Histone Purification Kit

(version B3)

Catalog No. 40025

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Introduction

Active Motif's Histone Purification Kit enables you to purify the core histone proteins and enrich for separate H2A/H2B and H3/H4 fractions while preserving their post-translational modifications such as acetylation, methylation, and phosphorylation states. This purification method is an alternative to acid precipitation methods and utilizes a purification resin and proprietary buffer system to purify the core histones and further enrich for a separate H2A/H2B fraction and a highly pure H3/H4 fraction from cells and tissue samples. Histones isolated by this method are suitable substrates for downstream assays and *in vitro* chromatin assembly.

Unlike standard acid extraction techniques, this kit uses proprietary technology to purify the core histones in either one population of H2A/H2B and H3/H4 together, or further separated into one H2A/H2B fraction and one H3/H4 fraction (Figure 1). Post-translational modifications such as phosphorylation, acetylation and methylation are preserved, so you can extract core histone proteins from your cell culture or animal model and determine which modifications are present and critical for your research system (Figure 2).

The Histone Purification Kit provides reagents for 10 histone purifications from as little as one 100 mm plate of cells to grams of tissue due to the robust binding capacity of the purification resin for histone proteins. The kit method is simple: first, an extract is made and applied to the purification column, then histones are eluted using a series of buffers, enabling the core histones to be purified together as one fraction by either gravity flow or centrifugation, or as separate H2A/H2B and H3/H4 fractions (gravity flow only). Total core histones may be quantified by an OD reading; the separate H2A/H2B and H3/H4 fractions may be quantified by BCA or Lowry protein assays.

product	format	catalog no.
Histone Purification Kit	10 rxns	40025

Kit Performance

The Histone Purification Kit is for research use only. Not for use in diagnostic procedures.

Yield of Histones:

The following yields are approximate. Results may vary according to cell or tissue type.

Adherent Cells: 0.4-0.5 mg total core histones (0.2 mg H2A/H2B and 0.2 mg H3/H4) from 3 x 10^7 cells (3 x 150 mm plates)

Suspension Cells: 0.4-0.5 mg total core histones (0.2 mg H2A/H2B and 0.2 mg H3/H4) from 10^8 cells

Tissue: 2.5 mg per gram of tissue

Core histones Purified from Cells or Tissue

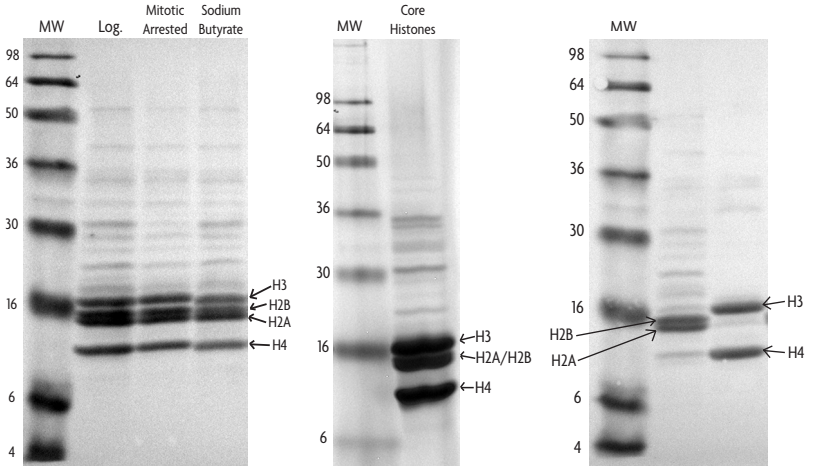


Figure 1: SDS-PAGE analysis of histone fractions.

Ten µg of sample were loaded per lane and run on a 16% Tris-glycine gel.

Left panel: Core histones purified from logarithmically growing (Log.), mitotic arrested or sodium butyrate treated HeLa cells. HeLa cells were treated with 100 nM paclitaxel overnight to induce mitotic arrested cells or 20 mM sodium butyrate to hyperacetylate histone tails.

