Histone Purification Microplate Kit

(version A2)

Catalog No. 40027

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Introduction

Active Motif's Histone Purification Microplate Kit enables you to purify the core histone proteins and enrich for histone fractions while preserving their post-translational modifications (*e.g.* acetylation, methylation, and phosphorylation). This purification method is an improvement over acid precipitation methods and utilizes a convenient spin column and a proprietary buffer system to purify the core histones from small volumes of cell and tissue samples. Histones isolated by this method are suitable substrates for downstream assays including analysis of histone modifications by Active Motif's Histone Modification ELISAs, Histone PTM Multiplex assay for Luminex® instruments, Western blot or mass spectrometry.

Unlike standard acid extraction techniques, this kit uses proprietary technology to purify the core histones and remove contaminating proteins that may affect downstream analysis. 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.

The Histone Purification Microplate Kit provides reagents to perform 96 histone purifications and is designed to increase the throughput of sample analysis. The purification columns are supplied as 8-well strips enabling both low and high throughput sample analysis.

product	format	catalog no.
Histone Purification Microplate Kit	96 rxns	40027

Kit Performance

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

Purity of the core histones isolated with the Histone Purification Microplate Kit

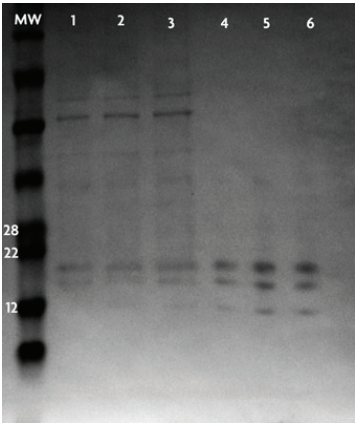
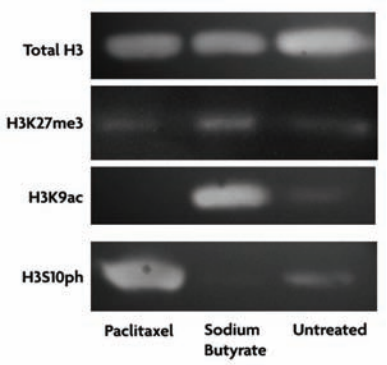


Figure 1: SDS-PAGE comparison of the purity of histone fractions isolated by crude acid extraction or purified using the Histone Purification Microplate Kit.

Histones were extracted from 2 x 10^5 HeLa cells using either crude acid extraction conditions (Lanes 1-3) or the Histone Purification Microplate Kit (Lanes 4-6). Nine µl of each biological replicate were loaded and run on a 4-20% SDS gradient gel, then stained with Ponceau. The crude acid extracted material shows contamination of other acid soluble proteins in the elution, while the Histone Purification Microplate Kit yields a pure fraction of core histones.

Histone Purification Microplate Kit preserves post-translational modifications

Active Motif's Histone Purification Microplate Kit preserves phospho, acetyl and methyl post-translational modifications (PTMs) on histones. The Western blot data shown below of paclitaxel-treated HeLa (lane 1), sodium butyrate-treated HeLa (lane 2), and unstimulated HeLa (lane 3) purified from 0.75 x 10^6 cells demonstrates that the PTMs are intact following purification of histones.



Determining Histone Yields

Active Motif's Histone Purification Microplate Kit provides a pure fraction of core histone proteins (H2A, H2B, H3 and H4) from as little as 0.9 x 10^5 cells. Due to the low sample volumes used in this assay, quantification of histone proteins is not possible using traditional methods for protein determination as the yields often fall below the detectable limits of those methods. We suggest using Active Motif's Histone H3 Total ELISA Kit to quantify the amount of histone H3 recovered.

