

ab185908 – ChIP-Seq High Sensitivity Kit

Instructions for Use

For selectively enriching a chromatin fraction containing specific DNA sequences from various species, particularly mammals, and to prepare a ChIP-Seq library for next generation sequencing.

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 on a genome-wide scale is important for understanding cellular processes.

Chromatin immunoprecipitation (ChIP) followed by next generation sequencing (ChIP-Seq) offers an advantageous tool for studying genome-wide protein-DNA interactions. It allows for detection that a specific protein binds to specific sequences in living cells. In particular, ChIP antibodies targeted against various transcriptional factors (TF) for genome-wide transcription factor binding site analysis by Chip-Seq is in high demand. Such analysis requires that ChIPed DNA contain minimal background for reliably identifying true TF-enriched regions. Currently used ChIP-Seq methods play an important role in identifying genome-wide protein-DNA interaction. However, these methods still have several drawbacks: 1) large amounts of cell/tissues are needed for obtaining a sufficient yield of library DNA, therefore these methods cannot be used for biological samples such as tumor biopsy and embryonic tissues whose amounts are limited; 2) the background levels of ChIPed DNA are high; and 3) the procedures are time consuming (>3 days) and inconvenient.

To address this issue, ab185908 combines microplate-based ultra ChIP and high sensitive DNA library construction technologies. This kit has the following features:

This kit has the following features:

- 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 of monoclonal or polyclonal form.

- Highly efficient enrichment of targeted DNA. Enrichment ratio of positive to negative control > 500.
- High sensitivity and flexibility: Can be used for both non-barcoded (singleplexed) and barcoded (multiplexed) DNA library preparation. The input cell number can be as few as 50,000 cells with a range from 50,000 to 1,000,000 cells. Broad range of cell/tissue samples can be used, including samples with limited amount.
- Fast and streamlined procedure: The procedure from cell/tissues to library DNA is less than 7 hours. No clean-up is required between each step from ChIPed DNA to size selection, and all reactions take place in the same tube, thereby saving time and preventing handling errors or loss of valuable samples. Gel-free size selection further reduces the preparation time.
- Highly convenient for use: The kit contains all required components for each step of ChIP-Seq, which are sufficient for both ChIP and ChIPed DNA library preparation, thereby allowing the ChIP-Seq to be the most convenient with reliable and consistent results.
- Minimized bias: Ultra HiFi amplification and optional PCR-free step allow achievement of reproducibly high yields of DNA library with minimal sequence bias and low error rates.

ab185908 ChIP-Seq High Sensitivity Kit is designed to selectively enrich a chromatin fraction containing specific DNA sequences from various species, particularly mammals, and to prepare a ChIP-Seq library for next generation sequencing using Illumina® platforms systems. The optimized protocol and components of the kit allow capture of low abundance protein/DNA complexes with minimized non-specific background levels and the ability to construct both non-barcoded (singleplexed) and barcoded (multiplexed) ChIP-Seq libraries quickly with reduced bias.

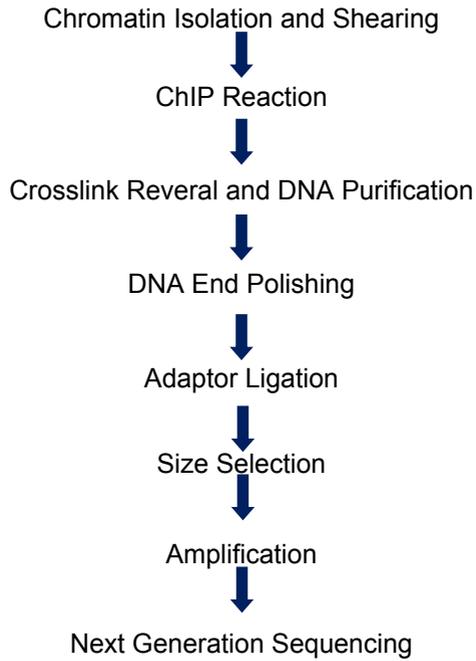
ab185908 contains all necessary reagents required for carrying out a successful ChIP-Seq starting from mammalian cells or tissues. In the ChIP

reaction, chromatin is isolated from cell/tissues and the target protein-DNA complex is immunoprecipitated using the antibody of interest. Immunoprecipitated DNA is then cleaned, released, and eluted. Included in the kit are a positive control antibody (RNA polymerase II), a negative control non-immune IgG, and GAPDH primers, which 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.

