

ab185913 – High-Sensitivity ChIP Kit

Instructions for Use

For the selective enrichment of chromatin fractions

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

Protein-DNA interaction plays a critical role for cellular functions such as signal transduction, gene transcription, chromosome segregation, DNA replication and recombination, and epigenetic silencing. Identifying the genetic targets of DNA binding proteins and knowing the mechanisms of protein-DNA interaction is important for understanding cellular processes.

Chromatin immunoprecipitation (ChIP) offers an advantageous tool for studying protein-DNA interactions. It allows for the detection of a specific protein bound to a specific gene sequence in living cells using PCR (ChIP-PCR), microarrays (ChIP-chip), or sequencing (ChIP-seq). For example, measurement of the amount of methylated histone H3 at lysine 9 (meH3-K9) associated with a specific gene promoter region under various conditions can be achieved through a ChIP-PCR assay, while the recruitment of meH3-K9 to the promoters on a genome-wide scale can be detected by ChIP-chip or ChIP-sequencing. In particular, ChIP that uses antibodies directly against various transcription factors (TF) for genome-wide transcription factor binding site analysis by next generation sequencing is in high demand. Such analysis requires that ChIPed DNA contain minimal background for reliably identifying true TF-enriched regions. High background in ChIP is mainly caused by the following weaknesses: ineffective wash buffer, insufficient cross-link reversal, inappropriate DNA fragment length and residual RNA interference.

Thus, for effectively capturing TF/DNA complexes which are often in low abundance, an ideal ChIP method requires having maximum sensitivity with minimized background levels. This method should also be able to enrich highly abundant protein/DNA complexes using a small amount of cells or tissues in a high throughput format. Abcam's High-Sensitivity ChIP Kit (ab185913) is designed to achieve these goals by maximizing sensitivity and minimizing non-specific background signals.

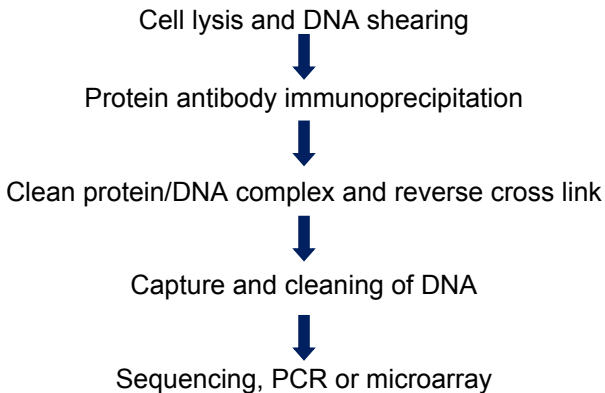
The High-Sensitivity ChIP Kit (ab185913) has the following advantages:

- Optimized buffers and protocol allow minimal ChIP background by overcoming the weaknesses that cause non-specific enrichment, thereby increasing sensitivity and specificity of the ChIP reaction.
- Increased antibody selectivity and capture efficiency through the use of unique chimeric proteins containing the maximum number of IgG binding domains coated on the strip-wells. This allows strong binding of any IgG subtype antibodies within a wide pH range regardless if they are in monoclonal or polyclonal form.
- 96-well plate format makes the assay flexible. Either (a) manual with one single reaction each time; or (b) high throughput with 24-48 reactions each time.
- Highly efficient enrichment. The enrichment ratio of positive to negative control is > 500 . An extremely low number of cells (as low as 2,000 cells per ChIP reaction) can be used for enriching highly abundant protein/DNA complexes.
- High reproducibility. Pre-optimized ChIP conditions make the ChIP procedure consistent.
- Wide downstream analysis compatibility. Compatible with various downstream analysis workflows including ChIP-PCR, ChIP-on-chip, and ChIP-seq.
- Fast 5 hour procedure, from input chromatin to ready-to-use DNA eluate.

The High-Sensitivity ChIP Kit is suitable for selective enrichment of a chromatin fraction containing specific DNA sequences which is in a high throughput format with high sensitivity and specificity using various mammalian cell/tissues. The optimized protocol and kit components reduce non-specific background ChIP levels to allow capture of low abundance protein/transcription factors and increased specific enrichment of target protein/DNA complexes. The target protein bound DNA prepared with the kit can be used for various downstream applications including PCR (ChIP-PCR), microarrays (ChIP-on-chip), and sequencing (ChIP-seq).

