [12]. Although we described the protocol for HeLa cells, it can be used with a variety of cells, including human and non-human cell lines, as well as cells from primary culture. For a schematic view of the procedure, *see* Fig. 1.

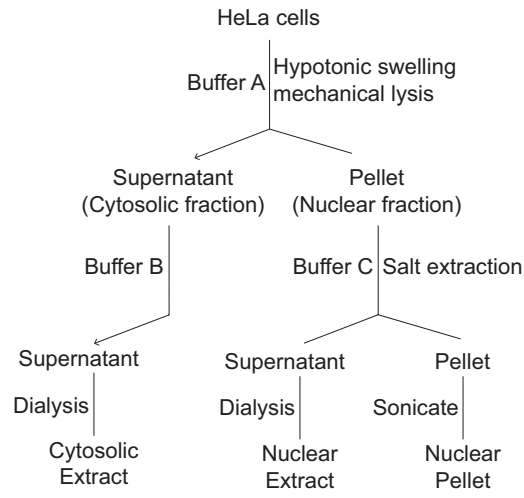


**Fig. 1** Subcellular fractionation scheme. After hypotonic swelling and mechanical lysis of the cells, the homogenate is centrifuged to recover the Cytosolic Extract in the supernatant, and Nuclear and Chromatin Extracts from the pellet. This pellet is salt extracted to obtain Nuclear Extract, while the unsoluble material corresponds to the Nuclear Pellet

*HeLa cell culture*

1. Seed $3 \times 10^6$ cells per 150 mm plates, 24 h before harvesting. To harvest, remove the culture medium by aspiration and wash the cells twice with warm PBS 1× (37 °C).
2. Add 5 mL of warm 0.01% Trypsin-EDTA. Incubate for 7 min, or until the cells detach from the plates, at 37 °C in a cell culture $CO_2$ incubator.
3. Add 5 mL of culture medium supplemented with 10% FBS (*see* **Note 9**). Recover the cells in a centrifuge tube.
4. Centrifuge the cells 8 min at 500 × *g*, at room temperature, and wash them twice with warm PBS 1×.
5. Keep the cell pellet, approximately 50 µL, on ice. Proceed quickly with the subcellular fractionation protocol.

*Subcellular fractionation to obtain cytosolic, nuclear, and chromatin extracts*

1. Add 250 µL of Buffer A to 50 µL of cell pellet, corresponding to five volumes of the cell pellet. Resuspend by a gentle up-and-down pipetting (*see* **Note 10**). Incubate the cells for 10 min on ice.
2. Centrifuge the cells at 10,000 × *g* 10 min, at 4 °C. Remove the supernatant carefully. Add 100 µL of Buffer A, corresponding to two volumes of the initial cell pellet (*see* **Note 11**). Resuspend the cells by gentle up-and-down pipetting. Transfer the cell suspension to the Dounce tissue grinder (*see* **Note 12**).

3. Homogenize the cells 10 times with the Dounce tissue grinder, using the "loose" type pestle (*see* **Note 13**). Transfer the homogenate to a new tube and centrifuge at 10,000 × *g* for 10 min, at 4 °C.
4. Take the supernatant carefully and leave it in a new tube. Keep the remaining pellet for nuclear soluble extraction (**step 7**).
5. Measure the volume of the supernatant recovered from **step 4**. Dilute Buffer B 10× in this supernatant to 1× concentration. In our example, for 200 µL of the recovered supernatant, add 22 µL of Buffer B 10×. Mix well and centrifuge at 13,800 × *g* 1 h, at 4 °C (*see* **Note 14**).
6. Take the supernatant from **step 5** and dialyze against 1000 mL of BC50 Buffer 2 h at 4 °C, using dialysis tubing of 1 K MWCO. Measure protein concentration (Bradford method [17]) and store the *Cytosolic Extract* at −80 °C.
7. To obtain the *Nuclear Extract*, take the pellet from **step 4** and add a volume of Buffer C equal to the volume obtained of C*ytosolic Extract*, in our case, 200 µL. Resuspend the pellet thoroughly by pipetting up-and-down, and incubate for 30 min at 4 °C in continuous agitation.
8. Centrifuge at 13,800 × *g* 30 min at 4 °C. Recover the supernatant carefully and transfer into a new tube, keeping the new pellet for nuclear pellet solubilization (*see* **step 10**).
9. Measure the volume of the supernatant recovered from **step 8** and dialyze against 1000 mL of BC50 Buffer 2 h at 4 °C. Measure protein concentration and store the *Nuclear Extract* at −80 °C.
10. To obtain soluble *Nuclear Pellet*, resuspend the pellet obtained from **step 8** in 200 µL of Buffer E (equal to the volume obtained from Cytosolic and Nuclear Extracts). Sonicate the pellet by three pulses of 30 s each time, 60% amplitude, or until complete solubilization. Store the *Nuclear Pellet* at −80 °C.

Western blot analyses using samples derived from cytosolic, nuclear, and chromatin extracts are shown in Fig. 2.

*Acid extraction of histones*

1. Measure the sample volume to use for the acid extraction (e.g., 200 µL of *Nuclear Pellet* obtained from the subcellular fractionation, **step 10**). Add one volume (200 µL) of 0.5 M HCl-10% Glycerol and resuspend thoroughly by pipetting up and down.
2. Incubate the sample with rotation for 1.5 h at 4 °C.

