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Chromatin Immunoprecipitation (ChIP) Assay Protocol V.4

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EXTERNAL LINK

https://www.biolegend.com/protocols/chromatin-immunoprecipitation-chip-assay-protocol/4306/

GUIDELINES

Tips and Trouble Shooting Guidelines:

1. To avoid low resolution with high background of the readout, make sure the size of DNA fragments from the chromatin sample are approximate 150-900bp. The time and condition for either sonication or Micrococcal Nuclease digestion may need to be determined empirically for individual cell types.
2. To avoid low signal of the readout:

- Make sure the starting cell number and chromatin sample is enough.
- Make sure cells are effectively lysed.
- Do not over cross-link. It will reduce the availability of epitopes and thus reduce the antibody binding capacity. Remember to treat cells with 0.65M glycine to quench the reaction after cross-linking.
- Make sure the DNA fragments from the chromatin samples are not too small. Do not sonicate or digest chromatin to a fragment size less than 200bp. It can result in breaks within the amplicon.
- Make sure the quantity of antibody included in the IP is enough. Check BioLegend's suggested use of Go-ChIP-Grade™ purified antibody per chromatin sample on the product datasheet. The quantity of antibody may need to be determined empirically for each experiment.
- Make sure to use ChIP-grade antibodies.

MATERIALS TEXT

Reagents:

- **Basic cell culture media:** respective to the cell line used and experimental design
- **Distilled water:** Invitrogen® UltraPure™ Distilled Water (catalog number 19977015) or equivalent product from other vendor.
- **Highthroughput (HT) Pro A or G 96 well plate or Spin Column** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **96-well collection plate:** USA scientific® TempPlate non-skirted 96-well PCR plate natural (catalog number 1402-9596) or equivalent product from other vendor.
- **QIAquick™ PCR purification kit** (Qiagen®; catalog number 28104) or equivalent product from other vendor.
- **Hypotonic Buffer** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **Digestion Buffer** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **Lysis Buffer** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **Column Conditioning Buffer** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **Wash Buffer 1** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **Wash Buffer 2** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **Wash Buffer 3** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **Elution Buffer** (Chromatrap® HT ChIP-qPCR kit; catalog number 500161 or 500162 or 500163)
- **5M NaCl:** dissolve 292g of NaCl in 800mL of H_2O. Adjust the volume to 1L with H_2O.
- **1M NaHCO3**: dissolve 12.6g of $NaHCO_3$in 100 mL of H_2O. Adjust the volume to 150 mL with H_2O.
- **Protease Inhibitor Cocktail** (PIC): Cell signaling Technology® (catalog number 5871) or equivalent product from other vendor.
- **Proteinase K: Qiagen®** (catalog number 19133) or equivalent product from other vendor.
- **Proteinase K stop solution:** 100mM Phenylmethylsulfonyl fluoride (PMSF). Weigh 1.742g PMSF (Sigma-Aldrich®, catalog

number 10837091001) and add DMSO (Sigma-Aldrich®, catalog number D2650) to a final volume of 100mL. Dissolve completely.

- **PBS:** HyClone™ Phosphate Buffered Saline (1X) (catalog number SH30256.01) or equivalent product from other vendor.
- **Formaldehyde:** VWR® 37% w/w aqueous solution (catalog number 97064-604) or equivalent product from other vendor.
- **0.65M Glycine:** Dissolve 488g of glycine into 1L of H_2O to make 6.5M glycine (10X). Dilute with PBS to make 1X before each experiment.
- **Micrococcal Nuclease:** Cell Signaling Technologies® (catalog number 10011S) or equivalent product from other vendor.
- **1.3-1.5% Agarose gel:** dissolve 1.3-1.5g of agarose powder into 100mL of TAE buffer by microwave heating. Visualize DNA under UV light by adding appropriate amount of Ethidium Bromide, or use an equivalent method. Add appropriate amount of Ethidium Bromide to cooled down gel solution to visualize DNA under UV light.
- **0.5M EDTA:** Dissolve 186.1g EDTA into 700mL of H_2O. Adjust the volume to 1L with H_2O.
- **100bp ladder:** NEB® (catalog number N0467S) or equivalent product from other vendor.

Other materials:

- Heat block
- Rotator
- Centrifuge
- Microcentrifuge
- 37°C incubator
- Nanodrop
- Sonicator
- UV light imager
- Rocking platform

Chromatin Sample Preparation

1 Culture between 1-15 million cells. Collect cells by spinning down at 500xg at 4°C for 5 minutes. Wash cells with PBS at room temperature.

2 Remove the PBS and add freshly made basic cell culture media (it should not contain any serum or large molecular weight proteins to avoid any interferences from those proteins) containing 1% formaldehyde (CH2O) to cross-link the DNA-protein complexes.

3 Incubate for 10 minutes at room temperature with gentle agitation on a rocking platform.

4 Remove the fixation solution by spinning down at 500xg at 4°C for 5 minutes. To quench the reaction, for cell count of 1-5 million, add 3mL of 0.65M glycine solution; for cell count of 5-10 million, add 4mL of 0.65M glycine solution; for cell count of 10-15 million, add 5mL of 0.65M glycine solution.

5 Incubate at room temperature for 5 minutes with gentle agitation on a rocking platform.

6 Remove the glycine solution by spinning down at 500xg at 4°C for 5 minutes. Discard the supernatant. The pellet can be frozen at -80°C after adding 1µL of Protease Inhibitor Cocktail (PIC).