Abcam's High-Sensitivity ChIP Kit contains all necessary reagents required for carrying out a successful chromatin immunoprecipitation starting from mammalian cells or tissues. This kit includes a positive control antibody (RNA polymerase II), a negative control non-immune IgG, and GAPDH primers that can be used as a positive control to demonstrate the efficacy of the kit reagents and protocol. RNA polymerase II is considered to be enriched in the GAPDH gene promoter that is expected to be undergoing transcription in most growing mammalian cells and can be immunoprecipitated by RNA polymerase II antibody but not by non-immune IgG. Immunoprecipitated DNA is then cleaned, released, and eluted. Eluted DNA can be used for various downstream applications such as CHIP-PCR, CHIP-on-chip, and CHIP-seq.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt.

Observe the storage conditions for individual prepared components in sections 9 & 10.

*For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

Check if Wash Buffer and ChIP Buffer contain salt precipitates before use. If so, warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved

5. MATERIALS SUPPLIED

Item	24 Tests	48 Tests	Storage Condition (Before Preparation)
Wash Buffer	25 mL	2 x 25 mL	4°C
Antibody Buffer	3 mL	6 mL	4°C
Lysis Buffer	14 mL	28 mL	RT
ChIP Buffer	6 mL	12 mL	4°C
DNA Release Buffer	8 mL	16 mL	RT
DNA Binding Solution	7 mL	14 mL	RT
Blocker Solution	2 mL	4 mL	4°C
DNA Elution Buffer	1 mL	2 mL	RT
Enrichment Enhancer*	55 µL	110 µL	-20°C
Protease Inhibitor Cocktail *	30 µL	60 µL	4°C
Non-Immune IgG (1 mg/mL)*	10 µL	20 µL	4°C
Anti-RNA Polymerase II (1 mg/mL)*	8 µL	16 µL	4°C
Proteinase K (10 mg/mL)*	60 µL	120 µL	4°C
RNAase A (10 mg/mL)*	30 µL	60 µL	-20°C
GAPDH Primer – Forward (20 µM)*	8 µL	16 µL	4°C
GAPDH Primer – Reverse (20 µM)*	8 µL	16 µL	4°C
8-well assay strips (with 1 frame)	3	6	4°C
8-well strip caps	3	6	RT
Adhesive covering film	1	2	RT
F-spin column	30	50	RT
F-collection tube	30	50	RT

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Sonicator
- 1.5 mL microcentrifuge tubes
- Vortex mixer
- Dounce homogenizer with small clearance pestle
- Variable temperature waterbath or incubator oven
- Thermocycler with 48 or 96-well block
- Centrifuge (up to 14,000 rpm)
- 0.2 mL or 0.5 mL PCR vials
- Orbital shaker
- 15 mL conical tube
- Cells or tissues
- Antibodies of interest
- Cell culture medium
- 37% Formaldehyde (if cross linked)
- 1.25 M Glycine solution (if cross linked)
- 100% Ethanol
- 1X PBS

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 Working Lysis Buffer

Add 6 μL of Protease Inhibitor Cocktail to every 10 mL of Lysis Buffer required.

9.2 Working ChIP Buffer

Add 1 μL of Protease Inhibitor Cocktail to every 1 mL of ChIP Buffer required.

10. SAMPLE PREPARATION

10.1 Antibody Binding to Assay Strip Well

10.1.1 Predetermine the number of Assay Strip Wells required for your experiment. Carefully remove any unneeded strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).

10.1.2 Set up the antibody binding reactions by adding the reagents to each well according to the following table:

Reagents	Sample (μL)	Positive control (μL)	Negative control (μL)
Antibody Buffer	50-80	50-80	50-80
Your antibodies	0.5-2	0	0
Anti-RNA Polymerase II	0	0.8	0
Non-Immune IgG	0	0	0.8

Note: The final amount of each component should be (a) antibodies of interest: 0.8 $\mu\text{g}/\text{well}$; (b) RNA Polymerase II: 0.8 $\mu\text{g}/\text{well}$; and (c) non-immune IgG: 0.8 $\mu\text{g}/\text{well}$.