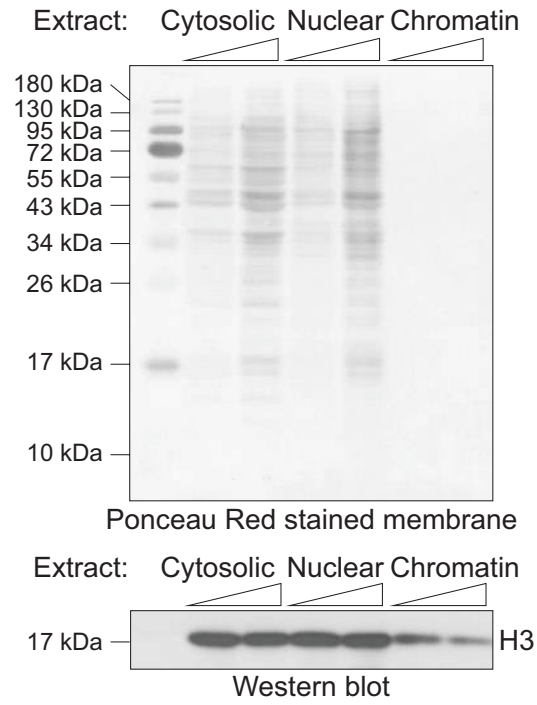


**Fig. 2** Subcellular fractionation analyzed by Western blot. After subcellular fractionation of HeLa cells aliquots derived from Cytosolic Extract (1 and 3% of the total Cytosolic Extract), Nuclear Extract (1 and 3% of the total Nuclear Extract), and Nuclear Pellet (0.005% and 0.015% of the total Nuclear Pellet) were loaded onto a 15% SDS-PAGE. First lane, molecular weight standards. Upper panel: Ponceau Red stained membrane after electrotransfer the gel. Lower panel: Western blot analysis against histone H3

3. Centrifuge at 13,800 × *g* for 10 min, at 4 °C. Take the supernatant and transfer into a new tube.
4. Add TCA to a final concentration of 25%. In our example, 130 μL of 100% TCA.
5. Incubate the sample with rotation for 1 h at 4 °C.
6. Centrifuge at 13,800 × *g* for 10 min, at 4 °C. Discard the supernatant.
7. Wash the pellet with 200 μL cold acetone and vortex 5 s. Centrifuge at 13,800 × *g* for 5 min and discard the supernatant. Repeat this step twice.
8. Let the pellet dry at room temperature for about 20 min. Resuspend the pellet in 50 μL of Buffer E and measure protein concentration (*see* **Note 15**). To analyze the purity of extracted histones, run 1 μg of protein in a 15% SDS-PAGE and stain the gel with Coomassie blue staining (*see* Subheading 3.2 and Fig. 3). Store histones at −80 °C.

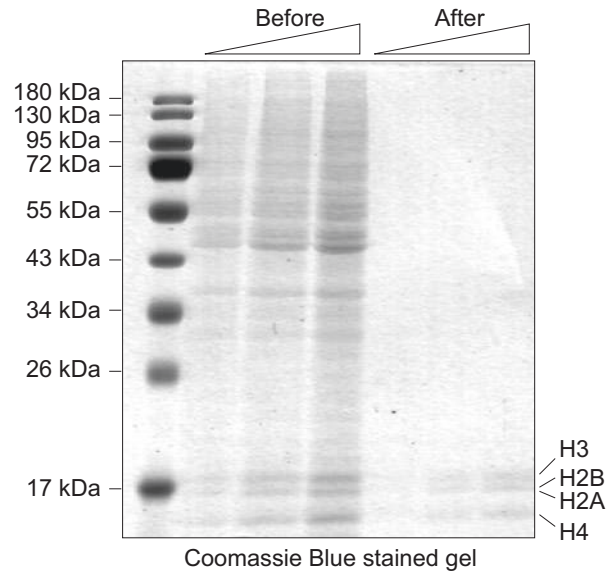


**Fig. 3** Proteins obtained from Nuclear Pellet before and after acid extraction. Increasing amounts of Nuclear Pellet aliquots before (0.4%, 0.8%, and 1.2% of the total Nuclear Pellet) and after (2.5%, 5%, and 7.5% of the total acid extraction recovered material) acid extraction were loaded on 15% SDS-PAGE and stained with Coomassie blue for protein visualization. First lane, molecular weight standards

*3.1.2 Recombinant Histones*

This protocol, based on Luger et al. [13], is designed to purify high amounts of histone proteins by using bacterial expression vectors. The purification does not rely on "tags," given that histones are purified from inclusion bodies. Since the proteins are expressed in bacteria, they lack PTMs, which makes them the ideal substrates for the evaluation of enzymes involved in the "de novo" establishment of histone PTMs [18]. Another advantage of this system is that histones (canonical and variant) can be mutated at any residue, to analyze, for example, the effect of PTMs over the nucleosome structure [19].

*Bacterial culture and histone expression*

1. Transform BL21 bacterial strain with the histone expression vector (e.g., pET-histone plasmid). For this, thaw 50 μL of chemically competent bacteria on ice, and incubate with 25 ng of the pET-histone plasmid for 30 min on ice. After this, incubate for 30 s at 42 °C and then place the tube on ice for 2 min. Add 250 μL of warm SOC medium and incubate in agitation for 1 h. Plate the transformed bacteria on LB-agar containing the appropriated antibiotic and incubate at 37 °C, overnight.
2. Inoculate 50 mL of LB-containing antibiotic with a single colony. Then, incubate at 37 °C, 250 r.p.m., overnight.

3. The next day, inoculate 1000 mL of LB-containing antibiotic with the 50 mL saturated culture obtained from **step 2**. Incubate in a shaker at 37 °C, 250 r.p.m., until 600 nm optical density (OD600) reaches a value between 0.6 and 0.8.
4. Take 1 mL sample from the culture and store it on ice for further analysis (*see* **Note 16**). Then, induce histone expression by adding 1 mL of 1 M IPTG, incubating in the shaker at 37 °C, 250 r.p.m., 2 h.
5. After induction, take 1 mL sample from the culture and store it on ice for further analysis. Then, harvest bacteria by centrifuging the culture at 15,000 × *g*, 10 min. The obtained pellet can be stored at −80 °C until performing the *Histone purification step*.
6. Using the samples from **steps 4** and **5**, verify histone induction. To do this, centrifuge the samples at 13,800 × *g*, eliminate the supernatant, and resuspend the pellet in 1/10 of the original volume using distilled water. Sonicate the samples by 3 pulses of 30 s each, 60% amplitude, with resting periods of 45 s between the pulses, and add *Laemmli Buffer* 5× to a final concentration of 1×. Run 15 μL of the samples in a 15% SDS-PAGE and stain gel with Coomassie Blue to visualize the induced histone band.

