10 Old Barn Road • Lake Placid, NY 12946
Technical Support: T: 800 548-7853 • F: 518 523-4513
email: techserv@upstatebiotech.com
Sales Department: T: 800 233-3991 • F: 781 890-7738
Licensing Dept.: 800 310-4659
www.upstatebiotech.com

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Certificate of Analysis

Anti-acetyl-Histone H4, ChIP Grade

(rabbit antiserum)
Catalog # 06-866
Lot # 23201

Immunogen: KLH-conjugated peptide [AGG$_{Ac}$KGG-$_{Ac}$KGMG$_{Ac}$KVGA$_{Ac}$KRHS-C] corresponding to amino acids 2-19 of *Tetrahymena* histone H4.

Specificity: Recognizes acetylated histone H4 of approximately 10kDa. Cross-reacts with acetylated histone H2B from *Tetrahymena* and weakly cross-reacts with acetylated histone H2B from HeLa cells. May cross-react with other acetylated proteins.

Cross-reactivity: Human and *Tetrahymena*. Other species not tested, but expected to cross-react since Histone H4 is well conserved.

Storage and Stability: Stable for 2 years at -20°C from date of shipment. Aliquot to avoid repeated freezing and thawing. For maximum recovery of product, centrifuge the original vial after thawing and prior to removing the cap.

Formulation: **200µl** of whole antiserum containing 0.05% sodium azide. Frozen solution.

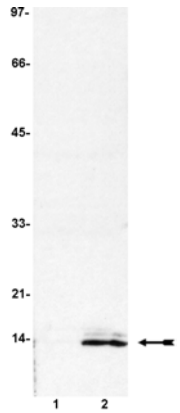
FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Quality Control Testing

Immunoblot Analysis: 1:2000 dilution of this lot detected acetylated histone H4 in *Tetrahymena* macronuclei and acid extracted proteins from HeLa cells treated with 5mM sodium butyrate. Sodium butyrate, an inhibitor of deacetylases, was used to enhance detection of acetylated histone H4.

Chromatin Immunoprecipitation: 5-10µl of a previous lot immunoprecipitated transcriptionally active chromatin containing acetylated histone H4 from 2 X 10^6 serum stimulated HeLa cells.

Immunocytochemistry: Not recommended. Use Catalog # 06-598.



Immunoblot Analysis.
Representative blot from a previous lot. Acid-extracted proteins from normal HeLa cells (Lane 1) and HeLa cells treated with 5mM sodium butyrate for 24 hours (Lane 2) were resolved by electro-phoresis, transferred to nitro-cellulose and probed with anti-acetyl Histone H4 (1:2000). Proteins were visualized using a goat-anti rabbit secondary antibody conjugated to HRP and a chemi-luminescence detection system. Arrow indicates acetylated histone H4 (10kDa).

Application References:

1. Alberts, A. S., *et al.*, Cell **92**: 475-487, 1998.
2. Braunstein, M., *et al.*, Genes & Devel. **7:** 592-604, 1993.
3. Lin, R., *et al.*, J. Cell Biol. **108**: 1577-1588, 1989.
4. Perry, C.A., *et al.*, Biochem. **32**: 13605-13614, 1993.

Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on acid-extracted protein from cells treated with or without sodium butyrate (see the protocol below) and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared PBS containing 3% nonfat dry milk (Catalog # 20-200), (PBS-MLK) for one hour at room temperature with constant agitation.
3. Incubate the nitrocellulose with **1:2000 dilution of anti-acetyl Histone H4**, **ChIP Grade** in freshly prepared PBS-MLK, overnight with agitation at 4°C.
4. Wash the nitrocellulose three times with water.
5. Incubate the nitrocellulose in the secondary reagent of choice (a **goat anti-rabbit** IgG, Catalog # 12-348, 1:5000 dilution was used) in PBS-MLK for 1.5 hours at room temperature with agitation.
6. Wash the nitrocellulose with water three times.
7. Wash the nitrocellulose in PBS-0.05% Tween 20 for 5 minutes.
8. Rinse the nitrocellulose with 4-5 changes of water.
9. Use detection method of choice (enhanced chemiluminescence was used).