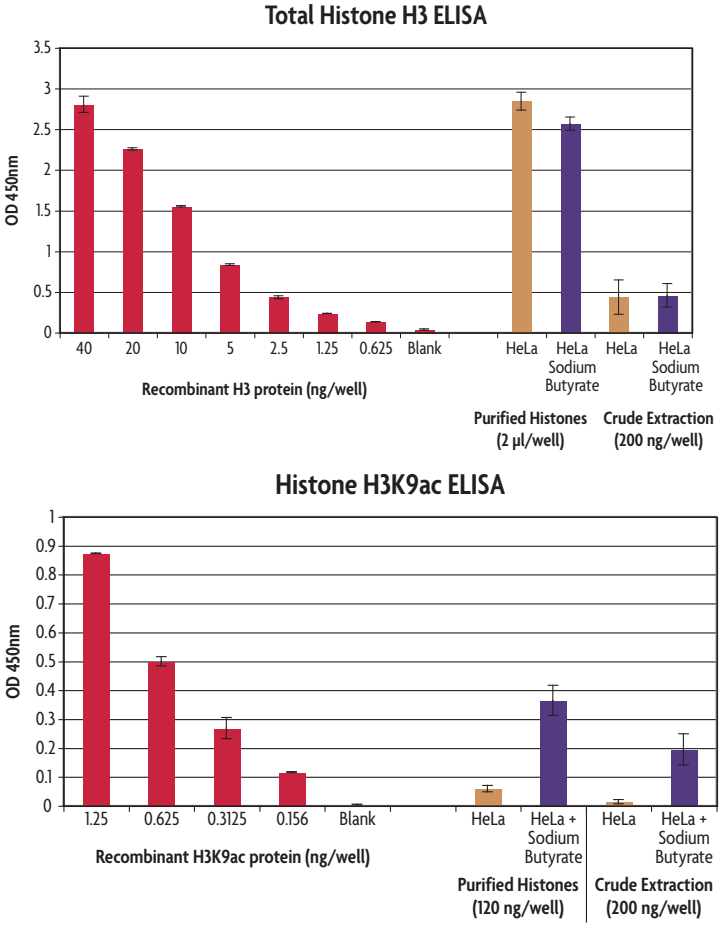


Figure 2: Determination of Histone H3 yields following Histone Microplate Purification.

Histones were extracted from 0.9 x 10^5 HeLa cells using the Histone Purification Microplate Kit. In the top image, the quantity of Total H3 recovered from the purification was determined using the Recombinant Histone H3 protein standard curve in the Total Histone H3 ELISA Kit (Catalog No. 53110) with 2 µl of purified histone sample or 200 ng crude acid extracted histone proteins. The Total H3 levels were consistent between both the untreated and treated HeLa cells as expected. The same samples were also run in the Histone H3K9ac ELISA (Catalog No. 53114) to show the increase in acetylation levels when the cells were treated with the HDAC inhibitor, sodium butyrate. In both assays, purified histones show greater sensitivity in the assay using less sample material than crude acid extracts.

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
Extraction Buffer	30 ml	4°C
5X Neutralization Buffer	8 ml	4°C
Equilibration Buffer	60 ml	4°C
Histone Wash Buffer	60 ml	4°C
Histone Elution Buffer	20 ml	4°C
Purification Columns	96 ea	Room Temp
Collection/Elution Microplates	4 ea	Room Temp

Additional materials required

- Cell culture plates and medium
- (Optional) 1 ml Dounce homogenizer for tissue samples (Active Motif Catalog No. 40401)
- Centrifuge with 96-well plate adaptor
- Rotating platform
- 1.7 ml microcentrifuge tubes
- Sterile water or Tris-EDTA, pH 8.0
- For precipitation of histones: Perchloric acid, 70% (Acros Organics, part no. 424030010)
- For washing of histones:
  4% perchloric acid
  0.2% HCl in acetone*
  100% acetone (VWR, part no. BDH1101)

* Add 0.5 ml of HCl stock (36.8%, Sigma, part no. H-7020) and adjust the volume with acetone until 92 ml. Store in a glass bottle at 4°C.

Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Section A: Prepare Sample Extracts

The yield of purified histones using this protocol is proportional to the amount of starting material used. For optimal recovery we recommend using at least 0.9 x 10^5 cells per purification. To determine cell number, prepare a mock well under the same growth conditions as your sampe. Count cells following cell scraping for an estimate of the number of cells per well available for purification. If the samples are to be treated with chemicals that may slow or inhibit growth, it may be necessary to pool multiple wells of cell culture material together for a single purification. We do not recommend exceeding 1 x 10^6 cells for a single purification column. Please determine the experimental set up for your culture conditions based upon these recommendations.

Table 1:

Culture plate	Estimated cell # post cell scrape*	Wash Medium Volume	Extraction Buffer Volume	Expected Histone H3 yield** (ng/µl)
6-well plate	0.75 x 10^6 cells	2 ml	300 µl	40 ng/µl
12-well plate	0.3 x 10^6 cells	2 ml	300 µl	18 ng/µl
24-well plate	0.9 x 10^5 cells	1 ml	200 µl	6 ng/µl

* Cell number estimates are based on HeLa cell confluency. Please adjust estimates based on cell type being tested.
** Expected yield is based on total Histone H3 measurements quantified using Active Motif's Total Histone H3 ELISA Kit (Catalog No. 53110). Yields may vary depending on the cell type tested and the density at the time of the extraction. Yields are provided as a guideline only. Please adjust the sample size or number of wells to use based on the needs of your downstream application.

For adherent cells:

1. Grow cells to 80-90% confluence in the appropriate medium. If treating the cells, perform the treatment as needed to harvest the cells at 80-90% confluency. Discard media and wash the cells 2 times with room temperature serum-free media using the volume recommendations provided in Table 1.
2. After the second wash, aspirate any remaining wash media and add **ice-cold** Extraction Buffer to each dish using the volume recommendations provided in Table 1. Using a plastic scraper (for 24-well plates, the wide end of a P200 pipette tip can be used to scrape the cells) and a pipet, collect the cell protein extracts and transfer to a new v-bottom microplate. Pipet up and down to homogenize the sample. Cover the plate with adhesive plate sealer. (If working with only a few samples, we recommend transferring the cells to microcentrifuge tubes.)

For suspension cells:

1. Grow cells to about 80-90% confluence in appropriate medium. If treating the cells, perform the treatment as needed to harvest the cells at 80-90% confluency. If necessary, gently scrape the cells from the sides of the dish while keeping them in their media.

2. Pellet the cells using room temperature centrifugation. If using culture plate centrifugation, use appropriate plate adaptor and spin covered at the maximum speed allowed for your plate adaptor for 10 minutes. Alternatively, transfer the cells and media to a 15 ml conical tube and centrifuge at 1000 x *g* for 5 minutes at room temperature.
3. Wash the cells twice with 2 ml room temperature serum-free media. After each wash, centrifuge as in Step 2 above, then discard the serum-free media.
4. Resuspend the cells in 200 µl **ice-cold** Extraction Buffer. Pipet up and down to homogenize the cells, then transfer the resuspended cells to a new v-bottom microplate. Cover the plate with adhesive plate sealer. (If working with only a few samples, we recommend transferring the cells to microcentrifuge tubes.)

For tissues:

1. Homogenize the tissue completely in an ice-cold 1 ml dounce homogenizer using 200 µl **ice-cold** Extraction Buffer. Keep the homogenate on ice.
2. Transfer the homogenate into a fresh microcentrifuge tube. Due to the limitations on centrifugation speeds using plate adaptors, we recommend using microcentrifuge tubes when working with tissue samples.