In the library preparation, ChIPed DNA fragments are end repaired and dA tailed (end polishing) simultaneously. Adaptors are then ligated to both ends of the polished DNA fragments for amplification and sequencing. Ligated fragments are size selected and purified using MQ binding beads, which allows quick and precise size selection of DNA. Size-selected DNA fragments are amplified with a high-fidelity PCR mix which ensures maximum yields from minimum amounts of starting material and provides highly accurate amplification of library DNA with low error rates and minimum bias.

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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.

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

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

5. MATERIALS SUPPLIED

For Library Preparation

Item	12 Tests	24 Tests	Storage Condition (Before Preparation)
10X End Polishing Buffer	30 µL	60 µL	-20°C
End Polishing Enzyme Mix	13 µL	26 µL	-20°C
End Polishing Enhancer	13 µL	26 µL	-20°C
2X Ligation Buffer	250 µL	500 µL	-20°C
T4 DNA Ligase	15 µL	30 µL	-20°C
Adaptors (50 µM)	15 µL	30 µL	-20°C
MQ Binding Beads	1.6 mL	2 x 1.6 mL	4°C
2X HiFi PCR Master Mix	160 µL	320 µL	-20°C
Primer U (10 µM)	15 µL	30 µL	-20°C
Primer I (10 µM)	15 µL	30 µL	-20°C
Elution Buffer	1 mL	2 mL	-20°C

GENERAL INFORMATION

For CHIP Reaction

Item	12 Tests	24 Tests	Storage Condition (Before Preparation)
Wash Buffer	12 mL	25 mL	4°C
Antibody Buffer	1 mL	2 mL	4°C
Lysis Buffer	7 mL	14 mL	RT
ChIP Buffer	6 mL	12 mL	4°C
DNA Release Buffer	7 mL	14 mL	RT
DNA Binding Solution	7 mL	14 mL	RT
Blocker Solution	1 mL	2 mL	4°C
DNA Elution Buffer	0.5 mL	1 mL	RT
Enrichment Enhancer	25 µL	50 µL	-20°C
Protease Inhibitor Cocktail (1000X)	15 µL	30 µL	4°C
Non-Immune IgG (1 mg/mL)	5 µL	10 µL	4°C
Anti-RNA Polymerase II	5 µL	10 µL	4°C
Proteinase K (10 mg/mL)	30 µL	60 µL	4°C
RNase A	15 µL	30 µL	-20°C
GAPDH Primer - Forward (20 µM)	5 µL	10 µL	4°C
GAPDH Primer - Reverse (20 µM)	5 µL	10 µL	4°C
8-Well Assay Strips (With Frame)	2	4	4°C
8-Well Strip Caps	2	4	RT
Adhesive Covering Film Strip	4	8	RT
F-Spin Column	15	30	RT
F-Collection Tube	15	30	RT

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Sonicator or enzymes for DNA fragmentation
- Vortex mixer
- Dounce homogenizer with small clearance pestle
- Variable temperature waterbath or incubator oven
- Thermocycler with 48 or 96-well block
- Centrifuge including desktop centrifuge (up to 14,000 rpm)
- Method to assess the quality of DNA library
- Orbital shaker
- Magnetic stand (96-well PCR plate format)
- Adjustable pipette and pipette tips
- 0.2 mL or 0.5 mL PCR vials
- 1.5 mL microcentrifuge tubes
- 15 mL conical tube
- Antibodies of interest
- Cells or tissues
- 100% Ethanol
- Distilled water
- Cell culture medium
- 37% Formaldehyde (if cross-linked)
- 1.25 M Glycine solution (if cross-linked)
- 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

9.1 Working Lysis Solution

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

9.2 Working CHIP Buffer

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

10. SAMPLE PREPARATION

Cell Collection and Cross-linking

10.1 For Monolayer or Adherent Cells:

- 10.1.1 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 ($\times 10^5$)
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

- 10.1.2 Centrifuge the cells at 1000 rpm for 5 min. Discard the supernatant.

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

Note: For cells that are not cross-linked, go directly to Step 10.1.8 after Step 10.1.3.