Acid Extraction of Proteins from Sodium Butyrate Treated HeLa Cells

1. Grow cells to 70% confluency in DMEM supplemented with 10% FBS.
2. Add sodium butyrate (100mM sterile stock solution), which inhibits histone deacetylases, to a final concentration of 5mM and continue to grow the cells for 24 hours.
3. Scrape the cells from the plate.
4. Pellet the cells by centrifugation at 200 x g for 10 minutes.
5. Decant the supernatant fraction.
6. Suspend the cells with 10-15 volumes of PBS and centrifuge at 200 x g for 10 minutes.
7. Decant supernatant fraction (PBS wash).
8. Suspend the cell pellet in 5-10 volumes of **lysis buffer.**
9. Add sulfuric acid to a final concentration of 0.2M (0.4N). **Use polypropylene tubes.**
10. Incubate on ice for 30 minutes.
11. Centrifuge at 11,000 x g for 10 minutes at 4°C.
12. Keep the supernatant fraction, which contains the acid soluble proteins, and discard the acid-insoluble pellet.
13. Dialyze the supernatant against 200ml 0.1M (0.1N) acetic acid, twice for 1-2 hours each.
14. Dialyze three times against 200ml H_20 for 1hour, 3 hours, and overnight, respectively. The protein can be quantified and lyophilized or stored at -70°C.

Lysis buffer:

10mM HEPES, pH 7.9
1.5mM $MgCl_2$
10mM KCl
*0.5mM DTT
*1.5mM PMSF

*Add PMSF and DTT just prior to use of the buffer.

CHROMATIN IMMUNOPRECIPITATION PROTOCOL

Part A. Optimization of DNA Shearing

Establish optimal conditions required for shearing cross-linked DNA to 200-1000 base pairs in length by following steps 1- 9 below. Vary the power setting and/or the number of 10-second pulses during sonication of the samples. Be sure to keep the sample on ice at all times (the sonication generates heat which will denature the DNA). Check the size of sonicated DNA by gel electrophoresis after reversion of cross-links. Our experience shows DNA is sheared to the appropriate length with 3-4 sets of 10-second pulses using a Cole Parmer, High Intensity Ultrasonic Processor/Sonicator, 50-watt model equipped with a 2mm tip and set to 30% of maximum power. **Once sonication conditions have been optimized, keep cell number consistent for subsequent experiments. The protocol below for the optimization of DNA Shearing is for one Chip assay (~1 x 10^6 cells per condition).**

Note: Steps 3- 7 should be done on ice.

1. Stimulate or treat 1 x 10^6 cells on a 10cm dish as appropriate. (Cells should be treated under conditions for which transcriptional activation of the gene of interest has been demonstrated). Include one extra dish (1 X 10^6 cells) to be used solely for estimation of cell number.
2. Cross link histones to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% and incubate for 10 minutes at 37ºC. (For example, add 100ul 37% formaldehyde into 10ml of growth medium on plate).
3. Aspirate medium, removing as much medium as possible. Wash cells twice using ice cold PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml aprotinin and 1μg/ml pepstatin A). **Note:** Add protease inhibitors to PBS just prior to use. PMSF has a half-life of approximately 30 minutes in aqueous solutions.
4. Scrape cells into conical tube.
5. Pellet cells for 4 minutes at 2000 rpm at 4ºC. Warm **SDS Lysis Buffer (Catalog # 20-163)** to room temperature to dissolve precipitated SDS and add protease inhibitors (inhibitors: 1mM PMSF, 1μg/ml aprotinin and 1μg/ml pepstatin A).
6. Resuspend cell pellet in 200μl of **SDS Lysis Buffer (Catalog # 20-163)** and incubate for 10 minutes on ice. **Note:** The 200μl of SDS Lysis Buffer is per 1 X 10^6 cells; if more cells are used, the resuspended cell pellet should be divided into 200μl aliquots so that each 200μl aliquot contains ~1 X 10^6 cells.
7. Sonicate lysate to shear DNA to lengths between 200 and 1000 basepairs being sure to keep samples ice cold (***Note:*** Once sonication conditions have been optimized following steps 1 to 9, proceed to Part B, step 1 below).
8. Add 8μl **5M NaCl (Catalog # 20-159)** and reverse crosslinks at 65ºC for 4 hours.
9. Recover DNA by phenol/chloroform extraction and run sample (example 5μl,10μl and 20μl samples) in an agarose gel to visualize shearing efficiency.

Part B. Experimental protocol.

If sonication conditions have been optimized (Part A), complete steps 1 through 7 and continue with the protocol below. For a negative/background control, prepare a sample to use as a no-antibody immunoprecipitation control in step 5 below. Additionally, transcriptionally unactivated DNA samples should be prepared as controls for PCR in section II.

1. Centrifuge samples (part A, step 7) for 10 minutes at 13,000 rpm at 4°C, and add 200μl of the sonicated cell pellet suspension to a new 2ml-microcentrifuge tube.

