ExactaChIP™

Human Acetyl-Histone H4 Chromatin Immunoprecipitation Kit

Catalog Number ECP5215

Reagents for the immunoprecipitation and detection of Acetyl-Histone H4 and Acetyl-Histone H4-associated DNA fragments.

This product contains sufficient materials to process 20 samples.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Chromatin is the structure composed of proteins (histones, enzymes, mediators, scaffold, and transcription factors), DNA, and RNA, which is found in the nucleus of eukaryotic cells. Chromatin mediates several central biological processes, such as cell-specific or tissue-specific expression patterns, DNA replication and repair, and mechanisms of gene expression. The understanding of *in vivo* mechanisms underlying gene expression is one of the most fascinating challenges of modern biology. The chromatin immunoprecipitation (ChIP) assay has become one of the most useful techniques in the study of gene expression mechanisms, identification of genetic networks, general transcription regulation proteins, and the identification of transcription factors and their target genes. This assay determines whether a certain protein-DNA interaction is present at a given location, condition, and time point. Protein-DNA complexes are fixed by formaldehyde crosslinking, the chromatin is sheared by sonication, and the protein of interest is immunoprecipitated, bringing along the protein-bound DNA fragments, which can then be detected by PCR. The use of an appropriate antibody for the immunoprecipitation step is considered the most critical factor for a successful ChIP assay (see Figure 1; references 1 - 6).

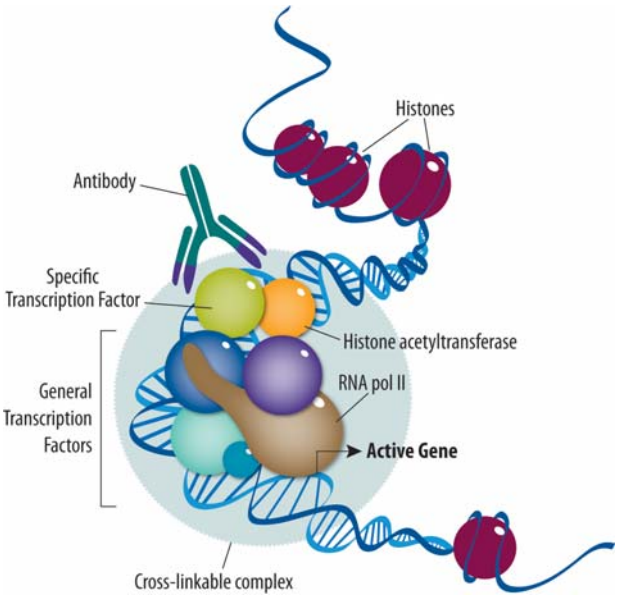

Figure 1: A schematic of a complex of DNA-bound proteins located at the promoter site of an active gene.

PRINCIPLE OF THE ASSAY

This ExactaChIP kit is designed to immunoprecipitate acetyl-histone H4-DNA complexes and to allow for the rapid PCR detection of acetyl-histone H4-bound DNA fragments. Epigenetics encompasses the cellular mechanisms that modulate gene expression without alterations in DNA sequence (as opposed to genetic changes). The three main categories of epigenetic modifications are: DNA methylation, RNA interference and histone post-translational modifications (7 - 9). Compelling evidence indicates that epigenetics in general, and histone modifications in particular, play a fundamental role in cancer, mammalian development and embryonic stem cell functions (10 - 13). Histones (H1 to H4) form the core chromatin structural proteins in eukaryotes, responsible for the formation of the nucleosomes and ultimately implicated in transcription, mitosis, spermatogenesis, and DNA repair. Histone modifications include acetylation, methylation, phosphorylation, and ubiquitination. Acetylation of histones H3 and H4 at various residues, mediated *in vivo* by Histone Acetyl Transferases (HAT), makes the nucleosome structures more accessible to nuclear factors, and are generally accepted as a hallmark of

transcriptionally active genes (14 - 16). Histone H4 acetylation at lysine residues 5, 8, 12, and 16, for example, have been correlated with promoters of actively transcribed genes (17 - 19). Therefore antibodies that recognize acetylated histones H3 and H4 constitute essential tools in studies investigating global expression patterns of eukaryotic genomes or the correlation between epigenetic signatures and human diseases (20 - 24).