The amounts of the positive control (Anti-RNA Polymerase II) and negative control (Non-Immune IgG) are sufficient for matched use with samples if two antibodies are used for each sample or one antibody is used for two of the same samples. If using one antibody of interest for each sample with matched use of the positive and negative control, extra RNA Polymerase II, Non-Immune IgG and 8-Well Strips will be required.

- 10.1.3 Seal the wells with Adhesive Covering Film Strips and incubate the wells at room temperature for 60-90 minutes on an orbital shaker (100 rpm). Meanwhile, perform the steps from 'Cell collection and Cross-linking' to 'Chromatin Shearing'

10.2 Cell Collection and Cross-Linking

Input Amount: In general, the amount of cells and tissues for each reaction can be 2×10^3 to 1×10^6 and 0.5 mg to 50 mg, respectively. For optimal preparation, the input amount should be 1 to 2×10^5 cells or 10 to 20 mg tissues since the enrichment of target proteins to genome loci may vary. For the target proteins that are low abundance transcription factors, the input amount should be 5 to 6×10^5 cells or 50-60 mg tissues.

Starting Materials: Starting materials can include various tissue or cell samples such as cells from flask or plate cultured cells, fresh and frozen tissues, etc.

Antibodies: Antibodies should be ChIP or IP grade in order to recognize fixed and native proteins that are bound to DNA or other proteins. If you are using antibodies which have not been validated for ChIP, then appropriate control antibodies such as an RNA Polymerase II should be used to demonstrate that the antibody and prepared chromatin are suitable for ChIP.

Internal Controls: Both negative and positive ChIP controls are provided in this kit.

10.2.1 For Monolayer or Adherent Cells:

- 10.2.1.1 Grow cells (treated or untreated) to 80%-90% confluence on a 6 well plate or 100 mm dish (the number of cultured MDA-231 cancer cells on an 80-90% confluent plate is listed in the table below as a reference), then trypsinize and collect them into a 15 ml conical tube. Count the cells in a hemocytometer.

Container	Cell number (x 10 ⁵)
96-well plate	0.3-0.6/well
24-well plate	1-3/well
12-well plate	3-6/well
6-well plate	5-10/well
60 mm dish	20-30
100 mm dish	50-100
150 mm dish	150-180

- 10.2.1.2 Centrifuge the cells at 1000 rpm for 5 minutes. Discard the supernatant.

- 10.2.1.3 Wash cells with 10 mL of PBS once by centrifugation at 1000 rpm for 5 minutes. Discard the supernatant.

Note: For cells that are not cross-linked, go directly to Step 10.2.1.9 after Step 10.2.1.3

- 10.2.1.4 Add 9 ml fresh cell culture medium containing formaldehyde to a final concentration of 1% (i.e. add 270 μ L of 37% formaldehyde to 10 mL of cell culture medium) to cells.

- 10.2.1.5 Incubate at room temperature (20-25°C) for 10 minutes on a rocking platform (50-100 rpm).

- 10.2.1.6 Add 1 mL of 1.25 M Glycine for every 9 mL of cross-link solution.

- 10.2.1.7 Mix and centrifuge at 1000 rpm for 5 minutes.

10.2.1.8 Remove medium and wash cells once with 10 mL of ice-cold PBS by centrifuging at 1000 rpm for 5 minutes. Discard the supernatant.

Note: *If the total solution volume is less than 1.5 mL, transfer the solution to a 1.5 mL microtube.*

10.2.1.9 Add Working Lysis Buffer to re-suspend the cell pellet (200 μ L/1 \times 10⁶ cells) and incubate on ice for 10 minutes.

10.2.1.10 Vortex vigorously for 10 seconds and centrifuge at 3000 rpm for 5 minutes. Then go to the 'Cell Lysis and Chromatin Extraction' section.

10.2.2 For Suspension Cells:

10.2.2.1 Collect cells (treated or untreated) into a 15 mL conical tube. (2 \times 10⁵ to 5 \times 10⁵ cells are required for each ChIP reaction). Count cells in a hemocytometer.

10.2.2.2 Centrifuge the cells at 1000 rpm for 5 minutes. Discard the supernatant.

10.2.2.3 Wash cells with 10 mL of PBS once by centrifugation at 1000 rpm for 5 minutes. Discard the supernatant.

Note: *For cells that are not cross-linked, go directly to Step 10.2.2.9 after Step 10.2.2.3.*

10.2.2.4 Add 9 mL fresh cell culture medium containing Formaldehyde to a final concentration of 1% (i.e., add 270 μ L of 37% Formaldehyde to 10 ml of cell culture medium) to cells.