*Histone purification from inclusion bodies*

1. Thaw the stored bacteria pellet in a warm bath (37 °C) and resuspend it in 50 mL of Inclusion Bodies Wash Buffer by pipetting up and down.
2. Perform 3 cycles of "freeze and thaw." To do this, keep the resuspended bacteria 20 min at −80 °C and then 5 min at 37 °C. Repeat twice.
3. Put the bacterial pellet on ice and sonicate by three pulses of 45 s each, 60% amplitude, with 45 s of rest between pulses.
4. Centrifuge the sonicated bacteria at 15,000 × *g*, 4 °C, 20 min and discard the supernatant.
5. Resuspend the pellet in 50 mL of Inclusion Bodies Wash Buffer plus 1% Triton X-100 and centrifuge at 12,000 × *g*, 10 min, 4 °C. Repeat this step once. It is difficult to resuspend the pellet at this step; however, the quality of histone purification is dependent on the efficiency of this step.
6. Resuspend the pellet in 50 mL of Inclusion Bodies Wash Buffer (without Triton X-100) and centrifuge at 12,000 × *g*, 10 min, 4 °C. Repeat this step once.
7. Resuspend the pellet, which contains the inclusion bodies, in 15 mL of Unfolding Buffer and incubate at 37 °C, with agitation, 1 h.

8. Eliminate undissolved material by 20 min centrifugation at 15,000 × *g*, 4 °C. Recover the supernatant and discard the pellet.
9. Dialyze the supernatant against 2 L of distilled water at 4 °C, using dialysis tubing of 1 K MWCO. Replace water every 2 h at least twice and then perform a final overnight dialysis step.
10. Recover the content inside the dialysis tubing and centrifuge at 12,000 × *g*, 4 °C, for 15 min.
11. Recover the supernatant and evaluate histone purification by performing 15% SDS-PAGE and stain the gel with Coomassie blue for visualization of the histone bands (*see* **Note 17**) (Fig. 4). Store the purified histones at −80 °C until use.

## 3.2 Analysis of PTMs

### 3.2.1 Western Blot

Western blot is one of the most utilized techniques in the field of cell biology, molecular biology, and biochemistry. This method, originated in the late 1970s and early 1980s [20–22], can be summarized in three steps: protein separation from a complex sample by gel electrophoresis, protein transfer from the gel to a solid membrane, and detection of the protein of interest (or PTMs), typically by antibodies. Although there are many protocols available in the literature, here we describe the conditions optimized for the analysis of histones and histone PTMs. For a more general

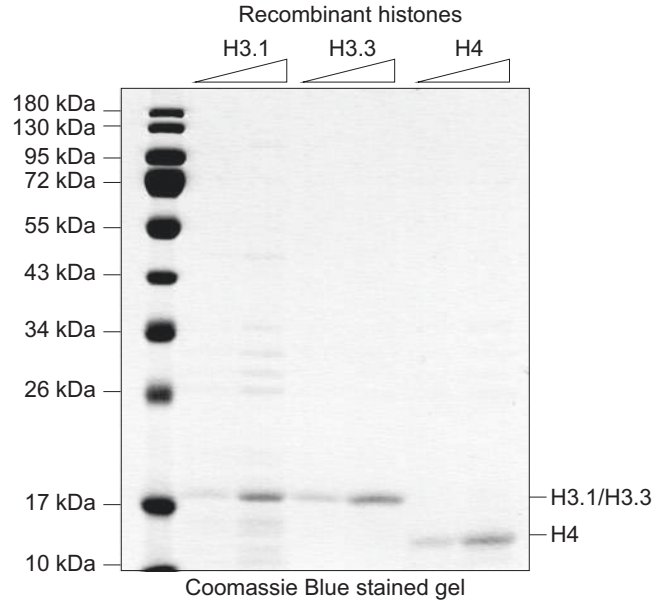


**Fig. 4** Recombinant histones purified from bacteria. After histone purification, 15% SDS-PAGE gel was loaded with 0.5 and 2 μg of H3.1, H3.3, and H4 proteins. Then, the gel was Coomassie blue stained for visualization of histones. H3.1 and H3.3 have the same electrophoretic migration. First lane, molecular weight standards

Western blot protocol, we recommend consulting the work by Mahmood and Yang [23].

*15% SDS-PAGE gel preparation*

1. Assemble clean glass plates into a casting frame to prepare a 1.5 mm thick mini-gel.
2. To prepare the resolving gel, mix 2 mL of distilled water, 2 mL of Lower Buffer, and 4 mL of 30% Acrylamide-0.8% Bis-acrylamide. Then, add 20 μL of 10% APS and 20 μL of TEMED. Mix well avoiding the formation of bubbles, and promptly proceed to the next step.
3. Place the resolving gel mix into the assembled glass plates, leaving space for the stacking gel. Then, gently overlay 1 mL of isopropanol on the surface of the gel mix. Allow the polymerization reaction to occur for at least 40 min.
4. Remove the isopropanol, wash the surface of the gel with distilled water and eliminate the excess of water by draining over a paper towel.
5. To prepare the stacking gel, mix 2.4 mL of distilled water, 1 mL of Upper Buffer and 600 μL of 30% Acrylamide-0.8% Bis-acrylamide. Then, add 20 μL of 10% APS and 20 μL of TEMED. Mix well, avoiding the formation of bubbles, and promptly place the mix on the top of the resolving gel. Immediately introduce the comb to form the loading wells.
6. Let the gel to polymerize for at least 20 min and do not remove the comb until loading the samples.