The most important advantage of this kit is that it can be performed in 4 - 5 hours, instead of the traditional 2 - 4 days (25 - 28), by avoiding the use of time-consuming crosslinking reversal and overnight antibody incubation. In addition, it is a straightforward method that is easy to learn. The kit contains a biotinylated anti-human Acetyl-Histone H4 antibody made against a peptide corresponding to the first 20 amino acids of human histone H4 acetylated at the lysine residues 5, 8, 12 and 16 and a biotinylated normal sheep IgG antibody to be used as a negative control for the immunoprecipitation step.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.

MATERIALS PROVIDED

Anti-Acetyl-Histone H4 Antibody (Part 965626) - 100 µg of lyophilized biotinylated sheep anti-human acetyl-histone H4 polyclonal antibody acetylated at K5, K8, K12, and K16 (sufficient for processing 20 samples).

Normal Sheep IgG (Part 965818) - 25 µg of lyophilized biotinylated sheep IgG.

Lysis Buffer (Part 895588) - 20 mL of a proprietary formulation.

Dilution Buffer (Part 895589) - 25 mL of a proprietary formulation.

Wash Buffer 1 (Part 895590) - 25 mL of a proprietary formulation.

Wash Buffer 2 (Part 895591) - 25 mL of a proprietary formulation.

Wash Buffer 3 (Part 895592) - 25 mL of a proprietary formulation.

Wash Buffer 4 (Part 895593) - 25 mL of a proprietary formulation.

Chelating Resin Solution (Part 895594) - 2.5 mL of resin in buffer.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C for up to 6 months from the date of receipt.</td></tr>
<tr><td rowspan="6">Opened/ Reconstituted Reagents</td><td>Anti-Acetyl-Histone H4 Antibody</td><td rowspan="2">May be stored for up to 1 month at 2 - 8° C.* Aliquot and store for up to 3 months at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Normal Sheep IgG</td></tr>
<tr><td>Lysis Buffer</td><td>May be stored at room temperature for up to 3 months.*</td></tr>
<tr><td>Dilution Buffer</td><td rowspan="3">May be stored for up to 3 months at 2 - 8° C.*</td></tr>
<tr><td>Wash Buffers 1 - 4</td></tr>
<tr><td>Chelating Resin Solution</td></tr>
</table>

*Provided this is within 6 months of the date of receipt.

OTHER SUPPLIES REQUIRED

Materials

- Pipettes and pipette tips
- 1.5 mL microcentrifuge tubes
- Falcon tubes

Reagents

- Formaldehyde (37%; Sigma, Catalog # 252549 or equivalent)
- Glycine (Sigma, Catalog # G6388 or equivalent)
- Leupeptin (Sigma, Catalog # L8511 or equivalent)
- Aprotinin (Sigma, Catalog # A4529 or equivalent)
- Phenylmethylsulfonyl fluoride (PMSF; Sigma, Catalog # P7626 or equivalent)
- Streptavidin beads (magnetic beads, R&D Systems, Catalog # MAG999 or equivalent; or agarose beads, Sigma, Catalog # 85881 or equivalent)
- PCR kit (Applied Biosystems, Catalog # 4311806 or equivalent)
- Phosphate-buffered saline (PBS; Invitrogen, Catalog # 10010-023 or equivalent)
- Dimethyl sulfoxide (DMSO; Sigma, Catalog # D2650 or equivalent)
- DNA purification kit (Qiagen, Catalog # 28144 or equivalent)
- Deionized or distilled water

Equipment

- Sonicator
- Ultrasonic bath
- Rocking or shaking device
- Eppendorf tube rotator
- Benchtop ultracentrifuge
- Refrigerated ultracentrifuge
- Benchtop centrifuge
- Water bath and heatblock
- PCR thermocycler

If using magnetic beads, the following is also required:

- Magnet (R&D Systems, Catalog # MAG997 or equivalent)

PRECAUTIONS

Formaldehyde is flammable, highly toxic, and potentially carcinogenic. Refer to the MSDS from the supplier for handling and hazard information.