Center panel: Core histones isolated from rat brain tissue.

Right panel: Separate H2A/H2B and H3/H4 fractions from HeLa cells.

Method Preserves Post-translational Modifications

Active Motif's Histone Purification Kit preserves phospho-, acetyl- and methyl- post-translational modifications on histones.

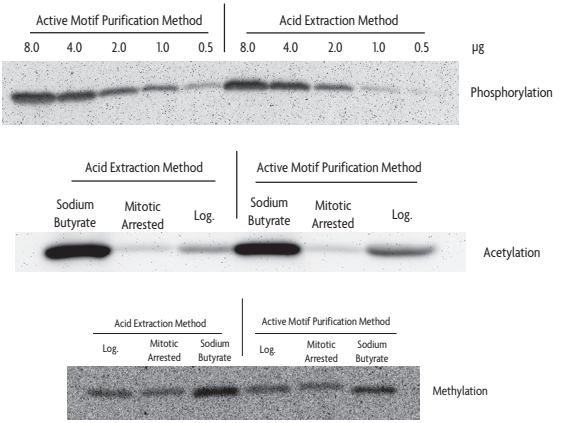


Figure 2: Western blots of histone modifications comparing standard acid extraction to the Histone Purification Kit.

Core histones were purified from HeLa cells by acid extraction and using the Histone Purification Kit. Ten µg were loaded per lane. **Top panel:** Incubation with Histone H3 phospho Ser28 rat mAb (Clone HTA28) (Catalog No. 39098) demonstrates that phosphorylation modifications are preserved in mitotic-arrested HeLa cells. **Middle** panel: Incubation with Histone H4 tetra-acetyl rabbit pAb (Catalog No. 39177) demonstrates that the acetylation state is preserved. **Bottom panel:** Incubation with Histone H3 trimethyl Lys4 rabbit pAb (Catalog No. 39159) demonstrates that the methylation state is preserved.

Sample Purification of HeLa Core Histones

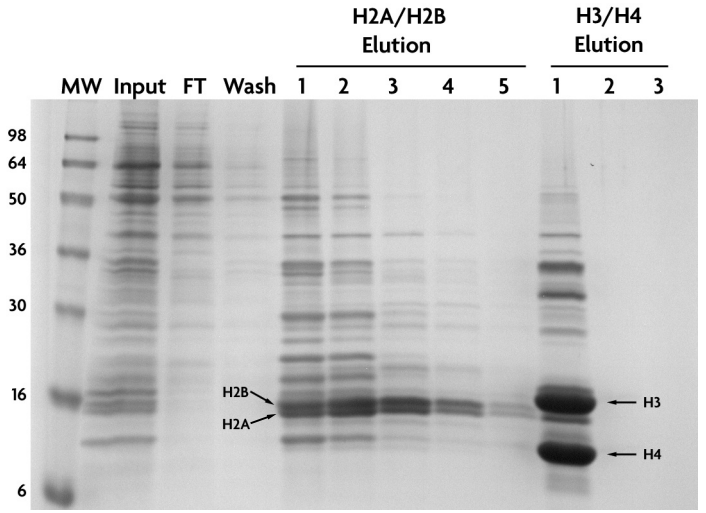


Figure 3: SDS-PAGE analysis of the complete histone purification process.

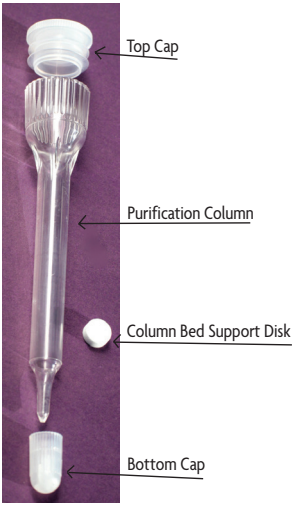
Core histones were purified from HeLa cells using the Histone Purification Kit. Fifteen µl of the input, flow through (FT) and 1st wash (Wash) fractions were loaded per lane. Eluates were precipitated and resuspended in 25 µl of sterile water. Five µl of each of the elution fractions were loaded per lane.