*Sample preparation for SDS-PAGE gel electrophoresis*

1. Measure the protein concentration of your samples.
2. Take the amount of sample needed and transfer into a new tube (*see* **Note 18**). Add *Laemmli* Buffer 5× to a final concentration of 1×.
3. Heat the samples 5 min at 100 °C.
4. Spin the samples 10 s to precipitate condensed water from the top. Maintain the tubes at room temperature until loading the gel.

*SDS-PAGE running*

1. Assemble the glass plates into the electrode gasket.
2. Place the assembled electrode gasket into the electrophoresis chamber. Fill the gasket and the electrophoresis chamber with Running Buffer 1× and gently remove the comb (*see* **Note 19**).
3. Load the samples into the gel's wells by using Hamilton syringes or 200 μL long pipette tips (*see* **Notes 20** and **21**),

saving one well for prestained protein ladder. Fill any non-used well with an equivalent Laemmli Buffer 1× volume (*see* **Note 22**).

4. Load prestained protein ladder, close the electrophoresis chamber, and run the gel at 110 V, room temperature, until the Bromophenol blue dye marker reaches the end of the gel. Then, proceed with the protein transfer to nitrocellulose or PVDF membrane.

*Electrophoretic protein transfer (Electroblotting)*

1. After finishing the SDS-PAGE running, recover the gel and remove the stacking gel by using a spatula. Place it into Transfer Buffer.
2. Cut two 7.5 × 10 cm pieces of 3 MM grade Whatmann® type filter paper and place into Transfer Buffer.
3. Cut one 7.5 × 10 cm piece of nitrocellulose or PDVF membrane (*see* **Note 23**). When using PVDF, activate the membrane with 100% methanol. Nitrocellulose membranes do not require methanol activation. Keep membranes in Transfer Buffer.
4. Assemble the protein-transferring sandwich by disposing the following components, in the indicated order, into a gel holder cassette:
   (a) A flat foam pad.
   (b) A piece of filter paper.
   (c) The nitrocellulose or PDVF membrane.
   (d) The acrylamide gel.
   (e) A piece of filter paper.
   (f) A flat foam pad.

   Eliminate any bubble between the layers of the sandwich (*see* **Note 24**).
5. Introduce the protein-transferring sandwich into the electrode module, with the gel facing toward the cathode and the membrane to the anode. Then, put the electrode module into the transferring chamber and fill it with Transfer Buffer. Place a cooling tray (−20 °C) inside the chamber and cap it, connecting the electrodes to a power supply.
6. Perform the electrotransfer 1.5 h at 400 mA, 4 °C (*see* **Note 25**).
7. Disassemble the transferring sandwich and stain the membrane with Ponceau to verify the transfer. To do this, incubate the membrane with 50 mL of Ponceau staining solution about 5 min. Remove the staining solution and wash the membrane with 100% methanol (PVDF) or T-TBS 1× (nitrocellulose).

*Membrane blocking and antibody incubation*

1. Block the membrane by incubating for 1 h in blocking solution (T-TBS 1% BSA or 5% Milk, depending on the antibody to use (*see* **Note 7**)).
2. Incubate the membrane with the primary antibody overnight at 4 °C and rotation. Dilute the antibody in T-TBS 1×, according to the manufacturer's instructions. Always use validated antibodies (*see* **Note 26**).
3. Wash the membrane 3 times with T-TBS 1×, 5 min each.
4. Incubate the membrane with the secondary antibody conjugated to HRP 1 h at room temperature and rotation. Dilute the antibody in T-TBS 1×, according to the manufacturer's instructions.
5. Wash the membrane 3 times with T-TBS 1×, 5 min each. Perform all the subsequent steps in the **dark room**.
6. Develop the assay by incubating the membrane with a chemiluminescence substrate mix (e.g., ECL from *Thermo Scientific*) 5 min. Prepare the mix according to the manufacturer's instructions.
7. Place the membrane between two transparent plastic sheets, removing the excess of the chemiluminescence substrate mix. Then, place the membrane in an autoradiographic cassette.
8. Place an autoradiographic film on top of the covered membrane for 1 min, or the required time, closing the autoradiographic cassette.
9. Remove and develop the film.

### 3.2.2 TAU Gel Electrophoresis Coupled to Western Blot

Three are the fundamental principles of TAU gels: First, with the exception of the linker histones, core histones associate to non-ionic detergents, such as Triton, increasing their "effective mass." Second, histones associate with non-ionic detergents at different degrees based on the amino acidic sequence composition. This differential binding is enhanced by urea, because this agent disrupts the interaction between detergent and histones. And third, histones acquire a positive net charge under acidic conditions, which is achieved by acetic acid. The present protocol can differentiate between the histone variants H3.1 and H3.3; and H2A.1, H2A.2, H2A.3, and H2A.4 [24]. By adapting the procedure described by Lennox and Cohen in 1989 [25], the present protocol utilizes mini-gels, facilitating the implementation of the technique as well as the electrotransfer to PVDF membranes for PTM analyses by Western blot.