10.2.2.5 Incubate at room temperature (20-25°C) for 10 minutes on a rocking platform (50-100 rpm).

10.2.2.6 Add 1 mL of 1.25 M Glycine for every 9 mL of cross-link solution.

10.2.2.7 Mix and centrifuge at 1000 rpm for 5 minutes.

10.2.2.8 Remove medium and wash cells once with 10 mL of ice-cold PBS by centrifuging at 1000 rpm for 5 minutes. Discard the supernatant.

10.2.2.9 Add Working Lysis Buffer to re-suspend the cell pellet (200 μ l/ 1×10^6 cells) and incubate on ice for 10 minutes.

Note: *If the total solution volume is less than 1.5 mL, transfer the solution to a 1.5 mL microtube.*

10.2.2.10 Vortex vigorously for 10 seconds and centrifuge at 3000 rpm for 5 minutes. Then go to the 'Cell Lysis and Chromatin Extraction' section.

10.2.3 For Tissues:

10.2.3.1 Put the tissue sample into a 60 or 100 mm plate. Remove unwanted tissue such as fat and necrotic material from the sample.

10.2.3.2 Weigh the sample and cut the sample into small pieces (1-2 mm³) with a scalpel or scissors.

Note: *For tissues that do not need to be cross-linked (eg, target proteins strongly associated with DNA, like histones) go directly to Step 10.2.3.10 after Step 10.2.3.2. However, for many targets cross-linking will be required so as to detect transient interactions.*

10.2.3.3 Transfer tissue pieces to a 15 mL conical tube.

10.2.3.4 Prepare cross-link solution by adding formaldehyde to cell culture medium to a final concentration of 1% (e.g., add 270 μ L of 37% formaldehyde to 10 mL of culture medium).

10.2.3.5 Add 1 mL of cross-link solution for every 50 mg tissues.

10.2.3.6 Incubate at room temperature for 15-20 minutes on a rocking platform.

10.2.3.7 Add 1 mL of 1.25 M glycine for every 9 mL of cross-link solution.

10.2.3.8 Mix and centrifuge at 800 rpm for 5 minutes. Discard the supernatant.

10.2.3.9 Wash cells with 10 mL of ice-cold PBS once by centrifugation at 800 rpm for 5 minutes. Discard the supernatant.

10.2.3.10 Transfer tissue pieces to a Dounce homogenizer.

10.2.3.11 Add 0.5 mL Working Lysis Buffer for every 50 mg of tissues.

10.2.3.12 Disaggregate tissue pieces with 20-40 strokes.

10.2.3.13 Transfer homogenized mixture to a 15 mL conical tube and centrifuge at 3000 rpm for 5 minutes at 4°C. If total mixture volume is less than 2 mL, transfer mixture to a 2 mL vial and centrifuge at 5000 rpm for 5 minutes at 4°C. Then go to the 'Cell Lysis and Chromatin Extraction' section.

10.3 Cell Lysis and Chromatin Extraction

10.3.1 Carefully remove supernatant.

10.3.2 Add CHIP Buffer to re-suspend the chromatin pellet (100 μ L/1x10⁶ cells or 50 mg tissue, 500 μ L maximum for each vial).

10.3.3 Transfer the chromatin lysate to a 1.5 mL vial and incubate on ice for 10 minutes and vortex occasionally.

10.4 Chromatin Shearing

10.4.1 Resuspend the chromatin lysate by vortexing.

10.4.2 Shear chromatin using one of the following methods.

10.4.2.1 Waterbath Sonication: Use 50 μ L of chromatin lysate per 0.2 mL tube or per PCR plate well. Shear 20 cycles under cooling condition, 15 seconds On, 30 seconds Off, each at 170-190 watts. OR follow supplier instructions for the sonicator you are using.

10.4.2.2 Probe Sonication: Use 300 μ L of chromatin lysate per 1.5 mL microcentrifuge tube. As an example, sonication can be carried out with a microtip, set to 25% power output. Sonicate 3-4 pulses of 10-15 seconds each, followed by 30-40 seconds rest on ice between each pulse. (The conditions of cross-linked DNA shearing can be optimized based on cells and sonicator equipment).

