Go-ChIP-Grade™ Protein G Enzymatic Kit

For Solid Phase Chromatin Immunoprecipitation Assay

Cat. No. 699904 (6 columns)

Go-ChIP-Grade™ Protein G Enzymatic Kit uses
Chromatrap® Technology

BioLegend, Inc

Biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

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Chromatrap® Technology: This patented format is unique and has been granted in the UK (Patent No. GB2482209), the US (Patent No. 9523681), China (Patent No. ZL 2011 8 0067254.X) Japan (Patent No. JP 6088434) and Australia (Patent No. AU 2011340263).

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Introduction

Go-ChIP-Grade™ Protein G Enzymatic Kit provides a quicker, easier, and more efficient way of performing Chromatin Immunoprecipitation (ChIP) assays. The kit offers all of the major components required from chromatin sample preparation to immunoprecipitation, protein-DNA complex purification, reverse-crosslinking, and DNA purification. The kit is based on the solid state technology from Chromatrap® that allows high-throughput screening from small cell numbers and low chromatin concentrations. The columns used in the kit contain discs of an inert, porous polymer to which Protein G has been covalently bound to maximize the capture efficiency of the target chromatin/antibody complex.

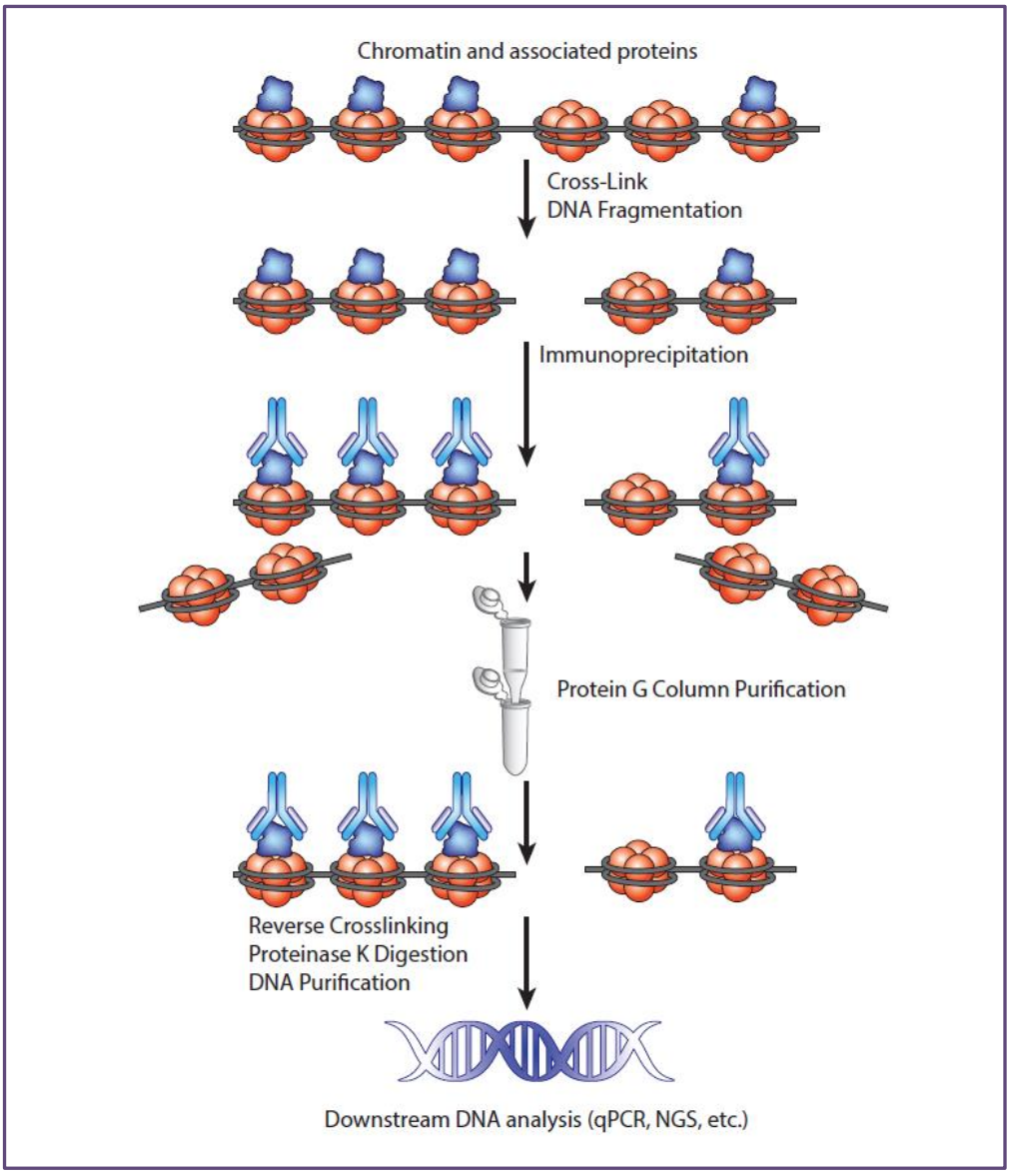


Figure 1. Principle of the ChIP assay

Advantages of the kit:

- The kit is compatible for use with ChIP-qPCR, ChIP-on-chip, and ChIP-seq
- Suitable for ChIP assays from as little as 1μg, to up to 50 μg of chromatin
- ChIP assay can be completed in 1-2 days
- The polymer disc is inert, reducing non-specific binding
- Kit provides reduced immunoprecipitation (IP) incubation times