*TAU acrylamide gel preparation (12% Acrylamide-0.2% Bisacrylamide, 6 M Urea, 6 mM Triton X-100, 5% Acetic Acid)*

1. Assemble the clean glass plates into a casting frame to prepare a 1.5 mm thick mini-gel.

2. To prepare the resolving TAU gel, mix 3 mL of 40% Acrylamide-0.67% Bis-acrylamide, 3.6 g of Urea and 0.5 mL of glacial Acetic acid. Add distilled water to a final volume of 9.6 mL and mix until the urea is completely dissolved.
3. Add 400 μL of 10% Triton X-100 and gently mix to avoid formation of bubbles (*see* **Note 27**).
4. Add 190 μL of 10% APS and 190 μL of TEMED and mix gently. Promptly proceed to the next step.
5. Place the resolving TAU gel mix into the assembled glass plates, leaving space for stacking gel. Then, gently overlay 1 mL of isopropanol on the surface of the gel mix. Allow the polymerization reaction to occur at least 40 min.
6. Remove the isopropanol, wash the surface of the gel with distilled water and eliminate the excess of water by draining over a paper towel.
7. To prepare the stacking TAU gel (5% Acrylamide-0.08% Bis-acrylamide, 3 M Urea, 6 mM Triton X-100, 5% Acetic Acid), mix 625 μL of 40% Acrylamide-0.67% Bis-acrylamide, 0.9 g of Urea and 250 μL of glacial Acetic acid. Add distilled water to a final volume of 4.8 mL and mix until the urea is completely dissolved.
8. Add 400 μL of 10% Triton X-100 and gently mix to avoid formation of bubbles (*see* **Note 27**).
9. Add 200 μL of 10% APS, 200 μL of TEMED and mix gently. Promptly place the mix on top of the resolving gel. Immediately place the appropriated comb to form the loading wells. Let it polymerize at least 40 min.
10. Assemble the glass plates into an electrode gasket and place the gasket into the electrophoresis chamber. Fill the gasket and chamber with TAU Running Buffer and gently remove the comb (*see* **Note 28**).
11. Close the chamber, connect the electrodes to the power supply in an **inverse manner**, that is the positive terminal of the gasket into the negative terminal of the power supply and vice versa (*see* **Note 29**). Prerun the gel overnight at 90 V (*see* **Note 30**).
12. The next morning, replace the TAU Running Buffer with fresh buffer, clean up the wells by gently pipetting TAU Running Buffer, and proceed to load the samples.

*Sample preparation for TAU gel electrophoresis*

1. Mix each sample with one volume of TAU Sample Buffer 2×. It is recommended to prepare the same volume for all the samples, up to 25 μL.

2. Keep the samples at room temperature until loading the gels. Do not heat the samples (*see* **Note 31**).

*TAU gel running*

1. Load 5 μL of 50 μg/μL of Cytochrome-C into the prerun TAU gel. Cytochrome-C is an H4 migration marker that can be visualized while running the gel by its brown color. Then, load the samples and fill any remaining well with TAU Sample Buffer 1×.
2. Close the electrophoresis chamber. Connect the electrodes to the power supply in the **inverse manner**, as detailed in **TAU acrylamide gel preparation, step 11**, and run the gel at 120 V, at room temperature, until the Cytochrome-C band migrates ¾ of the total gel length. This will take approximately 2 h. Once the running has finished, promptly proceed with the protein transfer to the PVDF membrane. Alternatively, stain the TAU gel with Coomassie blue without any preparation step (Fig. 5).

*Preparing TAU gels for electrotransfer*

1. Disassemble the electrophoresis chamber and gently take the TAU gel, removing the stacking gel.
2. Wash the gel twice 30 min with the TAU gel Wash Buffer 1.
3. Wash the gel once 30 min with TAU gel Wash Buffer 2.
4. Leave the gel in Transfer Buffer and proceed with Electroblotting, blocking of the membrane and incubation with primary and secondary antibodies as described previously (*see* Subheadings 3.2 and 3.2.1) (Fig. 6).

### 3.2.3 Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation relays on the recognition of chromatin fragments by specific antibodies to study proteins that are in contact with DNA. In a first step, cells are fixed using a cross-linker reagent to stabilize the interaction between proteins and DNA. Then, the fixed chromatin is disrupted by sonication to obtain chromatin fragments up to 500 bp. Chromatin fragments are then immunoprecipitated, and the DNA fragments purified and analyzed. The present protocol, adapted from Soutoglou and Talianidis, 2002 [26] describes a method optimized for the study of histone PTMs located on specific DNA sequences, like promoters. A scheme of the fundamental steps of this technique is shown on Fig. 7. Alternatively, when samples are obtained from cells expressing histone variants fused to a tag sequence, such as Flag-tag, antibodies directed against the tag sequence are utilized to explore the binding of variants to specific DNA sequences.

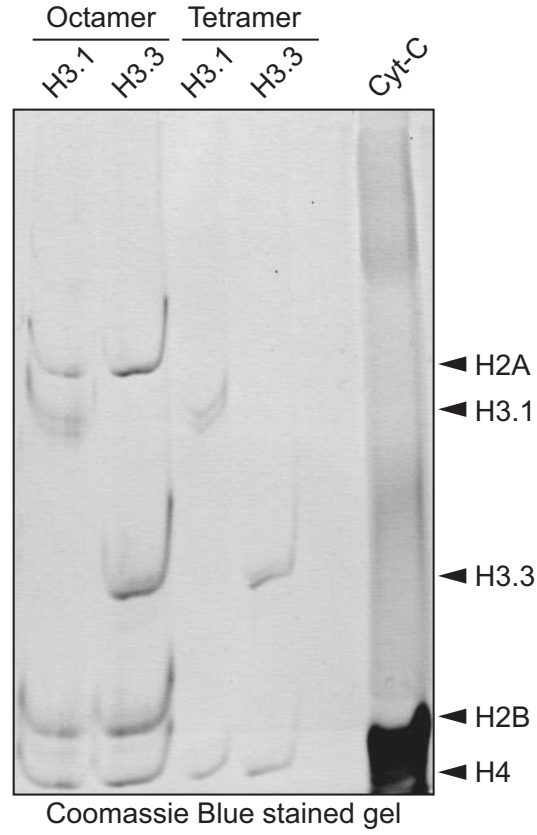


**Fig. 5** Analysis of recombinant histone variants by TAU gel electrophoresis. 5 μg of either octamer or tetramers H3-H4 proteins were loaded into the TAU gel and then Coomassie blue stained. The migration of the different histones is pointed on the right

*Cell fixation*

1. Seed $3 \times 10^6$ cells into a 150 mm plate, 24 h before harvest.
2. Collect the cells in a 15 mL conical tube and leave it on ice.
3. Centrifuge the tube at 800 × *g* 5 min and wash the cells with cold PBS 1×. Repeat this wash step once and centrifuge the cells at 800 × *g* 5 min.
4. Mix 100 μL of 37% formaldehyde solution and 3.6 mL of PBS 1× to freshly prepare 3.7 mL of 1% formaldehyde in PBS.
5. Add 500 μL of 1% formaldehyde to the cells to start the cross-linking. Then, transfer the cells to a 1.5 mL eppendorf tube and incubate 10 min at room temperature, with gentle agitation.
6. Quench the reaction by adding 25 μL of 2.5 M Glycine. Incubate with rotation 5 min at room temperature.