The Lysis Buffer is to be used at room temperature.

The Dilution Buffer and Wash Buffers are to be used at 2 - 8° C. It is important that the Dilution Buffer and Wash Buffers be kept cold prior to use.

REAGENT PREPARATION

Reconstitute the Anti-Acetyl-Histone H4 Antibody and Normal Sheep IgG in 100 µL of PBS.

ASSAY PROCEDURE

1. Fix the cells by adding 37% formaldehyde to a 1% final concentration (40 µL of formaldehyde per 1 mL of media) and, using a rocking or shaking device, swirl samples for 15 minutes at room temperature. **Note:** *The duration of the incubation and the final concentration of the formaldehyde can affect the efficiency of the procedure. A shorter incubation (e.g. 5 minutes) may improve shearing efficiency, however, it may affect the yield of precipitated DNA.*
2. Quench the formaldehyde by adding 1 M glycine to a final concentration of 125 mM (141 µL of glycine per mL of media). Swirl for 5 minutes at room temperature, pellet the cells, and remove the media. **Note:** *At this point, samples can be stored overnight at ≤ -70° C.*
3. Add protease inhibitors to the Lysis Buffer (10 µg/mL Leupeptin, 10 µg/mL Aprotinin, and 1 mM PMSF). Resuspend the cell pellet in 500 µL of Lysis Buffer per 5 x 10^6 cells. Pipette up and down to resuspend the cells, and incubate on ice for 10 minutes. **Note:** ***Keep samples on ice from this step forward.***
4. Sonicate the samples to shear chromatin to an average length of about 1 kilobase (optimization may be required; see the Technical Hints section for details). Transfer 500 µL of each sample to 1.5 mL microcentrifuge tubes. **Note:** *It is important to keep the samples on ice during this step.*
5. Centrifuge the lysates for 10 minutes using a refrigerated ultracentrifuge at 12,000 x g. Collect the supernatant in a clean tube, and discard the pellet.
6. Dilute the supernatant by adding 1 mL of Dilution Buffer (containing the same amount of protease inhibitors as in step 3), and add 5 µL of the Anti-Acetyl-Histone H4 Antibody to the samples. Use 20 µL of Normal Sheep IgG as a negative control. Incubate at room temperature for 15 minutes in an ultrasonic bath. **Note:** *Alternatively, incubate overnight at 2 - 8° C on a rotating device.*
7. Add 50 µL of Streptavidin beads (magnetic or agarose) to the samples and rotate for 30 minutes at 2 - 8° C, on a rotating device.
8. If using magnetic beads, collect the beads by leaving the tube in the magnet for 2 minutes. If using agarose beads, collect the beads by centrifugation at 12,000 x g for 1 minute.

   Perform 4 washes with 2 - 8° C Wash Buffers. For each wash, add 1 mL of Wash Buffer. Start with Wash Buffer 1, and finish with Wash Buffer 4. Pipette the beads up and down between each wash.
9. After the last wash, add 100 µL of Chelating Resin Solution directly to the beads (resuspend the resin solution by pipetting up and down for about 10 seconds). Boil for 10 minutes using a heatblock or a temperature controlled water bath.
10. Microcentrifuge at 12,000 x g for 1 minute at room temperature and transfer the supernatant (~80 µL) to a clean microcentrifuge tube.
11. Add 120 µL of deionized or distilled water to beads. Pipette up and down for 10 seconds, centrifuge for 1 minute, collect the new supernatant, and pool it with the supernatant from Step 10. **Note:** *At this point, samples can be stored at ≤ -20° C or ≤ -70° C.*
12. Clean up and concentrate the DNA preparation using a DNA purification kit. Resuspend the DNA in 50 µL of deionized or distilled water. **Note:** *This step increases the yield of PCR fragments by concentrating the DNA in a smaller volume, further eliminating impurities that might affect the PCR reaction. At this point, samples can be stored at ≤ -20° C or ≤ -70° C.*
13. Use 5 - 10 µL of DNA sample in PCR reactions (see the Technical Hints section for details).