Note: *When probe-based sonication is carried out, the shearing effect may be reduced if foam is formed in chromatin sample solution. Under this condition, discontinue sonication and centrifuge the sample at 4°C at 12,000 rpm for 3 minutes to remove the air bubbles.*

The isolated chromatin can also be sheared with various enzyme-based methods. Optimization of the shearing conditions, for example enzyme concentration and incubation time, is needed in order to use enzyme-based methods.

10.4.3 Centrifuge at 12,000 rpm at 4°C for 10 minutes after shearing.

10.4.4 Transfer supernatant to a new vial.

The chromatin solution can now be used immediately or stored at -80°C after aliquoting appropriately until further use. Avoid multiple freeze/thaw cycles.

Note: *The size of sonicated chromatin should be verified before starting the immunoprecipitation step.*

The following steps can be carried out to isolate DNA for gel analysis of DNA fragment size: (1) add 25 µL of each chromatin sample to a 0.2 mL PCR tube followed by adding 25 µL of DNA Release Buffer and 2 µL of Proteinase K; (2) incubate the sample at 60°C for 30 minutes followed by incubating at 95°C for 10 minutes; (3) spin the solution down to the bottom; (4) transfer supernatant to a new 0.2 mL PCR vial. Use 30-40 µL for DNA fragment size analysis along with a DNA marker on a 1-2% agarose gel; and (5) Stain with ethidium bromide or other fluorescent dye for DNA and visualize it under ultraviolet light.

The length of sheared DNA should be between 100-700 bp with a peak size of 300 bp.

11. ASSAY PROCEDURE

11.1 Preparation of ChIP Reaction

- 11.1.1 Carefully peel away the Adhesive Covering Film on the antibody binding wells to avoid contamination between each well.
- 11.1.2 Remove the antibody reaction solution and Non-Immune IgG solution from each well and wash the wells one time with 150 μ L of ChIP Buffer.
- 11.1.3 Set up the ChIP reactions by adding the reagents to the wells that are bound with antibodies (sample and positive control wells) or IgG (negative control well) according to the following chart:

Reagents	Sample (μ L)	Positive control (μ L)	Negative control (μ L)
ChIP Buffer	48-78	48-78	48-78
Chromatin	10-40	10-40	10-40
Enrichment Enhancer	2	2	2
Blocker Solution	10	10	10
Total	100	100	100

Note: The final amount of chromatin should be 2 μ g/well (2×10^5 cells may yield 1 μ g of chromatin); Sonicated chromatin can be further diluted with ChIP Buffer to desired concentration. For histone samples with sufficient chromatin ($> 0.5 \mu$ g), the Enrichment Enhancer is not required and 50-80 μ L of ChIP Buffer can be used. For low abundance targets, 2 μ L of Enrichment Enhancer and 88 μ L of chromatin can be used without adding ChIP Buffer.

Freshly prepared chromatin can be directly used for the reaction. Frozen chromatin samples should be thawed quickly at RT and then placed on ice before use. Store remaining chromatin samples at -20°C or -80°C if they will be not used within 8 hours. Input DNA control is only used for estimating the enrichment efficiency of ChIP and is generally not necessary since the positive and negative control can be used for estimating the same objective more accurately.

- 11.1.4 Cap wells with Strip Caps and incubate at room temperature for 60-90 minutes on an orbital shaker (100 rpm). For low abundance targets, the incubation time should extend to 2-3 hours or at 4°C overnight.

11.2 Washing of the Reaction Wells

- 11.2.1 Carefully remove the solution and discard from each well.
- 11.2.2 Wash each well with 200 μ L of the Wash Buffer each time for a total of 4 washes. Allow 2 minutes on an orbital shaker (100 rpm) for each wash. Pipette wash buffer out from the wells.
- 11.2.3 Wash each well with 200 μ L of the DNA Release Buffer one time by pipetting DNA Release Buffer in and out of the well.