- Elution chemistry is optimized for high quality and quantity of immunoprecipitated (IP'ed) DNA
- Kit is sensitive and provides selective enrichment of low chromatin loading

Kit Components (Table 1):

Go-ChIP-Grade™ Protein G Enzymatic Kit allows the user to perform 6 ChIP assays.

Kit Component	Quantity	Storage Temperature
Shearing cocktail	25μL	-20°C
Protease Inhibitor Cocktail (PIC)	50 μL	-20°C
Proteinase K stop solution	75μL	-20°C
Proteinase K	25μL	-20°C
Forward primer for GAPDH (human promoter region)	25μL	-20°C
Reverse primer for GAPDH (human promoter region)	25μL	-20°C
Chromatrap® Protein G spin columns	6 columns	4°C
Column conditioning buffer	15mL	4°C
Wash buffer 1	12mL	4°C
Wash buffer 2	12mL	4°C
Wash buffer 3	12mL	4°C
1.3M Glycine	5mL	4°C
Enzymatic Lysis Buffer	6mL	4°C
Digestion Buffer	2.5mL	4°C

ChIP-Seq Enzymatic Stop Solution	50µL	4°C
ChIP-Seq Elution Buffer	1mL	4°C
Hypotonic Buffer	2.5mL	4°C
5M NaCl	125µL	4°C
1M NaHCO3	125µL	4°C
Go-ChIP-Grade™ anti-RNA Polymerase II Antibody; clone 8WG16 (Positive control antibody)	50µl	4°C
Mouse IgG2a, k Isotype Control Antibody (Isotype control antibody)	50µl	4°C
1.5 ml Collection tubes	12	RT
DNA purification columns	6	RT
DNA binding buffer	3.75mL	RT
DNA wash buffer	3.75mL	RT
DNA elution buffer	500µl	RT

Storage

The kit is guaranteed to be stable for 6 months at the recommended storage temperature.

Precaution

The kit components are at 3 different storage temperatures (-20°C, 4°C, RT). Upon receipt, please ensure that the components are stored at the temperature listed in Table 1.

It is recommended that the Shearing Cocktail be aliquoted on receipt of the kit to minimize the number of freeze thaw cycles and to maintain the activity of the cocktail.