ASSAY PROCEDURE SUMMARY

Step 1: Crosslink and harvest cells (20 minutes).

Steps 2 - 4: Quench, lyse, and sonicate samples (30 minutes).

Steps 5 - 6: Centrifuge, dilute, and incubate with biotinylated antibody (25 minutes).

Step 7: Add Streptavidin beads and incubate (30 minutes).

Step 8: Collect the beads and wash (15 minutes).

Step 9: Add Chelating Resin Solution directly to the beads and boil (10 minutes).

Step 10: Centrifuge and transfer the supernatant to a clean tube (2 minutes).

Step 11: Add water to beads, centrifuge, collect supernatant, and combine with the previous supernatant (2 minutes).

Step 12: Clean up and concentrate DNA with a DNA purification kit (10 minutes).

Step 13: Run PCR reactions (2 - 3 hours).

Total = ~ 5 Hours

TYPICAL DATA

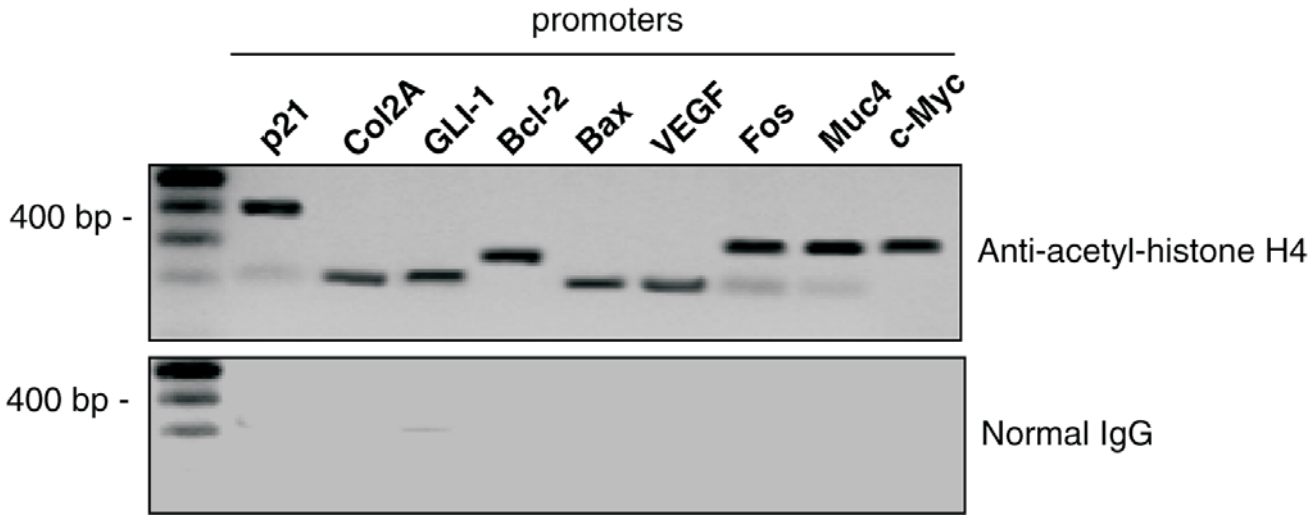

Figure 2: Jurkat T cells were stimulated for 30 minutes with 50 ng/mL PMA and 200 ng/mL ionomycin, fixed with formaldehyde, lysed, sonicated, and incubated with anti-human acetyl-histone H4 polyclonal antibody or normal IgG for 15 minutes in an ultrasonic bath. DNA was purified, and the promoters for nine different transcriptionally active genes were detected by standard PCR.