11.3 Reversal of Cross-Links, Release and Purification of DNA

- 11.3.1 Prepare RNase A solution by adding 1 μ L of RNase A to 40 μ L of DNA Release Buffer.
- 11.3.2 Add 40 μ L of DNA Release Buffer + RNase A to each well, and then cover with Strip Caps.
- 11.3.3 Incubate the wells at 42°C for 30 minutes.
- 11.3.4 Add 2 μ L of Proteinase K to each of the wells and re-cap the wells.
- 11.3.5 Incubate the wells at 60°C for 45 minutes.
- 11.3.6 Quickly transfer the DNA solution from each well to 0.2 mL strip PCR tubes. Cap the PCR tubes.
- 11.3.7 Incubate the PCR tubes containing DNA solution at 95°C for 15 minutes in a thermocycler.

- 11.3.8 Place the PCR tubes at room temperature. If liquid is collected on the inside of the caps, briefly spin the liquid down to the bottom. DNA prepared at this step can be directly used for PCR or go to Step 11.3.9 for further purification for use in ChIP-Seq.
- 11.3.9 Place a spin column into a 2 mL collection tube. Add 200 μ L of DNA Binding Solution to the samples and transfer the mixed solution to the column. Centrifuge at 12,000 rpm for 30 seconds.
- 11.3.10 Add 200 μ L of 90% ethanol to the column, centrifuge at 12,000 rpm for 30 seconds. Remove the column from the collection tube and discard the flowthrough.
- 11.3.11 Replace column to the collection tube. Add 200 μ L of 90% ethanol to the column and centrifuge at 12,000 rpm for 30 seconds.
- 11.3.12 Remove the column and discard the flowthrough. Replace column to the collection tube and wash the column again with 200 μ L of 90% Ethanol at 12,000 rpm for 1 minute.
- 11.3.13 Place the column in a new 1.5 mL vial. Add 20 μ L of DNA Elution Buffer directly to the filter in the column and centrifuge at 12,000 rpm for 30 seconds to elute the purified DNA.

Purified DNA is now ready for PCR, ChIP-chip and ChIP-seq use or storage at -20°C .

Note: *For real time PCR analysis, we recommend the use of 1-2 μ L of eluted DNA in a 20 μ L PCR reaction. If input DNA will be used, it should be diluted 10 fold before adding to the PCR reaction. Control primers (110 bp, for human cells) included in the kit can be used as a positive control. For end point PCR, the number of PCR cycles may need to be optimized for better PCR results.*

In general, the amplification difference between “normal IgG control” and “positive control” may vary from 3 to 8 cycles, depending on experimental conditions.

In general, at least 10 ng of ChIPed DNA is required for ChIP DNA library construction. This amount can easily be generated for high abundance targets from a single ChIP reaction well. However this may be difficult for low abundance target enrichment. For low abundance targets, we recommend pooling the DNA solution from several wells with the same ChIP reaction to gain 10 ng or more DNA amount. We also recommend performing qPCR to verify the quality of the ChIPed DNA.

12. ANALYSIS

12.1 Real Time PCR

Primer Design: The primers designed should meet the criteria for real time PCR. For example, the covered sequence region should be 50-150 bp in length. G/C stretches at 3' ends of primers should be avoided.

PCR Reaction: Real time PCR can be performed using your own proven method. As an example, the protocol is presented below:

Prepare the PCR Reactions: Thaw all reaction components including master mix, DNA/RNA free water, primer solution and DNA template. Mix well by vortexing briefly. Keep components on ice while in use and return to -20°C immediately following use. Add components into each well according to the following:

Component	Volume	Final concentration
Master Mix (2X)	10 μL	1X
Forward Primer	1 μL	0.4-0.5 μM
Reverse Primer	1 μL	0.4-0.5 μM
DNA Template	1-2 μL	50 pg – 0.1 μg
DNA/RNA free H ₂ O	6-7 μL	
Total volume	20 μL	

For the negative control, use DNA/RNA-free water instead of DNA template.

Program the PCR Reactions: Place the reaction plate in the instrument and set the PCR conditions as follows:

Cycle step	Temperature (°C)	Time	Cycle
Activation	95	7 minutes	1X
Cycling	95	10 seconds	40
	55	10 seconds	
	72	8 seconds	
Final extension	72	1 minute	1

Fold Enrichment Calculation: Fold enrichment (FE) can be calculated by simply using a ratio of amplification efficiency of the ChIP sample over that of Non-Immune IgG. Amplification efficiency of RNA Polymerase II can be used as a positive control.

$$\text{FE\%} = 2^{(\text{IgG CT} - \text{Sample CT})} \times 100\%$$

For example, if CT for IgG is “38” and the sample is “34”, then ...

$$\text{FE\%} = 2^{(38-34)} \times 100\% = 1600\%$$

12.2 Endpoint PCR

Primer Design: The primers designed should meet the criteria for endpoint PCR. For example, the covered sequence region should be 100-400 bp in length. PCR primer design tools (e.g., Primer3Plus) can be used to help in the selection of appropriate primer pairs.