10.1.4 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.

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

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

10.1.7 Mix and centrifuge at 1000 rpm for 5 min.

10.1.8 Remove medium and wash cells once with 10 mL of ice-cold PBS by centrifuging at 1000 rpm for 5 min.

10.1.9 Add Working Lysis Buffer to re-suspend the cell pellet (200 μ L/1x10⁶ cells) and incubate on ice for 10 min.

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

10.1.10 Vortex vigorously for 10 sec then centrifuge at 3000 rpm for 5 min. Go to Step 11.1.2, Cell Lysis and Chromatin Extraction.

10.2 For Suspension Cells:

10.2.1 Collect cells (treated or untreated) into a 15 mL conical tube (2 x10⁵ to 5x10⁵ cells are required for each ChIP reaction). Count cells in a hemocytometer.

10.2.2 Centrifuge the cells at 1000 rpm for 5 min. Discard the supernatant.

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

Note: For cells that are not cross-linked, go directly to Step 10.2.9 after Step 10.2.3.

10.2.4 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.

- 10.2.5 Incubate at room temperature (20-25°C) for 10 min on a rocking platform (50-100 rpm).
- 10.2.6 Add 1 mL of 1.25 M glycine for every 9 mL of cross-link solution.
- 10.2.7 Mix and centrifuge at 1000 rpm for 5 min.
- 10.2.8 Remove medium and wash cells once with 10 mL of ice-cold PBS by centrifuging at 1000 rpm for 5 min.
- 10.2.9 Add Working Lysis Buffer to re-suspend the cell pellet (200 μ L/1x10⁶ cells) and incubate on ice for 10 min.
- 10.2.10 Vortex vigorously for 10 sec and centrifuge at 3000 rpm for 5 min. Then go to Step 11.1.2.

10.3 For Tissues:

- 10.3.1 Put the tissue sample into a 60 or 100 mm plate. Remove unwanted tissue such as fat and necrotic material from the sample.
- 10.3.2 Weigh the sample and cut the sample into small pieces (1-2 mm³) with a scalpel or scissors.
Note: *For tissues that are not cross-linked, go directly to Step 10.3.11 after Step 10.3.2.*
- 10.3.3 Transfer tissue pieces to a 15 mL conical tube.
- 10.3.4 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).
- 10.3.5 Add 1 mL of cross-link solution for every 50 mg tissues.
- 10.3.6 Incubate at room temperature for 15-20 min on a rocking platform.
- 10.3.7 Add 1 mL of 1.25 M glycine for every 9 mL of cross-link solution.
- 10.3.8 Mix and centrifuge at 800 rpm for 5 min. Discard the supernatant.
- 10.3.9 Wash with 10 mL of ice-cold PBS once by centrifugation at 800 rpm for 5 min. Discard the supernatant.
- 10.3.10 Transfer tissue pieces to a Dounce homogenizer.
- 10.3.11 Add 0.5 mL Working Lysis Buffer for every 50 mg of tissues.

- 10.3.12 Disaggregate tissue pieces by 20-40 strokes.
- 10.3.13 Transfer homogenized mixture to a 15 mL conical tube and centrifuge at 3000 rpm for 5 min 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 min at 4°C. Then go to Step 11.1.2.

11. ASSAY PROCEDURE

For the best results please read the entire protocol before starting your experiment.

11.1 ChIP Reaction

11.1.1 Antibody Binding to Strip Wells

Antibodies should be ChIP-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 RNA Polymerase II should be used to demonstrate that the antibody and prepared chromatin are suitable for ChIP.

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

11.1.1.2 Setup the antibody binding reactions by adding the reagents to each well according to the following chart.

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 µg/well; (b) RNA Polymerase II: 0.8 µg/well; and (c) non-immune IgG: 0.8 µg/well.*

The amount of the positive control (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 are required and can be separately obtained

11.1.1.3 Seal the wells with Adhesive Covering Film Strips and incubate the wells at room temperature for 60-90 min on an orbital shaker (100 rpm). Meanwhile, perform steps 7 “Cell Collection and Cross-Linking”, 11.1.2 “Cell Lysis and Chromatin Extraction” and 11.1.3 “Chromatin Shearing”.