Section B: Prepare Crude Core Histone Extract

1. Leave the cells in Extraction Buffer for 2 hours (or overnight) on a rotating platform at 4°C.
2. If using microplates, use appropriate plate adaptor and spin at maximum speed allowed for you plate adaptor for 25 minutes at 4°C. If using microcentrifuge tubes, spin at maximum speed in a microfuge for 5 minutes at 4°C.
3. Transfer the supernatant, which contains the crude histones, to a new microplate or microcentrifuge tube. The pellet can be discarded or stored at -20°C for future analysis. If the pellet is to be stored, neutralize it by adding 50 µl of 1 M Tris HCl pH 8.0.
4. Neutralize and equilibrate the crude histones with 1/4 volume of 5X Neutralization Buffer. For example, add 50 µl of 5X Neutralization Buffer to 200 µl crude histones or 75 µl of 5X Neutralization Buffer to 300 µl crude histones. Store the crude histones at -80°C, or continue with Section C.

Section C: Purify Core Histones

1. Prepare the necessary number of purification columns. Remove columns that will not be used from the holder. Align the holder containing the purification columns being used over an empty v-bottom microplate for collection of the flow through. Equilibrate the spin columns by adding 200 µl Equilibration Buffer to each column, then centrifuging using a plate adaptor at 40 x *g* for 2 minutes at 4°C.
2. Discard the flow through and repeat the equilibration two more times by adding 200 µl

Equilibration Buffer to each column, then centrifuging using a plate adaptor at 40 x *g* for 2 minutes at 4°C. Discard flow through after each spin.

Note: Do not allow the column to dry out between equilibration centrifugations or the addition of the crude histones.

3. Immediately add 200 µl of the neutralized crude histones to the column and centrifuge at 40 x *g* for 2 minutes at 4°C. (Do not overload the column with the addition of more than a 200 µl volume.) Discard the flow-through. Repeat this step as many times as necessary to load the entire sample on the column.
4. Wash the columns with 200 µl Histone Wash Buffer and centrifuge at 40 x *g* for 2 minutes at 4°C. Discard the flow through. Repeat this step twice for a total of three washes.
5. To elute the purified histone, align the columns over a new v-bottom microplate. Add 100 µl of Histone Elution Buffer and centrifuge at 40 x *g* for 2 minutes at 4°C. Save the flow-through containing the eluted histone proteins. Perform a second elution into the same plate by adding 100 µl of Histone Elution Buffer and centrifuge at 40 x *g* for 2 minutes at 4°C. Save the flow-through containing the eluted histone proteins.
6. Transfer the eluted histone proteins (~ 200 µl) to labeled microcentrifuge tubes. Proceed with Section D.

   Note: The eluted fraction contains high levels of salts in the buffer that may interfere with downstream binding assays or other applications not compatible with high salt buffers. This protocol contains a precipitation step to remove the high salt buffers from the purified histone fraction. It is recommended to perform the precipitation step in microcentrifuge tubes to allow for high speed centrifugation to ensure complete recovery of the histone proteins. Centrifugation in the microplate will reduce efficiency and yield and is not recommended.

Section D: Precipitate Histone Proteins

Precipitation buffers should be prepared in advance and placed on ice. It is important to use ice-cold buffers and refrigerated centrifugation during the precipitation process to ensure complete recovery of the histone proteins.

1. Precipitate the histone proteins overnight at 4°C by adding perchloric acid to a final concentration of 4%. For example add 12 µl of 70% perchloric acid to each 200 µl combined elution, then vortex and quick spin.
2. On the following day, spin the samples at maximum speed in a microfuge for 1 hour at 4°C. Place the tubes in the microfuge in a marked direction as the pellet will not be visible and it is important to avoid disturbing the pellet in the following steps. A refrigerated microfuge is essential for proper precipitation of the histones. Centrifugation at warmer temperatures will reduce the recovery of the purified histones by half.