TECHNICAL HINTS

- Stimulation time and conditions should be determined and optimized by the investigator according to the cell type of interest.
- The sonicator settings need to be optimized by the investigator. For example, DNA of 1 kb average size can be obtained by setting a Heat Systems-Ultrasonics sonicator to 4% output power, 70% duty, output control 3, performing 4 rounds of 15 pulses (2 second pulses), resting the samples on ice water for 2 minutes between rounds, and keeping the samples on ice water at all times. Foaming should be avoided as it might decrease the shearing efficiency. Foaming can be caused by inappropriate position of the sonicator probe (too close to the surface or touching the bottom of the tube).
- To determine the DNA fragment average size, run an aliquot of sheared chromatin (after Step 5 of the Procedure) along with a DNA marker on a 1 - 2% (weight/volume) agarose gel, stained with Ethidium bromide, and visualize it under UV light.
- Incubation time with the biotinylated antibody can be changed to overnight at 2 - 8° C to increase efficiency of the immunoprecipitation, if needed. For example, this can be done in case of a limiting number of cells.
- Streptavidin magnetic beads or streptavidin agarose beads can be used with this kit.
- PCR reaction parameters used for the Typical Data section is: 43 cycles, including 5% DMSO with a cycle of 95° C for 9 minutes, 43 cycles at 94° C for 1 minute and 60° C for 1 minute, and one cycle at 60° C for 10 minutes.
- As a positive control for PCR, DNA prepared from samples prior to immunoprecipitation (whole cell lysates) can be used as total or input DNA.
- For other possible controls, use an irrelevant antibody, primers designed to anneal to promoters known not to be regulated by the protein of interest, or primers designed to anneal to regions of the gene known not to bind to the protein of interest.

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TROUBLESHOOTING GUIDE

Problem	Possible Cause	Possible Solution
Insufficient or degraded sheared chromatin	Over-crosslinked material	Reduce crosslinking time (5 - 15 minutes might be sufficient).
	Solution foamed during sonication	Sonicator tip should be localized in the tube as deep as possible without touching the bottom or walls of the tube. Immerse the tube in an appropriately sized ice water bath, which is most easily done by keeping the tip and tube fixed, placing the bath on a height-adjustable platform, and raising it into position. If there is significant foaming, remove bubbles by high speed centrifugation and resuspend all material.
	Insufficient amount of chromatin	Increase the amount of starting material.
	Under-sonicated	Increase the sonication time, sonicator power settings, and/or number of sonication rounds. Efficiency of sonication also varies, to some extent, according to sample volume and tube size.
	Inappropriate sonicator settings	Settings need to be optimized by the investigator. For example, DNA of 1 kb average size can be obtained by setting a Heat Systems-Ultrasonics sonicator to 4% output power, 70% duty, output control 3, performing 4 rounds of 15 pulses (2 second pulses), resting the samples in icy water for 2 minutes between rounds (to avoid DNA denaturation).
	Any of the above	Visualize the prepared samples in a preliminary experiment to determine the shearing efficiency.
PCR result: Non-specific, low signal, or no signal	Over-crosslinked material	Reduce crosslinking time (5 - 15 minutes might be sufficient).
	Under-crosslinked	Increase crosslinking time, sonication time, sonicator power settings, and/or number of sonication rounds.
	Insufficient amount of chromatin	Increase the amount of starting material.
	Inappropriate immunoprecipitation conditions	Check required reagents and temperatures of water baths and incubators. All wash steps are performed on ice. Wash Buffers 1 - 4 should be kept cold. Use room temperature Lysis Buffer to avoid SDS precipitation.
	PCR reaction did not work	Run genomic DNA as a positive control for primers and PCR reagents. The amount of sample used per reaction might be determined empirically.
	Insufficient number of PCR cycles or suboptimal parameters	Increase the number of PCR cycles and adjust the PCR parameters following the instructions provided by the PCR kit manufacturer.
	DNA secondary structures are preventing primer annealing	Use 5% DMSO in PCR reactions.