11.1.2 Cell Lysis and Chromatin Extraction

11.1.2.1 Carefully remove supernatant.

11.1.2.2 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).

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

11.1.3 Chromatin Shearing

11.1.3.1 Resuspend the chromatin lysate by vortexing.

11.1.3.2 Shear chromatin with one of the following methods:

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, 30 seconds ON, 30 seconds OFF, each at 170-190 watts. For more specific information please follow the waterbath sonicator supplier's instructions.

Probe-based Sonication: Use 300 μ L of chromatin lysate per 1.5 mL microcentrifuge tube. 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 the chromatin sample solution. Under this condition, discontinue sonication and centrifuge the sample at 4°C at 12,000 rpm*

for 3 min to remove the air bubbles then continue with sonication.

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.

11.1.3.3 Centrifuge at 12,000 rpm at 4°C for 10 min after shearing.

11.1.3.4 Transfer supernatant to a new vial.

11.1.3.5 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 immunoprecipitation step. The length of sheared DNA should be between 100-700 bps with a peak size of about 300 bps.

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 min followed by incubating at 95°C for 10 min; (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.

11.1.4 Preparation of ChIP Reaction

11.1.4.1 Peel away the Adhesive Covering Film on the antibody binding wells (from Step 11.1.1.2) carefully to avoid contamination between each well.

11.1.4.2 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.

11.1.4.3 Setup 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	50-80	50-80	50-80
Chromatin	10-40	10-40	10-40
Enrichment Enhancer	2	2	2
Blocker Solution	10	10	10

Note: *The final amount of chromatin should be 2 μ g/well (2 x 10⁵ cells may yield 1 μ g of chromatin); Sonicated chromatin can be further diluted with ChIP Buffer to desired concentration. For histone samples containing sufficient chromatin (> 0.5 μ 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 at -80°C if they will be not used within 8 hours.

An 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.

If you would like to include the input DNA control, the purified input DNA prepared at Step 11.1.2.3 Note can be used

11.1.4.4 Cap wells with strip cap and incubate at room temperature for 60-90 min on an orbital shaker (100 rpm). For low abundance targets, incubation time should be extended to 2-3 hours or at 4°C overnight.

11.1.5 **Washing of Reaction Cells**

11.1.5.1 **Carefully remove the solution** using a pipette and discard from each well.

11.1.5.2 Wash each well with 200 µL of fresh Wash Buffer each time for 4 times. Allow 2 minutes on an orbital shaker (100 rpm) for each wash. Pipette wash buffer out from the wells.

11.1.5.3 Wash each well with 200 µL of DNA Release Buffer one time by pipetting DNA Release Buffer into the well and then removing it.

11.1.6 **Reversal of Cross-Links, release and Purification of DNA**

11.1.6.1 Prepare RNase A solution by adding 1 µL of RNase A to 400 µL of DNA Release Buffer.

11.1.6.2 Add 40 µL of DNA Release Buffer-RNase A to each well, and then cover with a strip cap.

11.1.6.3 Incubate the wells at 42°C for 30 min.

11.1.6.4 Add 2 µL of Proteinase K to each well and re-cap the wells.

11.1.6.5 Incubate the wells at 60°C for 30 min.

11.1.6.6 Quickly transfer the DNA solution from each well to 0.2 mL strip PCR tubes. Cap the PCR tubes.

11.1.6.7 Incubate the PCR tubes containing DNA solution at 95°C for 15 min in a thermocycler.

11.1.6.8 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.

11.1.6.9 Place a spin column into a 2 mL collection tube. Add 200 µL of DNA Binding Solution to the samples and transfer mixed solution to the column. Centrifuge at 12,000 rpm for 30 seconds.

- 11.1.6.10 Add 200 μL 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.
- 11.1.6.11 Replace column to the collection tube. Add 200 μL of 90% ethanol to the column and centrifuge at 12,000 rpm for 30 seconds.
- 11.1.6.12 Remove the column and discard the flowthrough. Replace column to the collection tube and wash the column again with 200 μL of 90% ethanol at 12,000 rpm for 1 min.
- 11.1.6.13 Place the column in a new 1.5 mL vial. Add 11 μL of DNA Elution Buffer directly to the filter in the column and centrifuge at 12,000 rpm for 30 seconds to elute purified DNA.