3. Due to the small amount of sample material used in this assay, it may not be possible to see the pellet. Carefully remove and discard the supernatant trying to avoid the area where the pellet should have formed. Leave approximately 100 µl behind to avoid dislodging the pellet.
4. Gently wash the histone pellet with 1 ml ice-cold **4% perchloric acid**. Do not vortex the pellet. Centrifuge for 5 minutes at maximum speed in a refrigerated microfuge at 4°C. Carefully remove and discard the supernatant trying to avoid the area where the pellet should have formed. Leave approximately 100 µl behind to avoid dislodging the pellet. Repeat this step for a total of two washes.
5. Wash the histone pellet two times using 1 ml ice-cold **acetone containing 0.2% HCl**. Do not vortex the pellet. Centrifuge for 5 minutes at maximum speed in a refrigerated microfuge at 4°C. Carefully remove and discard the supernatant trying to avoid the area where the pellet should have formed. Leave approximately 100 µl behind to avoid dislodging the pellet
6. Wash the histone pellet two times using 1 ml ice-cold **acetone**. Do not vortex the pellet. Centrifuge for 5 minutes at maximum speed in a refrigerated microfuge at 4°C. Carefully remove and discard the supernatant trying to avoid the area where the pellet should have formed. Leave approximately 100 µl behind to avoid dislodging the pellet
7. Air dry the pellet (containing 100 µl acetone) until it is completely dry. This will take approximately 1 – 2 hours.
8. Resuspend in sterile water or TE. Flick the bottom of the tube gently with a finger. Let the pellet resuspend 10 to 20 minutes at room temperature, then gently vortex and centrifuge briefly. Check to make sure that pellet is completely resuspended. Store at -20°C or -80°C.
9. Quantification of histone proteins from small cell numbers is not possible using traditional methods for protein quantification, such as Bradford or absorbance at 230 nm, as the yield will fall below the detectable range for these methods. Instead, we suggest using Active Motif's Histone H3 Total ELISA Kit to quantify the amount of histone H3 recovered. ELISAs for specific histone modifications are also available to quantify the amount of a particular histone PTM within the sample. Alternatively, Active Motif's Histone H3 PTM Multiplex Assay can be used in combination with Total Histone H3 antibody-conjugated bead set to screen histone H3 levels.

    Total Histone H3 ELISA (Cat. No. 53110) – we suggest using a starting volume of 2 µl per well of purified histones extracted from 1 x 10^5 cells in the ELISA.

    Modification Histone ELISAs – we suggest using a starting volume of 20 µl per well of purified histones extracted from 1 x 10^5 cells in the ELISA.

    Histone H3 PTM Multiplex Assay (Cat. No. 33115) – we suggest using a starting volume of 10 µl per well of purified histones extracted from 1 x 10^5 cells in the assay.

Appendix

Section A: Troubleshooting Guide

Problem/question	Recommendation
Little or no histone is purified	Start with higher amounts of sample material. We recommend using between 0.9 x 10^5 to 1 x 10^6 cells per column. Additionally, use microcentrifuge tubes for the transfer of the cells in the extraction buffer. Higher centrifugation speeds can be obtained with microcentrifuge tubes which may result in an increase in histone yield. All precipitation steps should be performed using ice-cold reagents and a refrigerated microfuge. Yields of histones drops if these conditions are not followed.
	The pH of the neutralized sample may be too low/high for binding to the column. The optimal pH for the column is approximately pH 8. Check the pH using colorpHast pH-indicator strips (pH 0-14) by placing 1 µl of neutralized sample onto the yellow/green pH square used to test pH in the range of pH 5 to pH 10.
	Do not use gravity flow for the columns. The volumes used are too low for adequate flow-through. Use the recommendations for centrifugation of the columns.
In the precipitation step, there is no visible pellet after spinning the histone samples.	In general, the pellet is invisible at low cell numbers. Leave some of washing solutions in the tube 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 less volume to obtain more concentrated preparations.
Is it possible to eliminate H1 from the final preparation of histones?	Yes. Precipitate the histone proteins in perchloric acid 4% (H1 protein remains soluble). H1 protein can be recovered by re-precipitation of the soluble fraction using 20-30% trichloroacetic acid.
Do the histone proteins react with Coomassie dye while in solution?	No. Core histones react poorly with Coomassie dye while in solution, and H1 does not react at all. However, H1 and core histones are stained by Coomassie effectively in gel.

