upstate
cell signaling solutions
a Serologicals Company

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

Certificate of Analysis

Chromatin Immunoprecipitation (ChIP) Assay Kit

Catalog # 17-295
Lot # 31473

Kit Components

Salmon Sperm DNA/Protein A Agarose, Catalog # 16-157C, Lot # 30045. One vial containing **1.5ml** packed beads with **600μg** sonicated salmon sperm DNA, 1.5mg BSA and approximately 4.5mg recombinant Protein A. Provided as a 50% gel slurry for a final volume of **3ml** per vial. Suspended in TE buffer, pH 8.0, containing 0.05% sodium azide. Liquid suspension.

ChIP Dilution Buffer, Catalog # 20-153. Two vials, each containing **24ml** of 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl.

Low Salt Immune Complex Wash Buffer, Catalog # 20-154. One vial containing **24ml** of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl.

High Salt Immune Complex Wash Buffer, Catalog # 20-155. One vial containing **24ml** of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl.

LiCl Immune Complex Wash Buffer, Catalog # 20-156. One vial containing **24ml** of 0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1.

TE Buffer, Catalog # 20-157. Two vials, each containing **24ml** of 10mM Tris-HCl, 1mM EDTA, pH 8.0.

0.5M EDTA, Catalog # 20-158. One vial containing **250μl** of 0.5M EDTA, pH 8.0.

5M NaCl, Catalog # 20-159. One vial containing **500μl** of 5M NaCl.

1M Tris-HCl, pH 6.5, Catalog # 20-160. One vial containing **500μl** of 1M Tris-HCl, pH 6.5.

SDS Lysis Buffer, Catalog # 20-163. One vial containing **10ml** of 1% SDS, 10mM EDTA, 50mM Tris, pH 8.1.

FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Kit Description

Quantity: 22 chromatin immunoprecipitation (ChIP) assays.

Storage and Stability: Stable for 1 year at 4°C from date of shipment.

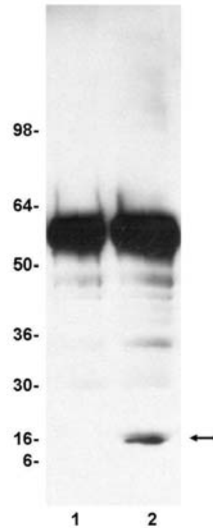
Use: The assay kit contains reagents optimized for immunoprecipitation of transcriptionally active chromatin from mammalian cells. Detection of the gene or promoter of interest in immunoprecipitated chromatin must be empirically determined by the researcher. Quantitative PCR or Southern slot-blot analysis, using promoter-specific primer or probe, is recommended.

Application References
1. Sundaraj, K.P., *et al.*, J. Biol. Chem. **279**: 6152-6162, 2004.
2. Detich, N., *et al.*, J. Biol. Chem. **278**: 27586-27592, 2003.
3. Gummow, B.M., *et al.*, J. Biol. Chem. **278**: 26572-26579, 2003.
4. Cervoni, N., & Szyf, M., J. Biol.Chem. **276**: 40778-40787, 2001.
5. Manabe, I., *et al.*, J. Clin. Invest. **107**: 823-834, 2001.

General References:
6. Luo, R.X., *et al.*, Cell **92**: 463-473, 1998.
7. Braunstein, M., *et al.*, Mol. Cell. Biol. **16**: 4349-4356, 1996.

Quality Control Testing

Chromatin Immunoprecipitation: 5-10µl of anti-acetyl Histone H4 polyclonal antibody (Catalog # 06-866) immunoprecipitated acetylated histone H4 as determined by subsequent immunoblot analysis using 0.2µg/ml of this antibody to detect histone from $1x10^6$ 3T3/A31 cells treated overnight with 5mM sodium butyrate.
Chromatin Immunoprecipitation may also be performed using 10µl of anti-acetyl Histone H3 polyclonal antibody (Catalog # 06-599) to immunoprecipitate acetylated histone H3.



Chromatin Immunoprecipitation: Representative blot from a previous lot. Anti-acetylated Histone H3 immunoprecipitated chromatin from lysates of quiescent 3T3/A31 cells (lane 1) or sodium butyrate treated 3T3/A31 cells (lane 2). The immunoprecipitate was resolved by electrophoresis, transferred to nitrocellulose and probed with anti-acetyl Histone H3 (0.2µg/ml, Catalog # 06-599). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system. Arrow indicates acetylated Histone H3 (17kDa).

Other components required but not included as part of kit are:

Reagents

- Cells, stimulated or treated as appropriate for the experimental system
- Antibody for chromatin immunoprecipitation
- 37% Formaldehyde
- PBS
- Dry ice
- Elution buffer: 1% SDS, 0.1M $NaHCO_3$
- Molecular Biology grade Proteinase K
- Glycogen or tRNA
- 50% Phenol/50%Chloroform (containing 1% isoamyl alcohol)
- 95% Ethanol
- 70% Ethanol

Equipment

- vortex mixer
- rotating wheel/platform
- shaking incubator
- timer
- variable volume (5-1000µl) pipettes + tips
- microfuge
- variable temperature water bath
- humidified 37ºC incubator
- cell scraper
- sonicator

Safety Warnings and Precautions: Formaldehyde should be used in a certified fume hood. Culture dishes containing formaldehyde should be placed in a sealed bag if returned to a humidified 37°C incubator to prevent exposing other cells in the incubator to formaldehyde. Ear protection must be worn during sonication. All chemicals should be considered potentially hazardous and handled with the principles of good laboratory practice.

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 37°C. (For example, add 270µl 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 4°C. 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 65°C 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 (from part A, step 7) for 10 minutes at 13,000 rpm at 4°C, and transfer the supernatant to a new 2ml-microcentrifuge tube. Discard pellet.
2. Dilute the sonicated cell supernatant 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 supernatant for a final volume of 2ml in each immunoprecipitation condition. **Note:** *If proceeding to PCR a portion of the diluted cell supernatant 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 material and needs to have the Histone-DNA crosslinks reversed by adding 1µl of 5M NaCl and heating at 65°C for 4 hours (see section II, step 3).*

3. To reduce nonspecific background, pre-clear the 2ml diluted cell supernatant with 75µl of **Salmon Sperm DNA/Protein A Agarose-50% Slurry (Catalog # 16-157C)** for 30 minutes at 4°C 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 4°C 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 # 16-157C****) for one hour at 4°C with rotation and proceed to step 7.*
6. Add 60µl of **Salmon Sperm DNA/Protein A Agarose Slurry (Catalog # 16-157C**) for one hour at 4°C with rotation to collect the antibody/histone complex.
7. Pellet agarose by gentle centrifugation (700 to 1000 rpm at 4°C, ~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) TE Buffer (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 65°C 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 by adding 1µl of 5M NaCl per 20µl sample and heating to 65°C for 4 hours*) 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 45°C.
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.