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Certificate of Analysis

Chromatin Immunoprecipitation (ChIP) Assay Kit

Catalog # 17-295
Lot # 20885

Kit Components

Salmon Sperm DNA/Protein A Agarose, Catalog # 16-157, Lot # 19620. Three vials, each containing **500ml** packed beads with **200mg** sonicated salmon sperm DNA, 500µg BSA, ~1.5mg recombinant protein A. Provided as a 50% gel slurry in TE buffer containing 0.05% sodium azide; final volume of 1ml per vial.

ChIP Dilution Buffer, Catalog # 20-153. Two vials, each containing **25ml** 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 **25ml** 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 **25ml** 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 **25ml** of 0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1.

1X TE Buffer, Catalog # 20-157. Two vials, each containing **25ml** of 10mM Tris-HCl, 1mM EDTA, pH 8.0.

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

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

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

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

FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Kit Description

Quantity: 20 chromatin immunoprecipitation (ChIP) assays and 2 negative controls per kit

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

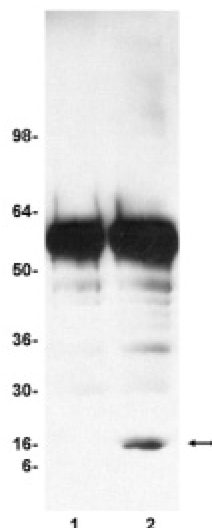
Use: The assay kit contains reagents optimized for immunoprecipitation of transcriptionally active chromatin from mammalian cells. Detection of the gene or promoter of interest in immunoprecipitated chromatin must be empirically determined by the researcher. Quantitative PCR or Southern slot-blot analysis,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

General References:

1. Luo, R.X., *et al.*, Cell. **92:** 463-473, 1998.
2. Braunstein, M., *et al.*, Mol. Cell. Biol. **16**: 4349-4356, 1996.
3. Braunstein, M., *et al.*, Genes & Devel. **7**: 592-604, 1993.
4. Alberts, A. S., *et al.*, Cell. **92:** 475-487, 1998.

Quality Control Testing

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



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 37ºC 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

Perform a mock experiment (steps 1-5) to establish optimal conditions required to shear cross-linked DNA to 200-1000 base pairs in length. Vary the power setting and/or the number of 10-second pulses during sonication of the samples. Chill samples between pulses. Check the size of sonicated DNA by gel electrophoresis after reversion of cross-links (step 14). Our experience shows DNA is sheared to the appropriate length with 3 sets of 10-second pulses using a Vibra cell sonicator equipped with a 2mm tip and set to 30% of maximum **power.**

1. Stimulate or treat 0.5-2 x 10^6 cells on 10cm dish as appropriate.[1] (Refer to Technical Tips on page four)
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.[2] Seal culture vessels if returning the cultures to an incubator containing other cells.
3. Aspirate medium, wash and scrape cells with ice cold PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1µg/ml aprotinin and 1µg/ml pepstatin A). We recommend using PMSF stock solutions less than one month old and adding PMSF to the buffer just prior to use since PMSF has a half-life of about 30 minutes in aqueous solutions.
4. Pellet cells for 4 minutes at 700 x g 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). Resuspend cell pellet in 200µl **SDS Lysis Buffer (Catalog # 20-163)** for 10 minutes on ice.[3]
5. Sonicate lysate to reduce DNA length to between 200 and 1000 basepairs. Cool samples on dry ice between pulses but do not freeze the samples. Remove debris by centrifugation for 10 minutes at 13,000 rpm at 4°C in a microcentrifuge.
6. Dilute supernatant fraction 10 fold in **ChIP Dilution Buffer (Catalog # 20-153)**. Add protease inhibitors as above. Keep a portion of this chromatin solution (1%) to quantitate the amount of DNA present in different samples before immunoprecipitation.
7. To reduce nonspecific background, pre-clear the chromatin solution with 80µl **of Salmon Sperm DNA/Protein A Agarose Slurry (Catalog # 16-157)** for 30 minutes at 4ºC with agitation.
8. Pellet beads by a brief centrifugation and collect supernatant fraction.
9. Add predetermined quantity of antibody to 1ml of chromatin solution (supernatant fraction of step 8) and incubate overnight at 4ºC with rotation. Save the other 1ml of chromatin solution for a no-antibody control.
10. Collect immune complexes with 60µl of **Salmon Sperm DNA/Protein A Agarose Slurry (Catalog # 16-157**) for one hour at 4ºC with rotation.
11. Prepare elution buffer (1%SDS, 0.1M $NaHC0_3$).[4]
12. Pellet beads by centrifugation.[5] Wash the beads for 3-5 minutes on a rotating platform with 1ml of each of the buffers listed below:[6]

    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**

13. Elute immune complexes by adding 250µl elution buffer to pelleted beads. Vortex briefly to mix and incubate at room temperature for 15 minutes with rotation. Spin down beads, carefully transfer the supernatant fraction (eluate) to another tube and repeat elution. Combine eluates.
14. Add 20µl **5M NaCl (Catalog # 20-159)** to the combined eluates and reverse crosslinks at 65ºC for 4 hours.[7]
15. 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 eluate and incubate for one hour at 45ºC.
16. Recover DNA by phenol/chloroform extraction and ethanol precipitation. Addition of an inert carrier, such as 20µg glycogen or yeast RNA is suggested. Wash pellets with 70% ethanol and allow to air dry.
17. Resuspend pellets in an appropriate buffer or water. Detect specific sequences from no antibody and immunoprecipitated samples by quantitative PCR or slot-blot. Include input and unbound DNA samples as controls. Conditions for PCR amplification must be determined empirically.

Technical Tips for ChIP Protocol

1. Cells should be treated under conditions for which transcriptional activation of the gene of interest has been demonstrated.
2. The formaldehyde crosslinking conditions listed in this protocol have been optimized for the anti-acetyl histone H3, antibody (Catalog # 06-599). For other antibodies, crosslinking conditions (duration and temperature) must be empirically determined.
3. An alternate lysis procedure can be used which may reduce background in the no antibody control immunoprecipitation.
   Resuspend cell pellet in 200µl 5mM Pipes pH 8.0, 85mM KCl, 0.5% NP40 containing protease inhibitors. Place on ice for 10 minutes. Pellet by centrifugation (5 minutes at 5000 rpm). Resuspend pellet in 200µl 1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1 containing protease inhibitors. Incubate on ice for 10 minutes.
4. Use of freshly prepared elution buffer is recommended; however, elution buffer prepared in advance may be used if it is no more than 1 month old.
5. Aliquots can be removed at this point which represent unbound DNA.
6. Following washing of the beads, immunoprecipitated histones can be assessed by immunoblot analysis after boiling of the samples in Laemmli buffer for 10 minutes.
7. Eluted material can be used as template in PCR reactions. We have successfully used 5µl of this material in a 100µl nested-PCR reaction.