For all liquid solutions, please mix well before use.

Materials to be provided by the End-User

Reagents and consumables:

- PBS (10x concentrate, BioLegend catalog number 926201)
- 37% formaldehyde, molecular biology grade (VWR® 37% w/w aqueous solution (catalog number 97064-604) or equivalent product from other vendors)
- Distilled water (Invitrogen® UltraPure™ Distilled Water (catalog number 19977015) or equivalent product from other vendors)
- 100bp ladder (NEB® (catalog number N0467S) or equivalent product from other vendors)
- Cell-scrapers
- Microcentrifuge tubes (1.5 ml)
- PCR plates
- Pipettes and tips (filter tips are recommended)
- Go-ChIP-Grade™ Purified Antibody or any other ChIP-validated Antibody for the target protein of interest, paired with matched isotype control antibody
- qPCR primer pairs for gene of interest
- 0.1% SDS solution (Invitrogen® UltraPure™ SDS Solution, 10% (catalog number 24730020) or equivalent product from other vendors)
- 1.3-1.5% Agarose gel (dissolve 1.3-1.5g of agarose powder into 100mL of TAE buffer by microwave heating. Add appropriate amount of Ethidium Bromide, or other DNA visualization dye, to cooled down gel solution to visualize DNA under UV light)
- 0.5M EDTA (Dissolve 186.1g EDTA into 700mL of water and solution up to 1L of water)
- Reservoirs for waste
- PowerUp™ SYBR™ Green Master Mix (ThermoFisher (catalog number A25741) or equivalent product from other vendors)
- 3M Sodium Acetate, pH 5 (Sigma-Aldrich Sodium Acetate, 3M, pH 5.2, Molecular Biology Grade (catalog number CAS-127-09-3) or equivalent product from other vendors)
- 95-100% Ethanol (Ethyl Alcohol, Pure (Sigma-Aldrich (catalog number E7023) or equivalent product from other vendors)

Equipment:

- Microcentrifuge (4°C)
- Agarose gel electrophoresis equipment
- Rocking platform
- Spectrophotometer/fluorometer for DNA quantification (Nanodrop)
- Labware for preparation of 1x buffer solution
- 37°C water bath
- 65°C heat block
- End to end rotator (4°C)
- qPCR machine

General Protocol:

Chromatin Sample Preparation:

All samples must be incubated on ice unless otherwise indicated in the protocol.

Step 1: Crosslinking

The following section describes the crosslinking steps for both adherent and suspension cell lines. Chromatin samples prepared from other sources (plant, yeast, tissue, FFPE samples, etc) will require optimization by the researchers.

1. Culture between 1-15 million cells (>95% viability). The cell number can be scaled up and the reagents need to be adjusted accordingly.
2. For adherent cells, collect cells using cell scrapers and spin down at 500xg at 4°C for 5 minutes. For suspension cells, spin down cells at 500xg at 4°C for 5 minutes. Discard the supernatant.
3. Wash cell pellet with PBS at room temperature and then spin down at 500xg at 4°C for 5 minutes.
4. Remove the PBS and add 5-30mL of basic cell culture media (it should not contain any serum or large molecular weight proteins to avoid interference from those proteins) containing 1% formaldehyde (CH_2O) to cross-link the DNA-protein complexes. The volume of basic cell culture media can be scaled up or down based on the starting cell number.