Section B: Related Products

Histone Antibodies

For an up-to-date list of over 140 antibodies against histones and modified histones, please visit **www.activemotif.com/histoneabs**.

Histone ELISAs	Format	Catalog No.
Total Histone H3 ELISA	1 x 96 rxns	53110
Histone H3 monomethyl Lys4 ELISA	1 x 96 rxns	53101
Histone H3 dimethyl Lys4 ELISA	1 x 96 rxns	53112
Histone H3 trimethyl Lys4 ELISA	1 x 96 rxns	53113
Histone H3 acetyl Lys9 ELISA	1 x 96 rxns	53114
Histone H3 dimethyl Lys9 ELISA	1 x 96 rxns	53108
Histone H3 trimethyl Lys9 ELISA	1 x 96 rxns	53109
Histone H3 phospho Ser10 ELISA	1 x 96 rxns	53111
Histone H3 acetyl Lys14 ELISA	1 x 96 rxns	53115
Histone H3 monomethyl Lys27 ELISA	1 x 96 rxns	53104
Histone H3 trimethyl Lys27 ELISA	1 x 96 rxns	53106
Histone H3 phospho Ser28 ELISA	1 x 96 rxns	53100

ChIP-IT® Kits	Format	Catalog No.
ChIP-IT® High Sensitivity	16 rxns	53040
ChIP-IT® Express	25 rxns	53008
ChIP-IT® Express Enzymatic	25 rxns	53009
ChIP-IT® Express Shearing Kit	10 rxns	53032
ChIP-IT® Express Enzymatic Shearing Kit	10 rxns	53035
ChIP-IT® Express HT	96 rxns	53018
Re-ChIP-IT®	25 rxns	53016
RNA ChIP-IT®	25 rxns	53024
Chromatin IP DNA Purification Kit	50 rxns	58002
EpiShear™ Multi-Sample Sonicator	110 V	53062
EpiShear™ Probe Sonicator	110 V	53051
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014
Siliconized Tubes, 1.7 ml	25 tubes	53036
ChIP-IT® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® Control qPCR Kit – Human	5 rxns	53026
ChIP-IT® Control qPCR Kit – Mouse	5 rxns	53027
ChIP-IT® Control qPCR Kit – Rat	5 rxns	53028
ChIP-IT® Control Kit – Human	5 rxns	53010
ChIP-IT® Control Kit – Mouse	5 rxns	53011
ChIP-IT® Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021
Bridging Antibody for Mouse IgG	500 µg	53017
Dounce Homogenizer	1 ml	40401
Dounce Homogenizer	15 ml	40415

ChIP-validated Antibodies

For an up-to-date list of over 135 ChIP-validated antibodies, please visit **www.activemotif.com/chipabs**.

Co-Immunoprecipitation	Format	Catalog No.
Universal Magnetic Co-IP Kit	25 rxns	54002
Nuclear Complex Co-IP Kit	50 rxns	54001

Modified Histones Array	Format	Catalog No.
MODified™ Histone Peptide Array	1 array	13001

Histone Purification & Chromatin Assembly	Format	Catalog No.
Histone Purification Kit	10 rxns	40025
Histone Purification Mini Kit	10 rxns	40026
Chromatin Assembly Kit	10 rxns	53500
HeLa Core Histones	36 µg	53501

Histone Acetyltransferase and Deacetylase Activity	Format	Catalog No.
HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100
Recombinant p300 protein, catalytic domain	5 µg	31205
Recombinant GCN5 protein, active	5 µg	31204
HDAC Assay Kit (Fluorescent)	1 x 96 rxns	56200
HDAC Assay Kit (Colorimetric)	1 x 96 rxns	56210