Problem	Possible Cause	Possible Solution
PCR result: Non-specific, low signal, or no signal	Inappropriate stimulation time and conditions (protein of interest not bound to DNA)	The investigator, according to the cell type of interest, should determine optimal stimulation conditions.
	Over-sonicated material	To confirm DNA suitability, check the overall appearance of the DNA fragments (it should be a DNA smear) and the average size. Run an aliquot of sheared chromatin (after Step 5 of the Assay Procedure) along with a DNA marker on a 1 - 2% (weight/volume) agarose gel, stained with Ethidium bromide, and visualize it under UV light.
	Inappropriate incubation time with antibody	For proteins of low abundance or small numbers of cells, incubation time can be increased to overnight at 2 - 8° C to increase the efficiency of the immunoprecipitation step.
	Primer design not optimal	Review primer design and composition. Use one of the many free primer design software packages that can be found on the web.
	PCR parameters not appropriate for the gene target	Adjust denaturation, annealing and extension times, as well as the number of cycles.
	Multiple bands observed in the PCR result	Check PCR parameters (number of cycles, temperatures, primers, enzyme, and magnesium concentration).
	Smeared product band by carry-over contamination	Set up PCR reactions in an area different from the area where PCR products are analyzed.
	Smeared product band due to reasons other than contamination	Check PCR parameters (number of cycles, temperatures, primers, enzyme, and magnesium concentration).
	Partial or complete loss of PCR product	Set up reactions in a separate area. Test a new aliquot of the sample.
	Many longer non-specific products	Decrease annealing time, extension time and temperature, primer, template, and polymerase amount. Increase annealing temperature and magnesium concentration. Check primers for repetitive sequences.
	Many shorter non-specific products	Increase annealing time and temperature, extension time and temperature, and magnesium concentration. Decrease primer, template, and polymerase amount. Check primers for repetitive sequences.
	Reaction was working but is not any longer	Check all PCR ingredients. Change dNTP solution (sensitive to freeze-thaw cycles). Check for primer reliability. Increase primer and template amount. Decrease annealing temperature.

Problem	Possible Cause	Possible Solution
PCR result: Non-specific, low signal, or no signal	PCR product is weak	Decrease the annealing temperature as low as possible. Increase primer, polymerase, and template amount. Use BSA (0.5 μg/mL). Check primer sequences for mismatches and/or increase the primer length by 5 nucleotides.
	Primers with different melting temperatures	Increase the length of the primer with a low melting temperature.
	Pipetting error	To minimize errors, prepare a “master mix”, containing all PCR ingredients (with the exception of template and primer, if using different primers) and add this mix to the DNA template sample. Use only calibrated pipettes.
	Polymerase degraded	Keep PCR ingredients on ice at all times. Add polymerase to the master mix after adding the PCR buffer.
	Non-mixed solution	Thaw completely and vortex the PCR reagents prior to adding them to the master mix. Magnesium chloride solutions, for example, form a concentration gradient when frozen.
	Too much dNTPs	Too much dNTP can inhibit the PCR reaction. The optimal range is 40 - 200 μM.
	Presence of inhibitors	Possible inhibitors include chloroform, phenol, EDTA, SDS, bromophenol blue, and ethanol. Use of DNA purification columns should eliminate inhibitors.
	Excess or insufficient template	Use a spectrophotometer to check for the presence and purity of the DNA samples. For 25 - 30 cycles, 10,000 copies of the target sequence are normally sufficient.
	Primer dimers	Primer dimers are bands of less than 100 bp that look like small PCR products. Use less primers, re-design to avoid self-annealing (primers should not share a large precentage of complimentary sequence), titrate magnesium concentration, and increase the annealing temperature. Adding DMSO up to 5% should help with primer dimers.
	Wrong magnesium concentration	The free magnesium ion serves as a co-factor for the DNA polymerase. Its concentration must exceed the dNTP concentration.
	Contamination (amplification in negative control)	Set up PCR reactions in an area different from the area where PCR products are analyzed, change gloves frequently, prepare your own sets of reagents and store them in aliquots, use sterile tubes, add all components of the reaction to the microfuge tube before adding the template DNA, and include a positive control as well as a negative control that contains all the components of the PCR except the template DNA.