5. Incubate for 10 minutes at room temperature with gentle agitation on a rocking platform. Too long of an incubation time might cause over-crosslinking, epitope damage and hard-to-fragment DNA afterwards; too short of an incubation time might cause failure of DNA-protein crosslinking, and therefore, less pull down. The incubation time might need to be adjusted if a new experiment/cell type/treatment is utilized.
6. Remove the fixation solution by spinning down at 500xg at 4°C for 5 minutes.
7. Dilute glycine solution (at 1.3M) at 1:1 ratio with PBS to reach a working concentration of 0.65M before use.
8. To quench the reaction, for a cell count of 1-5 million, add 3mL of 0.65M glycine solution; for a cell count of 5-10 million, add 4mL of 0.65M glycine solution; for a cell count of 10-15 million, add 5mL of 0.65M glycine solution.
9. Incubate at room temperature for 5 minutes with gentle agitation on a rocking platform.
10. Remove the glycine solution by spinning down at 500xg at 4°C for 5 minutes. Discard the supernatant. Proceed to step 11, or the pellet can be frozen at -80°C by adding 1µL of Protease Inhibitor Cocktail (PIC).

    Chromatin sample is very sensitive to temperature and protease activity. Incubate samples on ice unless otherwise indicated in the protocol and add PIC in the steps mentioned.

Step 2: Cell Lysis

11. Resuspend the cell pellet in Hypotonic Buffer and incubate the sample at 4°C for 10 minutes. For a cell count of 1-5 million, add 0.4mL of Hypotonic Buffer; for a cell count of 5-10 million, add 0.8mL of Hypotonic Buffer; for a cell count of 10-15 million, add 1mL of Hypotonic Buffer to the cell pellet.
12. Centrifuge the hypotonic slurries at 5,000xg at 4°C for 5 minutes to collect the nuclei.

Step 3: DNA Fragmentation by Enzymatic Digestion

13. For a cell count of 1-5 million, add 0.3mL of Digestion Buffer; for a cell count of 5-10 million, add 0.4mL of Digestion Buffer; for a cell count of 10-15 million, add 0.5mL of Digestion Buffer to the nuclei, and immediately add 2µL of PIC to each sample. Keep stock nuclei suspensions on ice while determining DNA concentration.
14. In order to determine the amount of shearing cocktail to be used, DNA concentration needs to be measured. Remove a 10µL sample of each stock nuclei suspension and add 490µL of 0.1% SDS, mix well and incubate on ice for 10 minutes. Estimate the concentration of total DNA on a spectrometer to calculate the total amount of chromatin in each stock nuclei suspension, in order to determine the volume of Shearing Cocktail to be used.
15. Add Shearing Cocktail to the stock nuclei suspension (from step 13) at a ratio of 1U Shearing Cocktail : 5µg chromatin and mix thoroughly. (Concentration of the Shearing Cocktail is 15 U/µl).

    Enzymatic digestion is very time and enzyme volume sensitive. It might be necessary to titer the shearing buffer or use a different incubation time to have optimal shearing conditions.

Example of how much shearing buffer to use:

- The DNA concentration is 10ng/µL from Nanodrop
- Total DNA is 10ng/µL X 50 (dilution rate) X 500 (total volume) = 250,000ng = 250µg
- For shearing cocktail, use 1U of shearing cocktail : 5µg DNA
- For a total of 250 µg of DNA, 50 U of shearing cocktail are needed (250/5)
- The concentration of shearing cocktail is 15U/µL, thus 3.33 µL of shearing cocktail is added (50/15 = 3.33)

16. Incubate for 5 minutes in a 37°C water bath. Immediately add Enzymatic Stop Solution to the nuclei suspension and place tubes on ice. For a cell count of 1-5 million, add 7.5µL of Enzymatic Stop Solution; for a cell count of 5-10 million,

add 10µL of Enzymatic Stop Solution; for a cell count of 10-15 million, add 12.5µL of Enzymatic Stop Solution.