Kit Components and Storage

Please store each component at the temperature indicated below. All components are guaranteed stable for 6 months from date of purchase when stored at the appropriate temperatures.

Reagents	Quantity	Storage / Stability
5X Neutralization Buffer	50 ml	4°C
Extraction Buffer	100 ml	4°C
Equilibration Buffer	200 ml	4°C
Histone Wash Buffer	100 ml	4°C
H2A/H2B Elution Buffer	100 ml	4°C
H3/H4 Elution Buffer	200 ml	4°C
Purification Resin	2 x 1.5 ml	4°C
Purification Column*	1	Room Temp to 4°C

* Column components include the purification column, top and bottom caps, and one column bed support disk, as shown in the photo below.



Additional materials required

- 1 M Tris HCl pH 8.0
- TE or sterile water
- For precipitation of histones: 70% perchloric acid, 100% Trichloroacetic acid (TCA)
- For washing after precipitation: 4% perchloric acid, 30% Trichloroacetic acid (TCA), acetone containing 0.2% HCl, 100% acetone

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Step 1: Prepare Sample Extracts

For adherent cells:

1. Grow cells to about 80-90% confluence in appropriate medium. Discard media and wash the cells 2 times with 10 ml (90 mm dish), 2 ml (35 mm dish) or 20 ml (150 mm dish) of pre-warmed (37°C) serum-free media.
2. After the 2nd wash, aspirate any remaining wash media and add 0.8 ml ice-cold Extraction Buffer to each 90 mm dish (0.3 ml to each 35 mm dish or 1.5 ml to each 150 mm dish). Using a plastic scraper and a pipet, collect the cell protein extracts in a 15 ml tube (or in 1.7 ml microcentrifuge tubes). Pipet the cells up and down to homogenize them into the solution.

For suspension cells:

1. Grow cells to about 80-90% confluence in appropriate medium. If necessary, gently scrape the cells from the sides of the dish while keeping them in their media.
2. Pipet the cells with the media and transfer to a 15 ml tube.
3. Centrifuge at 3000 rpm for 5 minutes at room temperature.
4. Wash the cells twice with ~20 ml pre-warmed (37°C) serum-free media. Centrifuge each time after washing and discard the serum-free media.
5. Resuspend the cells in ice-cold Extraction Buffer (0.5 ml per 150 cm^2 flask yields good concentrated extracts). Pipet up and down to homogenize the cells well, then transfer to a microcentrifuge tube.

For tissues:

1. Homogenize the tissue completely in an ice-cold dounce homogenizer using as little Extraction Buffer as possible, as this will help ensure a highly concentrated extract. Keep the homogenate on ice.
2. Pour the homogenate into a tube. Continue with Step 2: Prepare Crude Core Histone Extract on page 6.

Step 2: Prepare Crude Core Histone Extract

1. Leave the cells in Extraction Buffer for 2 hours to overnight on a rotating platform at 4°C. (Time course experiments performed on some cell lines have shown that most histones are extracted in as little as 10 minutes.)
2. Transfer the cell extracts to fresh tubes and centrifuge in a microcentrifuge at maximum RCF for 5 minutes at 4°C.
3. Transfer the supernatant, which contains the crude histones to a new tube. The pellet can be discarded or stored at -20°C for future analysis (see Appendix, Section B). If the pellet is to be stored, neutralize it by adding ~100 µl 1M Tris HCl pH 8.0.
4. Store the crude histones at -80°C, or continue with the next step.
5. Neutralize and equilibrate the crude histones with 1/4 volume of 5X Neutralization Buffer. For example: Add 0.25 ml of 5X Neutralization Buffer to 1 ml crude histones, 0.5 ml (5X) to 2 ml, 1 ml (5X) to 4 ml of crude, *etc*.
6. Check the pH. If still acidic, add additional 5X Neutralization Buffer until the pH reaches 8.