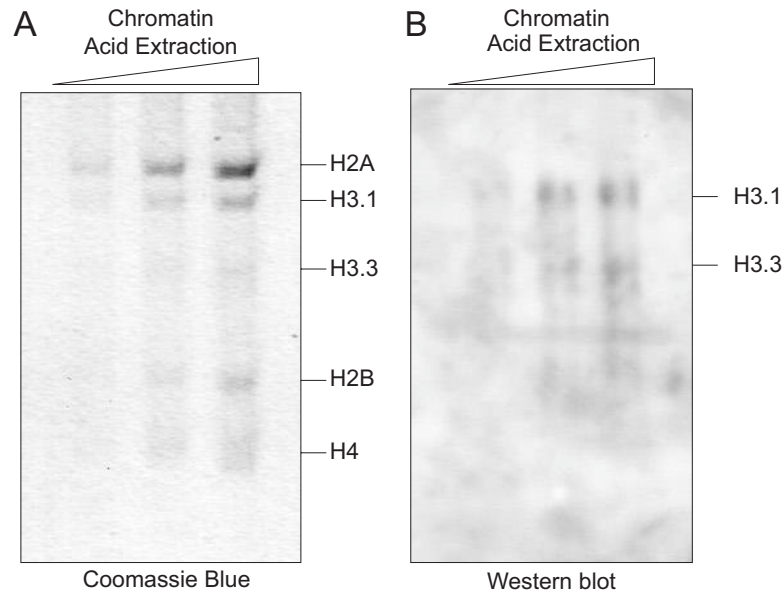


**Fig. 6** Analysis of histone variants derived from Nuclear Pellet by TAU gel electrophoresis analysis coupled to Western blot. (**a**) 2.5%, 5%, and 7.5% of the total extracted Nuclear Pellet sample were loaded into the TAU gel and then Coomassie blue stained. (**b**) 0.1%, 0.2%, and 0.3% of the total extracted Nuclear Pellet sample were loaded into the TAU gel and then electrotransferred. Then, the membrane was Western blotted against histone H3. The migration of the different histones is pointed on the right

7. Centrifuge the cells 5 min at 400 × *g*, 4 °C. Discard the supernatant.
8. Mix 450 µL of cold PBS 1× with 50 µL of protease inhibitors cocktail and wash the cells with this solution. Repeat this step once. If EGS fixation is required, continue to **EGS fixation**. Otherwise, proceed with **Cell lysis and chromatin fragmentation.**

*EGS fixation (Optional).*

EGS is a crosslinker that contains amine-reactive ends around a 12-atom spacer arm, having a longer cross-linking radius in comparison to formaldehyde. Therefore, EGS fixation is utilized to analyze protein complexes associated with DNA.

1. Freshly prepare a solution of 50 mg/mL of EGS (EGS 50×) in 50% Acetic acid, diluted in water. To facilitate the dissolution of EGS, pre-warm the 50% Acetic acid solution between 60 and 70 °C.
2. Dilute *EGS 50×* to a final concentration of 1× in PBS 1×, at room temperature. Keep at 37 °C.

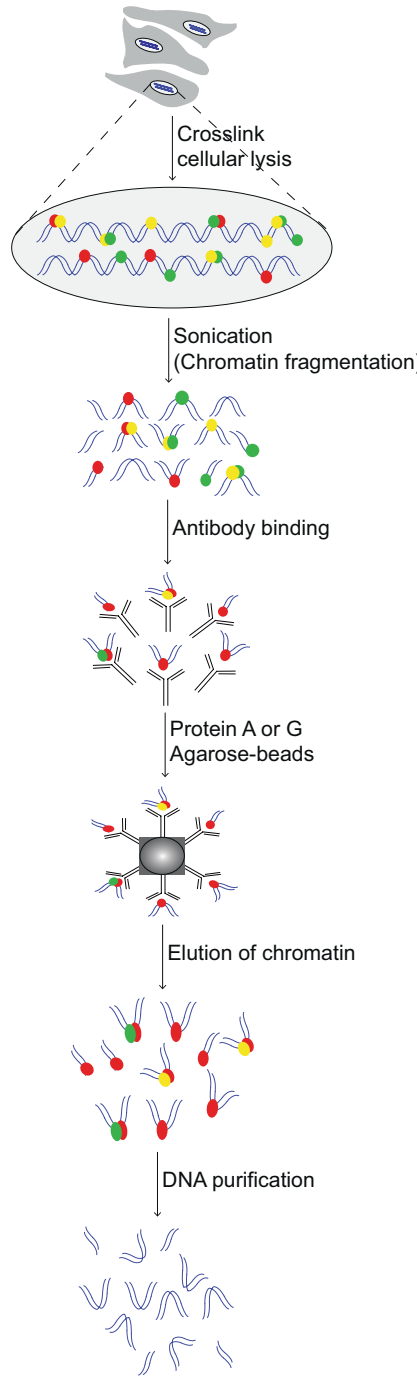


**Fig. 7** Steps of Chromatin Immunoprecipitation. Fixed cells are lysed and sonicated to obtain chromatin fragments up to 500 bp. Fragments are then immunoprecipitated by using specific antibodies and then agarose beads conjugated to protein A or G. Chromatin fragments are eluted, proteins degraded and DNA purified and quantified for further analyses. Adapted from [27]

3. Add 500 µL of EGS 1× to the cell pellet. Incubate for 1 h at room temperature with rotation.
4. Wash the cells 3 times with 500 µL of PBS 1× at room temperature, centrifuging 5 min the cells at 400 × *g*.