17. Pellet nuclei by centrifugation at 12,000xg at 4°C for 5 minute. Discard the supernatant.
18. Pre-warm the Lysis Buffer in a 37°C water bath mixing occasionally to dissolve any precipitates. Bring Lysis Buffer back to room temperature before use. For a cell count of 1-5 million, add 0.3mL of Lysis Buffer; for a cell count of 5-10 million, add 0.3-0.5mL of Lysis Buffer; for a cell count of 10-15 million, add 0.5-1mL of Lysis Buffer to the nuclei suspension. Resuspend nuclear pellet in Lysis Buffer and incubate the sample on ice for 10 minutes to lyse the nuclei.
19. Centrifuge the sample at 16,000xg at 4°C for 10 minutes. Transfer the supernatant to a clean dry microcentrifuge tube.
20. Add 1µL of PIC to each sample and mix.
21. Chromatin samples are now ready for ChIP Assay. If the samples are not to be used immediately, store at -80°C. It is recommended that the shearing efficiency is analyzed at this stage.

Addendum: Shearing Efficiency Analysis

i. Take a 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 in a 65°C heat block for two hours. More proteinaceous samples may need a longer incubation time up to overnight if necessary.

ii. 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.

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

iv. Use a nanodrop to quantify the DNA in the samples. This will be used to determine the volume of chromatin to load in ChIP assay.

v. To check shearing efficiency, ensure 150-900bp fragments have been obtained, by running each DNA sample on a 1.3-1.5% agarose gel and visualizing against a marker of known size DNA fragments (e.g., 100bp ladder).

Step 4: Chromatin Immunoprecipitation

22. Mix the Chromatin Sample, Protease Inhibitor Cocktail (PIC), and antibodies (either your antibody of target protein of interest or matched isotype control) at optimal concentrations*, and add Column Conditioning Buffer to make slurries at a final volume of 1mL. Gently rotate at 4^{o}C for one hour or up to overnight.
23. Set aside the equivalent amount of chromatin sample in a microcentrifuge tube and make up to 100 µL with Column Conditioning Buffer, label as input. This input sample will be processed alongside the samples from reverse crosslinking (step 35) and will be used as an input control in the downstream analysis.

*Please refer to BioLegend or other manufacturer's datasheet for optimal concentration of ChIP-validated antibody.

It is recommended to use the positive antibody control and matched isotype controls that are included in this kit. The suggested dilution of the Go-ChIP-Grade anti-RNA Polymerase II Antibody; Clone 8WG16 (positive control) is 1:300 – 1:500 by volume.

The amount of chromatin sample to be used depends on several factors such as the abundance of the target protein, and needs to be determined empirically by the end-users.

Example of how to make IP slurries:

If we have 100ng/µL of chromatin sample from HeLa cells,

Add the individual reagents in this order:

- 966µL of Conditioning Buffer
- 2µL of PIC
- 3µg (3/0.1=30µL) of Chromatin sample
- 1µg (2µL) of Go-ChIP-Grade™ Purified anti-RNA Polymerase II Antibody (8WG16)

Total volume is 1mL

Step 5: Column Conditioning, Washing and Elution

24. Prepare the Spin Column by adding 600µL of Column Conditioning Buffer in each column and allow it to flow through by gravity (approximately 15 minutes). Alternatively, centrifuge the Spin Column with slow speed to fasten the flow through process.
25. Discard the flow-through and repeat this conditioning step for a second time.
26. Discard the flow-through.
27. Remove the slurries from the rotator following 4^{o}C incubation, and briefly spin down to remove residual liquid from the caps.
28. Load the entire 1mL slurries and allow to flow completely through the Spin Column at room temperature (approximate 15-20 minutes).
29. Add 600µL of Wash Buffer 1 to each column and centrifuge at 4000xg for one minute at room temperature. Discard the flow through and repeat once.
30. Add 600µL of Wash Buffer 2 to each column and centrifuge at 4000xg for one minute at room temperature. Discard the flow through and repeat once.
31. Add 600µL of Wash Buffer 3 to each column and centrifuge at 4000xg for one minute at room temperature. Discard the flow through and repeat once.
32. Spin dry at top speed for one minute at room temperature to remove any remaining liquid from the membrane of the column. Place a clean collection tube beneath the column.
33. Pre-warm the Elution Buffer in a 37^{o}C water bath to dissolve the precipitates and use it at room temperature. Add 50µL of Elution Buffer to each column. Incubate at room temperature for 15 minutes.
34. Centrifuge the column at 16,000xg for one minute at room temperature to collect the eluted chromatin-protein complex.