Step 3: Prepare Column for Purification

1. Prepare the column by securing the bottom cap on the column tip and placing the column in a stand.
2. Using the end of a Pasteur pipette, push the filter disc to the bottom of the column.
3. Add 1 ml sterile water and mark the top water level on the outer surface of the column. Allow the water to drain.
4. Next, add a sufficient volume of purification resin to reach the mark made in the previous step. About 1.5 ml of resin slurry will pack to obtain a column of 1 ml.
5. Allow the resin to settle in the column for 10 minutes.
6. Before first use, clean the packed column with 9 ml of sterile water. Place the column into a 15 ml tube and add 3 ml of sterile water, very carefully as not to disturb the resin bed. Centrifuge for 3 minutes at 50 x *g* at 4°C. Discard the flow-through. Repeat this step two more times for a total of three washes.
7. Next, equilibrate the column with 9 ml of total Equilibration Buffer. Place the column into a 15 ml tube and add 3 ml of Equilibration Buffer. Centrifuge for 3 minutes at 50 x *g* at 4°C. Discard the flow-through. Repeat this step two more times for a total of three washes.

Step 4: Purifying Core Histones

1. After centrifugation to remove the equilibration buffer, replace the bottom cap on the column tip and add 0.5 ml of sample from Step 2. Place the column in a stand and resuspend the column matrix by pipetting up and down to remove and trapped air bubbles. Allow the column resin to settle for 5 minutes.
2. Remove the bottom cap and place the column in a 15 ml tube. Add 3 ml more of the sample from Step 2 to the column. Be careful not to disturb the column matrix or introduce any air bubbles into the resin bed*. Centrifuge the column as before (50 x *g* for 3 minutes at 4°C).
3. Repeat section 2 above until the entire sample from Step 2 has been loaded on the column. Combine all the flow-through samples in the same tube. The flow-through can be stored and analyzed to confirm the histones were retained on the column, if desired (unbound fraction).
4. Wash the column with 9 ml total of histone wash buffer. Place the column into a new 15 ml tube, and add 3 ml of histone wash buffer. Centrifuge the column as before (50 x *g* for 3 minutes at 4°C). Transfer the flow-through to a new tube for future analysis, if desired (1st wash). Repeat wash step two more times, for a total of three washes. Collect 2nd and 3rd washes.
5. If a separate H2A/H2B fraction is not desired, skip this step and proceed with section 6. To collect a separate fraction of H2A/H2B dimers, place the column in a stand and add 3 ml H2A/H2B Elution Buffer to the column. Collect fractions in microcentrifuge tubes by gravity flow.** Discard the first 0.5 ml, and collect the remaining eluate in 1 ml fractions. Continue eluting H2A/H2B dimers by adding more H2A/H2B elution buffer (for a total of 10 ml) while saving the eluate in additional 1 ml fractions. The bulk of the H2A/H2B proteins will be in fractions 2-5.
6. To elute the H3/H4 tetramers (or all core histones together if the "separate H2A/H2B elution step" in section 5 above was skipped) place the column into a new 15 ml tube, add 1 ml of H3/H4 Elution Buffer and centrifuge the column as before (50 x *g* for 3 minutes at 4°C). Transfer the eluate containing the H3/H4 tetramers (or total core histones) to a new microcentrifuge tube.
7. Repeat elution step three more times (for a total of four elutions) and save each eluate in a new microcentrifuge tube. Do not pool the eluates. The H3/H4 tetramers (or total core histones) will be recovered mainly in fractions 1-3 (the 1st and 2nd fractions being the most concentrated). At this point the proteins are ready for use, but contain high levels of salts in the buffer that may interfere with downstream binding assays or other applications. If desired, the proteins can be used without precipitation after being dialyzed against the desired buffer, or desalted using columns designed for this purpose (*e.g.* Zeba spin columns, Thermo-Fisher #89889). To further concentrate the proteins and remove the salt, proceed with precipitation step (Step 5, below).