2. Dilute the sonicated cell pellet suspension 10 fold in **ChIP Dilution Buffer (Catalog # 20-153)**, adding protease inhibitors as above. This is done by adding 1800μl ChIP Dilution Buffer to the 200μl sonicated cell pellet suspension for a final volume of 2ml in each immunoprecipitation condition. **Note:** *If proceeding to PCR a portion of the diluted cell pellet suspension 1% (~20μl) can be kept to quantitate the amount of DNA present in different samples at the PCR protocol, Part B, section II, step 6. This sample is considered to be your input/starting material and needs to have the Histone-DNA crosslinks reversed by heating at 65°C for 4 hours (see section II, step 3.)*

3. To reduce nonspecific background, pre-clear the 2ml diluted cell pellet suspension with 80µl of **Salmon Sperm DNA/Protein A Agarose-50% Slurry (Catalog # 16-157)** for 30 minutes at 4ºC with agitation.

4. Pellet agarose by brief centrifugation and collect the supernatant fraction.

5. Add the immunoprecipitating antibody (the amount will vary per antibody) to the 2ml supernatant fraction and incubate overnight at 4ºC with rotation. *For a negative control, perform a no-antibody immunoprecipitation by incubating the supernatant fraction with 60µl of* ***Salmon Sperm DNA/Protein A Agarose- 50% Slurry (Catalog # 16-157****) for one hour at 4ºC with rotation and proceed to step 7.*

6. Add 60µl of **Salmon Sperm DNA/Protein A Agarose Slurry (Catalog # 16-157**) for one hour at 4ºC with rotation to collect the antibody/histone complex.

7. Pellet agarose by gentle centrifugation (700 to 1000 rpm at 4ºC, ~1min). Carefully remove the supernatant that contains unbound, non-specific DNA. Wash the protein A agarose/antibody/histone complex for 3-5 minutes on a rotating platform with 1ml of each of the buffers listed in the order as given below:

a) Low Salt Immune Complex Wash Buffer (Catalog # 20-154), **one wash**
b) High Salt Immune Complex Wash Buffer (Catalog # 20-155), **one wash**
c) LiCl Immune Complex Wash Buffer (Catalog # 20-156), **one wash**
d) 1X TE (Catalog # 20-157), **two washes**

After step 7 above, the sample is now a protein A/antibody/histone/DNA complex ready for either an Immunoprecipitation/Immunoblot assay (Section I) or Polymerase Chain Reaction (PCR) assay (Section II):

Section I. Immunoprecipitation/Immunoblot protocol to detect histone.

1 Following washing of the beads in part B, step 7, immunoprecipitated histones can be analyzed by immunoblot analysis. Add 25µl of 1X Laemmli buffer per sample and boil for 10 minutes. Load 20µl per lane and perform immunoblot procedure as described per appropriate antibody.

Section II. PCR protocol to amplify DNA that is bound to the immunoprecipitated histone.

1. Freshly prepare elution buffer (1%SDS, 0.1M $NaHCO_3$).

2. Elute the histone complex from the antibody by adding 250µl elution buffer to the pelleted protein A agarose/antibody/histone complex from step 7d above. Vortex briefly to mix and incubate at room temperature for 15 minutes with rotation. Spin down agarose, and carefully transfer the supernatant fraction (eluate) to another tube and repeat elution. Combine eluates (total volume = ~500µl).

3. Add 20µl 5M NaCl (Catalog # 20-159) to the combined eluates (500µl)and reverse histone-DNA crosslinks by heating at 65ºC for 4 hours. At this step the sample can be stored and -20°C and the protocol continued the next day.

   Note: Include the input/starting material (*the sample saved from Part B, step 2, which has had the Histone-DNA crosslinks reversed*) as well as a transcriptionally-unactivated DNA sample as negative and background controls for the PCR reaction. *Previously, a 5µl sample has been used in a nested PCR reaction. However, the amount of sample used per reaction must be determined empirically (e.g., titrate the sample at this step by using 1, 2, 5, or 10µl per PCR reaction)*. If PCR results are poor, complete steps 4, 5 and 6 below to purify the DNA sample. NOTE: Handle the samples carefully, some DNA may be lost during the purification steps.

4. Add 10µl of 0.5M EDTA (Catalog # 20-158), 20µl 1M Tris-HCl, pH 6.5 (Catalog # 20-160) and 2µl of 10mg/ml Proteinase K to the combined eluates and incubate for one hour at 45ºC.

5. Recover DNA by phenol/chloroform extraction and ethanol precipitation. Addition of an inert carrier, such as 20µg glycogen or yeast tRNA, helps visualize the DNA pellet. Wash pellets with 70% ethanol and air dry.

6. Resuspend pellets in an appropriate buffer for PCR or slot-blot reactions. PCR or slot-blot conditions must be determined empirically.