Step 6: Reverse Crosslinking

35. To each eluted sample, add 5µL of 1M $NaHCO_3$, 5µL of 5M NaCl and 50µL of Distilled Water. The input control from step 23 which has not been through the IP process must be reintroduced from this step. To the input sample, add 5µL of 1M $NaHCO_3$, 5µL of 5M NaCl. Mix thoroughly and incubate at 65^{o}C on the heat block for two hours. More proteinaceous samples may need a longer incubation time (up to overnight incubation).

Step 7: Digestion with Proteinase K and DNA Purification

36. 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.
37. Add 2µL of Proteinase K Stop Solution to each sample, vortex briefly and perform a short spin down.
38. Add 5 volumes of DNA Binding Buffer to 1 volume of sample and mix. DNA Binding Buffer contains an intrinsic pH indicator. DNA adsorption requires a pH ≤ 7.5. The pH indicator in the buffer will appear yellow within this range. If the pH is ≥ 7.5, the binding mixture will turn orange or violet, and the DNA adsorption will be inefficient. In this case, add 10 µL of 3M sodium acetate, pH 5, to adjust the pH until the color of the mixture turns yellow.
39. Place a DNA purification column in the collection tube provided and transfer sample onto the column. Centrifuge at 16,000xg for 1 minute. Discard the flow through.
40. To prepare DNA Wash Buffer, add 15mL ethanol (95-100%) to the DNA Wash Buffer Concentrate before use
41. Add 700 µL DNA Wash Buffer onto DNA purification column and centrifuge at 16,000xg for 1 minute. Discard the flow through.
42. Centrifuge at 16,000xg for another 1 minute to remove residual wash buffer.
43. Place DNA Purification Column in a clean 1.5mL microcentrifuge tube.
44. To elute DNA, add 50 µL DNA Elution Buffer to the center of the membrane and incubate for 1 minute. Centrifuge at 16,000xg for 1 minute. Transfer the eluted DNA to a clean microcentrifuge tube.

Step 8: qPCR to Quantify DNA

Prior to downstream analysis, we recommend analyzing the IP'ed DNA by qPCR using at least one positive and one negative control to validate the IP. This kit contains a positive control antibody (Go-ChIP-Grade™ anti-RNA Polymerase II Antibody; clone 8WG16) and a matched isotype control antibody (Mouse IgG2a, k Isotype Control Antibody) with primers for human GAPDH.

45. Prepare the qPCR reaction mix as follows for a 10µl reaction volume:
    – 5 µl of a 2x SYBR™ Green qPCR mix
    – 2.5 µl primer mix (combine primers 1:1)
    – 2.5 µl IP'ed or input DNA

Primer concentrations may need to be adjusted. We recommend a final concentration of 1 µM in the reaction mix for each primer.

Program the thermal cycler as follows for GAPDH primers provided in this kit.