Purified DNA is now ready for ChIPed DNA library preparation after verifying the quality and amount of the ChIPed DNA by qPCR or a suitable fluorescence method.

Note: *For real time PCR analysis, we recommend the use of 1 μL of eluted DNA in a 20 μL PCR reaction. If input DNA will be used, it should be diluted 10 fold before adding to PCR reaction. Control primers (110 bp, for human cells) included in the kit can be used as a positive control.*

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

Optimally, 10 ng of ChIPed DNA are required for ChIP DNA library construction. This amount can be easily generated for high abundance targets from a single ChIP reaction well. However, this may be difficult for low abundance target enrichment. We recommend pooling the DNA solution from several low abundance ChIP reaction wells to gain 10 ng or more of DNA.

11.2 ChIPed DNA Library Preparation

11.2.1 DNA End Polishing

11.2.1.1 Prepare the End Repair reaction in a 0.2 mL PCR tube according to Table below:

Component	Sample (µL)
ChIPed DNA (from Step 11.1.6)	10
10X End Repair Buffer	1.5
End Repair Enzyme Mix	1
End Polishing Enhancer	1
Distilled Water	1.5
Total Volume	15

11.2.1.2 Mix and incubate for 20 min at 25°C and 20 min at 72°C in a thermocycler (without heated lid).

Note: *The amount of fragmented DNA can be 0.2-100 ng with an optimal amount of 10-50 ng.*

11.2.2 Adaptor Ligation

11.2.2.1 Prepare the Adaptor Ligation reaction in a 0.2 mL PCR tube according to Table below:

Component	Sample (µL)
End Polished DNA(Step 11.2.1)	15
2X Ligation Buffer	17
T4 DNA Ligase	1
Adaptors	1
Total Volume	34

11.2.2.2 Mix and incubate for 15 min at 25°C in a thermocycler (without heated lid).

Note: *(1) The pre-annealed adapters included in the kit are suitable for both non-barcoded (singleplexed) and barcoded (multiplexed) DNA library preparation and are fully*

compatible with Illumina® platforms, such as MiSeq® or HiSeq™ sequencers. (2) If using adaptors from other suppliers (both single-end and barcode adaptors), make sure they are compatible with Illumina® platforms and add the correct amount (final concentration 1.5-2 µM, or according to the supplier's instruction).

11.2.3 Size Selection of Ligated DNA

If the starting DNA amount is less than 50 ng, the size selection is not recommended and alternatively, clean-up of ligated DNA can be performed prior to PCR amplification according to 11.2.4 protocol.

11.2.3.1 Resuspend MQ Binding Beads by vortex.

11.2.3.2 Add 14 µL of resuspended MQ Binding Beads to the tube of ligation reaction. Mix well by pipetting up and down at least 10 times.

11.2.3.3 Incubate for 5 minutes at room temperature.

11.2.3.4 Put the tube on an appropriate magnetic stand until the solution is clear (about 2 minutes). Carefully transfer the supernatant containing DNA to a new tube. (Caution: Do not discard the supernatant.) Discard the beads that contain the unwanted large fragments.

11.2.3.5 Add 10 µL resuspended beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

11.2.3.6 Put the PCR tube on an appropriate magnetic stand until the solution is clear (about 2 minutes). Carefully remove and discard the supernatant. (Caution: Be careful not to disturb or discard the beads that contain DNA).

11.2.3.7 Keep the PCR tube in the magnetic stand and add 200 µL of freshly prepared 90% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.

11.2.3.8 Repeat Step g one time, for total of two washes.

11.2.3.9 Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.

- 11.2.3.10 Resuspend the beads in 12 μ L Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.2.3.11 Capture the beads by placing the tube in the magnetic stand for 4 minutes or until the solution is completely clear.
- 11.2.3.12 Transfer 11 μ L to a new 0.2 mL PCR tube for PCR amplification.