*Cell lysis and chromatin fragmentation*

1. Add 50 µL of protease inhibitors cocktail 10× to 450 µL of Cell Lysis Buffer (*see* **Note 8**). Use this solution to resuspend the cell pellet. Incubate for 10 min on ice.
2. Homogenize the cells with a Dounce tissue grinder, 10 times, using the loose pestle.
3. Centrifuge the lysate for 1 min at 5400 × *g*, at 4 °C. The nuclei will remain in the pellet.
4. Mix 25 µL of protease inhibitors cocktail 10× and 225 µL of Nuclei Buffer. Use this solution to resuspend the nuclei pellet. Incubate for 10 min on ice. Add 225 µL of Sonication Buffer and 25 µL of protease inhibitors cocktail 10×.
5. Perform chromatin fragmentation by sonicating the resuspended nuclei by 80 pulses of 15 s each time, 60% amplitude, with pauses of 15 s between each pulse of sonication.
6. Centrifuge the sonicated samples for 10 min at 17,000 × *g*, at 4 °C. Transfer the supernatant into a clean tube and discard the pellet. Repeat this step once. Store this *Chromatin sample* at −80 °C or proceed with the immunoprecipitation step (*see* **Note 32**).

*Preparation of protein A (or G) agarose beads*

1. Take twice the volume of a 50% slurry agarose beads required for the immunoprecipitation (*see* **Note 33**). In our example, take 50 µL of protein A-agarose beads and centrifuge 5 min at 1500 × *g*, 4 °C. Discard the supernatant.
2. Wash the beads by adding 100 µL of PBS 1× and centrifuge 5 min at 1500 × *g*, 4 °C. Discard the supernatant and repeat this step once.
3. Add one volume of PBS 1× to the beads. In our example, add 25 µL of PBS 1×. This is the *Beads solution*. Keep at 4 °C or use immediately.

*Immunoprecipitation*

1. Measure the DNA concentration of the *Chromatin sample,* using a mixture of Nuclei Buffer and Sonication Buffer (1:1) as blank. Aliquot the *Chromatin sample* in fractions of 1 µg of DNA per tube and add Sonication Buffer, plus protease inhibitors, to a final volume of 500 µL. Keep one of these aliquots as *Input*.

2. Perform a "pre-clearing step" to each *Chromatin sample*. To do this, add 50 μL of the *Beads solution* and 2 μg of immunoglobulin (e.g., rabbit IgG (*see* **Note 34**)) to each *Chromatin sample*. Incubate with rotation for 2 h at 4 °C.
3. Centrifuge 5 min at 1500 × *g*, 4 °C, and transfer the supernatant into a clean tube. Discard the pellet.
4. Add 1 μg of antibody to the precleared *chromatin sample*. Incubate with rotation overnight at 4 °C (*see* **Note 35**).
5. Add 10 μL of *Beads solution* and incubate in rotation 1 h at 4 °C. Then, centrifuge 2 minutes at 1500 × *g*, 4 °C, and discard the supernatant.
6. Add 500 μL of Sonication Buffer and incubate 5 min, with rotation, at 4 °C. Centrifuge 5 min at 1500 × *g*, 4 °C, and discard the supernatant (*see* **Note 36**).
7. Add 500 μL of IP Wash Buffer and incubate for 5 min, with rotation, at 4 °C. Centrifuge for 5 min at 1500 × *g*, 4 °C, and discard the supernatant. Repeat this step once (*see* **Note 36**).
8. Wash the beads with 500 μL of TE Buffer (pH 8.0). Incubate the beads for 5 min, with rotation, at 4 °C. Centrifuge 5 min at 1500 × *g* and discard the supernatant (*see* **Note 36**).

*DNA recovery from the immunoprecipitation*

1. Add 100 μL of Elution Buffer to the beads obtained from the last step of the immunoprecipitation (*see* **Note 37**).
2. From now on, include the Input sample. Vortex gently 15 s and incubate for 15 min at 65 °C. Vortex gently 15 s once more.
3. Centrifuge 1 minute at 17,000 × *g*. Transfer the supernatant into a new tube and discard the pellet. Add 4 μL of 5 M NaCl.
4. Add 1 μL of 20 mg/mL of RNase A and incubate overnight at 65 °C.
5. Add 2.5 μL of Proteinase K to a final concentration of 1 U/mL. Then, add Proteinase K Buffer 10× to a final concentration of 1×. Incubate for 2 h at 50 °C and then add 95 μL of Buffer TE (pH 8.0) (*see* **Note 38**).
6. Purify the DNA by phenol-chloroform extraction or by using the purification kit of your choice (*see* **Note 39**).
7. Use the purified DNA for real-time qPCR, using primers directed against the sequence of interest.