7 To lyse the cells, for cell count of 1-5 million, add 0.4mL of Hypotonic Buffer; for cell count of 5-10 million, add 0.8mL of Hypotonic Buffer; for cell count of 10-15 million, add 1mL of Hypotonic Buffer to the cell pellet. Resuspend the cells and incubate at 4°C for 10 minutes.

8 Centrifuge the hypotonic slurries at 5,000xg at 4°C for 5 minutes to collect the nuclei.

9 For cell count of 1-5 million, add 0.3mL of Digestion Buffer; for cell count of 5-10 million, add 0.4mL of Digestion Buffer; for cell count of 10-15 million, add 0.5mL of Digestion Buffer to the nuclei, immediately followed by adding 2µL of PIC to each sample.

10 Add Micrococcal Nuclease to each sample to digest the DNA. Mix by inverting the tube several times and incubate at 37°C for 20 minutes. Mix by inversion every 3-5 minutes. The amount and incubation time of Micrococcal Nuclease required to digest the genomic DNA to an optimal 150 900 bp length may need to be determined empirically for individual cell types.

11 Stop digestion by adding 10µL of 0.5M EDTA per sample and place the sample on ice.

12 Pellet nuclei by centrifugation at 12,000xg at 4°C for 1 minute. Discard the supernatant.

13 Resuspend nuclear pellet in Lysis Buffer and incubate the sample on ice for 10 minutes to lyse the nuclei. Alternatively, sonicate to shear the DNA. The time and strength for sonication may need to be determined empirically.

14 Centrifuge the sample at 16,000xg at 4°C for 10 minutes. Transfer the supernatant to a clean dry microcentrifuge tube.

15 Add 1µL of PIC to each sample and mix.

16 Chromatin sample is now ready for ChIP Assay. If the sample is not to be used immediately, store at -80°C. It is recommended that the shearing efficiency is analyzed at this stage to ensure that 150-900bp fragments are obtained during shearing the DNA to increase the ChIP efficiency.

Addendum: Shearing Efficiency Analysis

17 i. Take 50µL aliquot of sheared chromatin from each sample into a new microcentrifuge. Add 5µL of 1M $NaHCO_3$, 5µL of 5M NaCl and 50µL of Distilled Water. Mix thoroughly and incubate at 65°C (heat block) for two hours or incubation can be extended for overnight.

18 ii. Remove the sample from heat block, add 1µL of Proteinase K to each sample, vortex briefly and perform a spin down at 500xg at 4°C for 5 minutes. Incubate at 37°C for one hour.

19 iii. Add 2µL of Proteinase K Stop Solution to each sample, vortex briefly and perform a short spin down.

20 iv. Purify the DNA by QIAquick™ PCR purification kit, according to manufacturer's manual.

21 v. Quantify the DNA in the sample using a Nanodrop. This will be used to determine the volume of chromatin to load in ChIP assay.

22 vi. To ensure 150-900bp fragments have been obtained during shearing the DNA, run each DNA sample on a 1.3-1.5% agarose gel and visualize against a marker of known size DNA fragments (e.g., 100bp ladder).

23 Mix the Chromatin Sample, Protease Inhibitor Cocktail (PIC), optimal quantity of BioLegend's Go-ChIP-GradeTM Purified antibodies and add Column Conditioning Buffer to make final 1mL slurries. Gently rotate at 4°C for one hour or incubation can also be extended to overnight.

24 Prepare the high-throughput (HT) Protein A or G 96 well plate or Spin Column by adding 600µL of Column Conditioning Buffer in each well or column and allow it to flow through via gravity (~15 minutes).

25 Discard the flow-through and repeat steps 18 and 19.

26 Remove the slurries from the rotator following 4°C incubation and briefly spin down to remove residual liquid from the caps.

27 Load the entire 1mL slurries and allow to flow completely through the high-throughput (HT) Protein A or G 96 well plate or Spin Column at room temperature (approximate 15-20 minutes).

28 Add 600µL of Wash Buffer 1 to each well or column and centrifuge at 2,000xg for plate, or 4,000xg for column, for one minute at room temperature. Discard the flow through and repeat once.

29 Add 600µL of Wash Buffer 2 to each well or column and centrifuge at 2,000xg for plate, or 4,000xg for column, for one minute at room temperature. Discard the flow through and repeat once.

30 Add 600µL of Wash Buffer 3 to each well or column and centrifuge at 2,000xg for plate, or 4,000xg for column, for one minute at room temperature. Discard the flow through and repeat once.

31 Spin dry at 4,000xg for the plate or 16,000xg for the column for one minute at room temperature to remove any remaining liquid from the membrane of the plate or column. Place a clean 96 well collection plate or a collection tube beneath the plate or column.

32 Add 50µL of Elution Buffer to each well or column. Incubate at room temperature for 15 minutes.

33 Centrifuge the plate or column at 4,000xg for the plate, or 16,000xg for the column, for one minute at room temperature to collect the eluted chromatin-protein complex.

34 To each eluted sample, add 5µL of 1M $NaHCO_3$, 5µL of 5M NaCl and 50µL of Distilled Water. Mix thoroughly and incubate at 65°C on the heat block for two hours or the incubation time can be extended to overnight.

35 Remove the sample from the heat block, add 1µL of Proteinase K to each sample, vortex briefly and perform a short spin down. Incubate at 37°C for one hour.

36 Add 2µL of Proteinase K Stop Solution to each sample, vortex briefly and perform a short spin down.

37 Purify the DNA by QIAquick™ PCR purification kit, according to manufacturer's manual. Purified DNA is ready for downstream real-time qPCR analysis.