PCR Reaction: Endpoint PCR can be performed using your own proven method. It is important to stop the PCR reaction at the exponential phase by setting up an appropriate number of PCR cycles in order to make a reliable comparison of enrichment efficiency obtained from different ChIP reactions. Thus, the optimized number of PCR cycles should be determined empirically.

PCR Product Analysis: Endpoint PCR products can be analyzed by separating amplicons on a 1-2% agarose gel, followed by staining with ethidium bromide and visualizing with UV-illumination.

13. TROUBLESHOOTING

Problem	Cause	Solution
Little or no PCR products generated from both sample and positive control wells	Poor chromatin quality due to insufficient amount of cells, or insufficient or over cross-linking.	The optimal amount of chromatin obtained per ChIP reaction should be 2-4 µg (about 2-4 x 10 ⁵ cells). Appropriate chromatin cross-linking is also required. Insufficient or over-crosslinking will cause DNA loss or increased background. During the cross-linking step of chromatin preparation, ensure that the cross-linking time is within 10-15 minutes, the final concentration of formaldehyde is 1%, and the quench solution is 0.125 M glycine
	Poor enrichment with antibody: some antibodies used in ChIP might not efficiently recognize fixed protein	Increase the antibody amount and use ChIP-grade antibodies validated for use in ChIP.
	Inappropriate DNA fragmenting condition.	If chromatin is from a specific cell/tissue type, or is differently fixed, the shearing conditions should be optimized to allow DNA fragment size to be between 200-800 bp

RESOURCES

	<p>Incorrect temperature and/or insufficient time during DNA release.</p>	<p>Ensure the incubation times and temperatures described in the protocol are followed correctly</p>
	<p>Improper PCR conditions, including improper PCR programming, PCR reaction solutions, and/or primers</p>	<p>Ensure the PCR is properly programmed. If using a homebrew PCR reaction solution, check if each component is correctly mixed. If using a PCR commercial kit, check if it is suitable for your PCR. Confirm species specificity of primers. Primers should be designed to cover a short sequence region (70-150 bp) for more efficient and precise amplification of target DNA region (the binding site of the protein of interest)</p>
	<p>Improper sample storage</p>	<p>Chromatin sample should be stored at – 80°C for no longer than 6 months, preferably less than 3 months. Avoid repeated freeze/thaw cycles. DNA samples should be stored at –20°C for no longer than 6 months, preferably less than 3 months</p>

RESOURCES

<p>No difference in signal intensity between negative and positive control wells</p>	<p>Insufficient washing</p>	<p>Check if washing recommendations at each step is performed according to the protocol. If the signal intensity in the negative control is still high, washing stringency can be increased in the following ways:</p> <ol style="list-style-type: none"> 1. Increase wash time at each wash step: after adding Wash Buffer, leave it in the wells for 3-4 min and then remove it. 2. Add an additional one wash with Wash Buffer, respectively: the provided volume of Wash Buffer is sufficient for 4 extra washes for each sample
	<p>Too many PCR cycles; Plateau phase of amplification caused by excessive number of PCR cycles in endpoint PCR may mask the difference of signal intensity between negative control and positive control</p>	<p>Decrease the number of PCR cycles (i.e., 32-35 cycles) to keep amplification at the exponential phase. This will reduce high background in endpoint PCR and allow differences in amplification to be seen. Real time PCR is another choice in such cases</p>

RESOURCES

Little or no PCR products generated from sample wells only	Poor enrichment with antibody; Some antibodies used in ChIP might not efficiently recognize fixed protein	Poor enrichment with antibody; Some antibodies used in ChIP might not efficiently recognize fixed protein
	PCR primers are not optimized	Confirm species specificity of primers. Primers should be designed to cover a short sequence region (70-150 bp) for more efficient and precise amplification of target DNA region (the binding site of the protein of interest)

14. NOTES

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