## 4 Notes

1. PMSF is not stable in aqueous solution, having a half-life of 35 min in this medium [28]. For this reason, this compound is added to the buffers shortly before use.
2. Some investigators prefer DTT over β-mercaptoethanol because it is less irritant, but both are used for the same purpose: preventing protein oxidation. When replacing one by another, 0.5 mM DTT is equivalent to 10 mM β-Mercaptoethanol.
3. Dissolve EDTA in 400 mL of distilled water, add NaOH pellets until reaching the desired pH and then complete volume to 500 mL. EDTA will not dissolve until the pH is above 8.0.
4. When prepared in anhydrous isopropanol, PMSF is stable for months [28].
5. The antibiotic needed will depend on the resistance marker associated with the plasmid in which the gene coding for the recombinant protein is cloned. Always consult the plasmid datasheet.
6. If not protected from UV light, acrylamide will enter into polymerization reactions, forming clusters that precipitate and reduce the actual concentration of the stock acrylamide solution. To avoid this, use amber bottles to store acrylamide solution or wrap conventional bottles with foil.
7. The use of T-TBS 5% Milk or T-TBS 1% BSA as blocking solutions is dependent on the antibody employed. Some antibodies give stronger signals when the membrane is blocked with BSA. This is common for the analysis of histone PTMs. In contrast, blocking with milk might help to reduce the recognition of unspecific bands. Therefore, we highly recommend testing the best blocking solution for each antibody.
8. Commercially available protease inhibitors cocktails can be replaced by homemade cocktails. Prepare 1000× aprotinin (10 mg/mL), 1000× leupeptin (5 mg/mL) and 1000× pepstatin (7 mg/mL) and dilute them to 1× concentration in the desired buffer.
9. Inactivation of the trypsin is achieved thanks to the presence of fetal bovine serum in the culture medium. Thus, it is important to wash the cells with PBS 1× before adding trypsin.
10. Handle carefully the cells to minimize their premature disruption and leaking the nuclear content.
11. The cell pellet increases twice its initial size due to the swelling of the cells in Buffer A.

12. It is recommended to precool the Dounce tissue grinder on ice before the homogenization.
13. The "loose" type pestle allows the disruption of the plasma membrane without affecting the nuclear membrane integrity. Ten up-and-down cycles are sufficient for the mechanical disruption of the cells.
14. In the original protocol, cytosolic extract is the soluble fraction recovered after centrifugation at 100,000 × *g*. However, the present protocol is adapted to work by using refrigerated microcentrifuges available in almost any laboratory. When possible, centrifuge the extract at 100,000 × *g*.
15. When starting from 50 μL of cell pellet, 100 μL of a final concentration of at least 1 μg/μL is obtained.
16. This sample reveals the proteins synthesized by the bacteria before IPTG mediated protein induction.
17. About 2 mg of histone proteins can be obtained from 1000 mL of bacterial culture. 20 μL of the final 15 mL histone solution should be enough to visualize the purified histones.
18. To perform Western blot analyses, the optimal amount of protein to load into the gel depends of several things. This includes the sample source (e.g., Cytosolic Extracts or Nuclear Pellet) and the sensitivity of the antibody employed for detection (e.g., Antibodies against histones or histone PTMs). As a reference, 5–20 μg of proteins derived from cytosolic, nuclear and whole cell extracts, and 50–200 ng of proteins derived from solubilized chromatin or acid extractions are sufficient for the analysis of histones H3 and H4, and their PTMs.
19. Although it is possible to remove the comb from the glass plates on dry, removing the comb while immersed into Running Buffer 1× facilitates the process.
20. Based on our experience, avoid loading protein samples on the wells located at the gel side edges. Protein bands from those lanes usually run with a "smile" shape.
21. Samples can be loaded using common 200 μL yellow pipette tips, but it is recommended to use long pipette tips or Hamilton syringes to facilitate the loading process.
22. Filling any non-used well with Laemmli Buffer 1× helps to keep the proteins in one lane, without expanding to the other lanes.
23. PVDF is more resistant than nitrocellulose membrane. In our experience, we prefer PVDF when analyzing histone proteins.
24. To ensure that bubbles are completely eliminated, assemble the sandwich in a deep tray filled with sufficient Transfer Buffer

as to cover all the sandwich layers. Use a 15 mL conical tube as a roller to flatten the layers and remove bubbles.

25. To keep the temperature low, place the transferring chamber into a box filled with ice.
26. The most critical aspect in validating results obtained from Western blot analyses (especially against histone PTMs) is the quality of the antibody utilized. Rothbart and collaborators [29] have created a database which summarizes the specificity of over 100 commercially available antibodies against histones.
27. Since Triton X-100 is a detergent, abrupt agitation will produce a persistent foam. If that happens, let the mixture rest until bubbles disappear.
28. The TAU stacking gel is more "gooey" than the SDS-PAGE gel, therefore, remove the comb carefully.
29. Under TAU conditions, proteins are positively charged thanks to the acetic acid. Therefore, the proteins migrate toward the negative electrode.
30. The prerunning step is necessary to eliminate all the remaining APS from the gel to avoid protein oxidation. Furthermore, this prerunning step "ages" the gel to eliminate residual free radicals from the acrylamide polymerization reaction [25].
31. Since TAU Sample Buffer has high concentration of urea to induce protein denaturation, no heat is needed. Moreover, heating proteins in the presence of high concentrations of urea induces protein carbamylation, a modification that affects protein charge and, consequently, affects its electrophoretic migration on TAU gels [30].
32. After sonication, the expected size of chromatin fragments is shorter than 500 bp. Confirm the effectiveness of the sonication step by running the DNA on an agarose gel. This sonication protocol has been standardized for human cell lines. When chromatin samples are derived from other sources the correct sonication protocol requires some adjustments.
33. The use of either protein A or G agarose beads will depend on the isotype of the antibody used. As a guide, see Bonifacino et al., 2001 [31].
34. The immunoglobulin used will depend on the antibody used to immunoprecipitate.
35. When evaluating histone PTMs, 1 μg of antibody is enough for immunoprecipitation. When evaluating non-histone proteins bound to DNA, the amount of antibody must be standardized.

36. Pour buffer on the tube's wall and shake it by taping with your fingers. Do not pipet the beads at this step.
37. Use not more than 7 days old Elution Buffer, to avoid changes on the pH.
38. Remove the tubes using a warm water recipient, in order to avoid SDS precipitation. When adding TE Buffer, the final volume in the tube should be 200 μL.
39. Do not use TE Buffer or DNA Elution Buffer from commercial kits. These buffers often come with EDTA that reduces the PCR efficiency.

## Acknowledgments

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