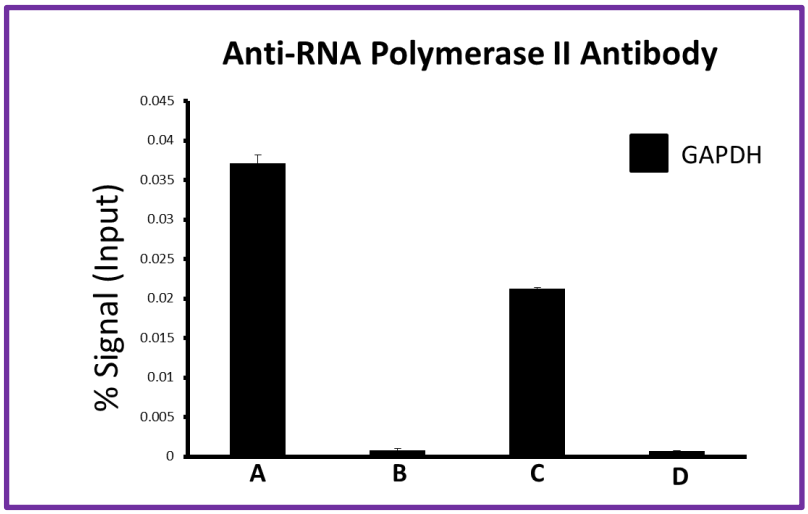
2 minutes at 95°C	
10 seconds at 95°C 30 seconds at 60°C 15 seconds at 72°C	40 cycles

These conditions may require optimization depending on the primers, reagent and qPCR system used.

Step 9: Data Acquisition and Analysis

Purified DNA is ready for downstream analysis, such as Next-Generation Sequencing (NGS), qPCR analysis, or ChIP-on chip analysis.

Example Data:



ChIP was performed using Go-ChIP-Grade™ Protein G Enzymatic Kit by loading 3µg of cross-linked chromatin samples from HeLa cells with either A) 1:300 dilution of Go-ChIP-Grade™ Purified anti-RNA Polymerase II Antibody (Clone 8WG16), B) equal amount of Purified Mouse

IgG2a, k Isotype Control Antibody, or C) competitor's ChIP-grade Purified anti-RNA Polymerase II Antibody and D) equal amount of matched Isotype Control Antibody as recommended by the manufacturer. The enriched DNA was purified and quantified by real-time qPCR using primers targeting human GAPDH gene region. The amount of IP'ed DNA in each sample is represented as signal relative to the 5% of total amount of input chromatin.

Troubleshooting and FAQs:

1. What cell types have been validated with this protocol?

Human and mouse cell lines such as HeLa, Jurkat, Daudi, THP-1, NIH3T3, and HEK 293T have been validated using this protocol. Other cell types (plant, yeast, tissue, FFPE samples, etc) may require optimization.

2. **How do I determine optimal number of cells for ChIP assay?**

An important consideration when performing ChIP assay is the amount of chromatin that will need to be loaded to the column in order to elute sufficient IP'ed DNA for downstream analysis. Sufficient cell numbers should be used so that at least 3µg of chromatin can be used per IP. Start with 1-15 million cells. However, the cell number can be scaled up and the reagents need to be adjusted accordingly.

3. How much antibody for target protein should be used per ChIP?

This should be determined empirically by the end-user. Refer to the datasheet for our Go-ChIP-Grade™ Purified Antibodies, or other ChIP-validated antibodies. The suggested dilution of the Go-ChIP-Grade anti-RNA Polymerase II Antibody; Clone 8WG16 (positive control) is 1:300 – 1:500 by volume.

4. What is causing high background?

The quality of the ChIP antibody has a major impact on the success of the assay. Use only ChIP-validated antibodies. Make sure to have good quality of chromatin samples with fragments between 150-900bp. Insufficient DNA shearing leads to longer DNA fragment length that can cause high background in downstream experiments. Insufficient wash steps can also leave traces of

non-specific chromatin alongside enriched DNA. If background remains high, include an additional wash step during the IP protocol.

5. Why do I not have any enrichment?

Make sure to only use ChIP-validated antibodies and follow the instructions for the amount of antibody to be used or titer the antibody for your experiment. It is also recommended to include ChIP validated positive and negative controls (included in the kit) to validate the efficiency of your ChIP assay. Good quality of chromatin samples, and chromatin fragment size between 150 – 900 bp are also critical. Other factors can include cells were not effectively lysed, cells may have been cross-linked for too long that can reduce antibody binding due to decreased epitope availability, and not enough starting material (chromatin sample per IP) used.

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