11.2.4 Clean Up of Ligated DNA (Optional)

- 11.2.4.1 Resuspend MQ Binding Beads by vortex.
- 11.2.4.2 Add 34 μ L of resuspended beads to the PCR tube of ligation reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.
- 11.2.4.3 Incubate for 5 minutes at room temperature to allow DNA to bind to beads.
- 11.2.4.4 Put the PCR tube on an appropriate magnetic stand until the solution is clear (about 2 minutes). Carefully remove and discard the supernatant. (Caution: Be careful not to disturb or discard the beads that contain DNA.)
- 11.2.4.5 Keep the PCR tube in the magnetic stand and add 200 μ L of freshly prepared 90% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.
- 11.2.4.6 Repeat Step 11.2.4.5 two times for total of three washes.
- 11.2.4.7 Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.
- 11.2.4.8 Resuspend the beads in 12 μ L Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.2.4.9 Capture the beads by placing the tube in the magnetic stand for 4 minutes or until the solution is completely clear.
- 11.2.4.10 Transfer 11 μ L to a new 0.2 mL PCR tube for PCR amplification.

11.2.5 Library Amplification

11.2.5.1 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 PCR tube/well according to the following table.

Component	Sample (µL)
HiFi PCR Master Mix (2X)	12.5
Primer U	1
Primer I	1
Adaptor Ligated DNA	10.5
Total Volume	25

Note: Use of Primer I included in the kit will generate a singleplexed library. For multiplexed library preparation, replace Primer I with user defined barcodes (Illumina® compatible) instead of Primer I.

11.2.5.2 Place the reaction plate in the PCR instrument and set the PCR conditions as follows.

Cycle Step	Temp (°C)	Time (seconds)	Cycle #
Activation	98	30	1
Cycling	98	20	Variable*
	55	20	
	72	20	
Final Extension	72	120	1

***Note:** PCR cycles may vary depending on the input DNA amount. In general, use 12 PCR cycles for 200 ng, 13 cycles for 100 ng, 15 cycles for 50 ng, 17 cycles for 10 ng, and 22 cycles for 1 ng DNA input. Further optimization of PCR cycle number may be required by the end user.

11.2.6 Clean up of Amplified Library DNA

11.2.6.1 Resuspend MQ Binding Beads by vortex.

11.2.6.2 Add 25 µL of resuspended beads to the PCR tube of amplification reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

11.2.6.3 Incubate for 5 minutes at room temperature to allow DNA to bind to beads.

11.2.6.4 Put the PCR tube on an appropriate magnetic stand until the solution is clear (about 2 minutes). Carefully remove and discard the supernatant. (Caution: Be careful not to disturb or discard the beads that contain DNA.)

11.2.6.5 Keep the PCR tube in the magnetic stand and add 200 µL of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 1 min, and then carefully remove and discard the ethanol.

11.2.6.6 Repeat Step 11.2.6.5 two times for total of three washes.

11.2.6.7 Open the PCR tube cap and air dry beads for 10 minutes while the tube is on the magnetic stand.

- 11.2.6.8 Resuspend the beads in 22 μ L Elution Buffer, and incubate at room temperature for 2 minutes to release the DNA from the beads.
- 11.2.6.9 Capture the beads by placing the tube in the magnetic stand for 4 minutes or until the solution is completely clear.
- 11.2.6.10 Transfer 20 μ L to a new 0.2 mL PCR tube..

Quality of the prepared library can be assessed using an Agilent® Bioanalyzer® or other comparable methods. Library fragments should have the correct size distribution (e.g., 300-400 bps at peak size) without adaptors or adaptor-dimers.

To check the size distribution, dilute library 5-fold with water and apply it to an Agilent® high sensitivity chip. If there is presence of <150 bp adaptor dimers or of larger fragments than expected, they should be removed. To remove fragments below 150 bp or above 500 bp, use 0.8X MQ Binding Beads according to sub-steps a through l of Step 11.2.3 - "Size Selection of Ligated DNA".

Store the prepared library at -20°C until ready to use for sequencing.