* If some air bubbles are trapped at the top of the column, replace the bottom cap, add 0.3 ml of Wash Buffer to the top of the column and flick the column gently with the finger or slowly pipet up and down to allow the bubbles to come out the column into the wash buffer. Allow the resin to settle in the column for 5 minutes and remove the air bubbles by pipetting them out from the column in wash buffer.

** Purification of separate H2A/H2B fractions should be performed by gravity flow only. Elution of H3/H4 tetramers or total core histones can be carried out by either centrifugation or gravity flow.

8. After elution of the histones, the column must be cleaned and prepared for subsequent usage. To clean the column, add 3 ml of H3/H4 elution buffer and spin down as before. Repeat this step once more, for a total of two cleaning steps. Discard the flow-through fractions, as there are no histones left on the column.
9. Wash the column with 9 ml of sterile water. Place the column into a 15 ml tube and add 3 ml of sterile water (very carefully as not to disturb the resin bed). Centrifuge for 3 minutes at 50 x *g* at 4°C. Discard the flow-through. Repeat this step two more times for a total of three washes.
10. Equilibrate the column with 9 ml of total Equilibration Buffer. Place the column into a 15 ml tube and add 3 ml of Equilibration Buffer. Centrifuge for 3 minutes at 50 x *g* at 4°C. Discard the flow-through. Repeat this step two more times for a total of three washes. Store the packed column upright at 4°C with the gel bed submerged under 1-2 ml of Equilibration Buffer and the cap securely in place on top of the column. For long-term storage, sodium azide can be added to the buffer to a final concentration of 0.02%.

Step 5: Precipitation of Histone Proteins

1. Precipitate all core histones (or H2A/H2B and H3/H4 fractions) with 4% perchloric acid final concentration, overnight at 4°C. For example, add 60 µl of 70% perchloric acid to each 1 ml fraction. This step will eliminate most of the residual H1, as H1 remains soluble well above 4% perchloric acid. Vortex. Store at 4°C overnight.

   Note: H1 may be isolated from the H2A/H2B fraction or from the total core histone fraction by re-precipitating with 30% TCA. See Appendix, Section A for the protocol to isolate H1 protein.

2. Next day: Centrifuge the tubes in a microcentrifuge at maximum RCF for 1 hour at 4°C. Carefully remove the supernatant and discard.
3. Wash the pellet twice with 1 ml 4% perchloric acid to eliminate salt. Do not vortex, centrifuge in a microcentrifuge at maximum RCF for 5 minutes at 4°C. Remove the supernatant, leaving about 50-100 µl of each wash solution to avoid disturbing the pellet.
4. Wash twice as above with acetone containing 0.2% HCl.
5. Wash twice as above with 100% acetone.
6. Air dry for ~20 minutes, until the pellet is completely dry.
7. Resuspend in sterile water or TE. Flick the bottom of the tube with your finger to mix gently. Leave at room temperature for 20 minutes, then vortex, checking first to verify the pellet is in solution. Centrifuge briefly to collect all material. Measure protein concentration in Step 6. Store at -20°C or -80°C.

Step 6: Quantify Yield of Histone Proteins

Total core histone proteins (H2A/H2B and H3/H4 all together) can be quantified by measuring the absorbance at 230 nm. An OD of 0.42 of a sample diluted 1:10 indicates a concentration of 1 mg/ml of core histone proteins.

The core histone proteins react poorly with Coomassie dye in solution, and H1 does not react at all, so it is not advisable to use the Bradford method for calculating protein concentration. However, they will react with Coomassie in an SDS-PAGE gel, so it is possible to quantify the histone proteins in a gel, having run known quantities of histone proteins as standards.