Recombinant Methylated, Acetylated & Phosphorylated Histone Proteins	Format	Catalog No.
Recombinant Histone H2A	50 µg	31251
Recombinant Histone H2B	50 µg	31252
Recombinant Histone H3 (C110A)	100 µg	31207
Recombinant Histone H3 biotinylated	25 µg	31271
Recombinant Histone H3 phospho Thr3 (H3T3ph)	25 µg	31274
Recombinant Histone H3 monomethyl Lys4 (H3K4me1)	50 µg	31208
Recombinant Histone H3 dimethyl Lys4 (H3K4me2)	50 µg	31209
Recombinant Histone H3 trimethyl Lys4 (H3K4me3)	50 µg	31210

Recombinant Histone Proteins (continued)	Format	Catalog No.
Recombinant Histone H3 acetyl Lys9 (H3K9ac)	25 µg	31253
Recombinant Histone H3 monomethyl Lys9 (H3K9me1)	50 µg	31211
Recombinant Histone H3 dimethyl Lys9 (H3K9me2)	50 µg	31212
Recombinant Histone H3 trimethyl Lys9 (H3K9me3)	50 µg	31213
Recombinant Histone H3 phospho Ser10 (H3S10ph)	25 µg	31272
Recombinant Histone H3 acetyl Lys14 (H3K14ac)	25 µg	31254
Recombinant Histone H3 monomethyl Lys14 (H3K14me1)	50 µg	31256
Recombinant Histone H3 dimethyl Lys14 (H3K14me2)	50 µg	31257
Recombinant Histone H3 trimethyl Lys14 (H3K14me3)	50 µg	31258
Recombinant Histone H3 acetyl Lys18 (H3K18ac)	25 µg	31273
Recombinant Histone H3 monomethyl Lys18 (H3K18me1)	50 µg	31259
Recombinant Histone H3 dimethyl Lys18 (H3K18me2)	50 µg	31260
Recombinant Histone H3 trimethyl Lys18 (H3K18me3)	50 µg	31261
Recombinant Histone H3 acetyl Lys23 (H3K23ac)	25 µg	31255
Recombinant Histone H3 monomethyl Lys23 (H3K23me1)	50 µg	31262
Recombinant Histone H3 dimethyl Lys23 (H3K23me2)	50 µg	31263
Recombinant Histone H3 trimethyl Lys23 (H3K23me3)	50 µg	31264
Recombinant Histone H3 monomethyl Lys27 (H3K27me1)	50 µg	31214
Recombinant Histone H3 dimethyl Lys27 (H3K27me2)	50 µg	31215
Recombinant Histone H3 trimethyl Lys27 (H3K27me3)	50 µg	31216
Recombinant Histone H3 monomethyl Lys36 (H3K36me1)	50 µg	31217
Recombinant Histone H3 dimethyl Lys36 (H3K36me2)	50 µg	31218
Recombinant Histone H3 trimethyl Lys36 (H3K36me3)	50 µg	31219
Recombinant Histone H3 monomethyl Lys79 (H3K79me1)	50 µg	31220
Recombinant Histone H3 dimethyl Lys79 (H3K79me2)	50 µg	31221
Recombinant Histone H3 trimethyl Lys79 (H3K79me3)	50 µg	31222
Recombinant Histone H4	50 µg	31223
Recombinant Histone H4 monomethyl Lys5 (H4K5me1)	50 µg	31265
Recombinant Histone H4 dimethyl Lys5 (H4K5me2)	50 µg	31266
Recombinant Histone H4 trimethyl Lys5 (H4K5me3)	50 µg	31267
Recombinant Histone H4 monomethyl Lys16 (H4K16me1)	50 µg	31268
Recombinant Histone H4 dimethyl Lys16 (H4K16me2)	50 µg	31269
Recombinant Histone H4 trimethyl Lys16 (H4K16me3)	50 µg	31270
Recombinant Histone H4 monomethyl Lys20 (H4K20me1)	50 µg	31224
Recombinant Histone H4 dimethyl Lys20 (H4K20me2)	50 µg	31225
Recombinant Histone H4 trimethyl Lys20 (H4K20me3)	50 µg	31226

For an up-to-date list of Recombinant Histone Proteins, please visit **www.activemotif.com/recombhis**.

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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