12. ANALYSIS

Typical Results

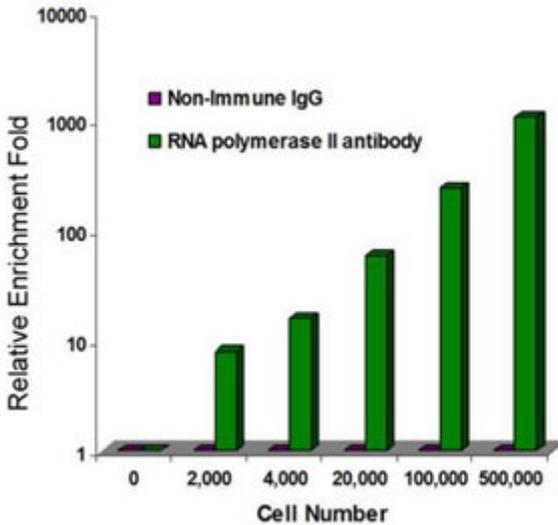


Fig. 1. High sensitive ChIP: The sheared chromatin isolated from different number of MBD-231 cells was used for ChIP-qPCR analysis of RNA polymerase II enrichment in GAPDH promoters.

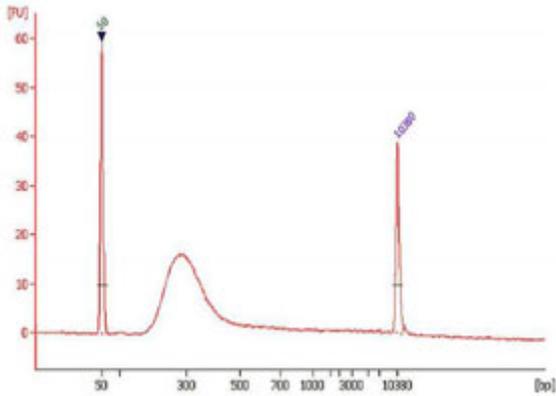


Fig. 2. Size distribution of library fragments. Ten nanograms of DNA was ChIPed by RNA polymerase II enrichment and used for DNA library preparation.

13. TROUBLESHOOTING

For ChIP Reaction:

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 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 min, 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

RESOURCES

	Inappropriate DNA fragmenting condition	If chromatin is from specific cell/tissue types, or is differently fixed, the shearing conditions should be optimized to allow DNA fragment size to be between 100-700 bp
	Incorrect temperature and/or insufficient time during DNA release	Ensure the incubation times and temperatures described in the protocol are followed correctly
	Improper PCR conditions, including improper PCR programming, PCR reaction solutions, and/or primers.	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 the target DNA region (the binding sites of the protein of interest).

RESOURCES

	Improper sample storage	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.
No difference in signal intensity between negative and positive control wells	Insufficient washing	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: <ol style="list-style-type: none">1. Increase wash time at each wash step: after adding WB, 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 WashBuffer is sufficient for 4 extra washes for each sample.

RESOURCES

	<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>
<p>Little or no PCR products generated from sample wells only</p>	<p>Poor enrichment with antibody: some antibodies used in ChIP might not efficiently recognize fixed protein</p>	<p>Increase the antibody amount and use ChIP-grade antibodies validated for use in ChIP</p>
	<p>PCR primers are not optimized.</p>	<p>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 sites of the protein of interest)</p>

RESOURCES

For ChIPed DNA Library Preparation:

Problem	Cause	Solution
Low yield of library	Insufficient amount of starting DNA.	To obtain the best results, the amount of input DNA should be 100-200 ng. For a library directly used for sequencing without amplification, 500 ng or more is needed
	Insufficient purity of starting DNA.	Ensure that RNA is removed by RNase A treatment before starting library preparation protocol
	Improper reaction conditions at each reaction step.	Check if the reagents are properly added and incubation temperature and time are correct at each reaction step including DNA End Polishing, Adaptor Ligation, Size Selection and Amplification
	Improper storage of the kit.	Ensure that the kit has not exceeded the expiration date. Standard shelf life, when stored properly, is 6 months from date of receipt

RESOURCES

Unexpected peak size of trace: presence of <150 bp adaptor dimmers or presence of larger fragments than expected	Improper ratio of MQ Binding Beads to DNA volume in size selection	Check if the correct volume of MQ Binding Beads is added to DNA solution accordingly. Proper ratios should remove the fragments with unexpected peak sizes
	Insufficient ligation	Too much and too little input DNA may cause insufficient ligation, which can shift peak size of the fragment population to be shorter or larger than expected. Make sure that ligation reaction is properly processed with the proper amount of input DNA
	Over-amplification of library	PCR artifacts from over-amplification of the library may cause the fragment population to shift higher than expected. Make sure to use proper PCR cycles to avoid this problem

14. NOTES

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