Appendix

Section A: Purification of Histone H1

1. Because H1 protein remains soluble in 4% perchloric acid, some of the H1 can be recovered by re-precipitating the soluble perchloric acid fractions (from the H2A/H2B fraction or the total core histone fraction). Add 100% TCA to a final concentration of 30% TCA, then incubate overnight at 4°C.
2. Next day: Centrifuge in a microcentrifuge at maximum RCF for 1 hour at 4°C.
3. Wash twice with 1.5 ml 30% TCA. Do not vortex the pellet, centrifuge in a microcentrifuge at maximum RCF for 5 minutes at 4°C. Remove the supernatant without disturbing the pellet.
4. Wash twice with acetone containing 0.2% HCl.
5. Wash twice with 100% acetone.
6. Air dry for ~20 minutes, until the pellet is completely dry.
7. Resuspend in sterile water or TE. Flick the bottom of the tube with your finger to mix gently. Leave at room temperature for 20 minutes, then vortex, checking first to verify the pellet is in solution. Centrifuge briefly to collect all material. Measure protein concentration by Lowry or BCA assay, or by SDS-PAGE with known standards. Store at -20°C or -80°C.

Section B: Troubleshooting Guide

Problem/question	Recommendation
The column flow is stopped or intermittent.	Remove air bubbles from the purification resin. Flick the side of the purification column to gently force the air bubbles out. If bubbles are still present, pipette the resin up and down and allow the column to settle again.
	Be sure the input material does not contain large un-homogenized particles, which can impede the flow of buffer through the purification resin.
There is no visible pellet after spinning the precipitated fractions.	In general, a small white pellet will be visible. Try not to disturb the pellet to prevent the pellet from pouring out of the tube during the wash steps, and take care to avoid aspirating the pellet when pipetting. Leave some of the wash buffer to avoid disturbing the pellet; do not vortex it. Also, the histones will not precipitate well if the preparation is too dilute. Elute the sample in a lower volume to obtain more concentrated preparations.
Neutralizing the pellet from Step 2 for further analysis.	Do not vortex the pellet or mix by pipetting. Leave for 10 minutes, then pour out the neutralizing Tris buffer without disturbing the pellet. Resuspend the pellet in 2X SDS-PAGE sample buffer (0.5 ml per two 150 mm dishes). If the solution is yellow (still acidic) add more 1M Tris HCl pH 8.0 to neutralize it (for example 50 µl or less until it turns blue in color). Store at -20 to -80°C or analyze by SDS-PAGE.
How do I monitor for protein in the washes and flow-through?	The purification can be monitored by sampling 10 µl of the washes and flow-through and adding to 200 µl of Bradford reagent to check for blue color. Fractions can also be analyzed by SDS-PAGE, loading 15 µl per lane.
Is it possible to do all the protocol by gravity-flow instead of centrifugation?	Yes, but this is an improved method that takes less time and avoids some clogging problems by preventing air bubbles being trapped into the column.
Is it possible to elute H2A/H2B by spinning-down?	Yes, but with a reduction in the amount of H2A/H2B recovered.
Air bubbles are trapped in the column	The centrifugation method avoids most clogging problems associated with air bubbles trapped in the column bed. Be sure the crude histone preparation does not contain any impurities or cell debris, which can impede column flow. Be careful not to introduce air bubbles that might be present in the crude histone sample into the column. To remove trapped air bubbles from the column, replace the column bottom cap, add some wash buffer (0.3 ml) to the top of the column and gently flick the column gently, or slowly pipette the column resin up and down. Let the column resin settle for 5 minutes and remove out the air bubbles by pipetting away the top layer of buffer.
Are there additional methods to clean the column resin?	Although all the proteins should be removed after washing with H3/H4 Elution Buffer (Step 4, number 8), if deep column cleaning is desired, a washing step with 3 ml 0.2N NaOH is recommended (repeat twice for a total of three washes). Then repeat Step 3, numbers 6 and 7, column rinsing and equilibration.
Do histone proteins react with Coomassie dye in solution?	No, the core histones react poorly with Coomassie dye in solution, and H1 does not react at all. However, H1 and core histones are effectively stained with Coomassie in SDS-PAGE gels.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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