Version 1a Last updated 31 August 2021

ab270812 ChIP Kit (Histones, ChIP-seq)

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A robust ChIP protocol suitable for the investigation of histone modifications within chromatin from cells and tissues.

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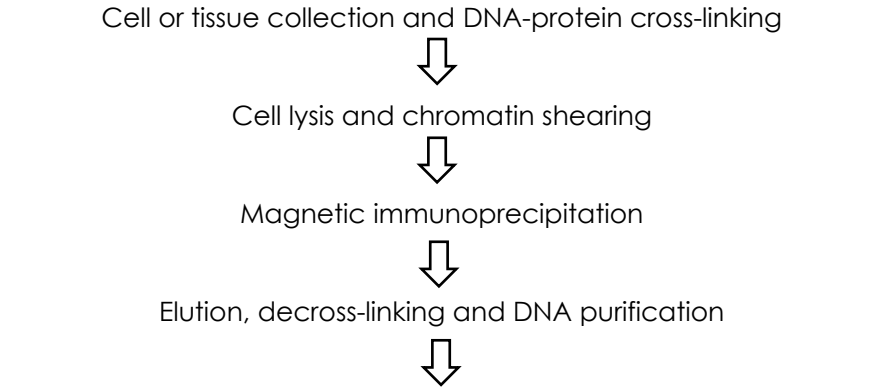
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1. Overview

ChIP Kit (Histones, ChIP-seq) (ab270812) provides a robust ChIP protocol suitable for the investigation of histone modifications within chromatin from cells and tissues. The protocol involves protein-DNA cross-linking with formaldehyde, followed by cell lysis and fragmentation of the cross-linked chromatin. The subsequent immunoprecipitation of chromatin is performed with an antibody (user supplied) specific to a target histone modification. Protein A magnetic beads are used to isolate the protein-DNA complexes of interest. The immunoprecipitated DNA is eluted and purified using unique IPure magnetic beads. The eluted DNA is ideal for ChIP-seq library preparation. Additionally, the kit includes a negative (IgG) and a positive (H3K4me3) control antibody and primer pairs amplifying H3K4me3 positive and negative loci.



2. Materials Supplied and Storage

Store kit at +4°C immediately on receipt. Please see table below for individual storage conditions.

Component	Quantity 24 tests	Quantity 10 tests	Storage
5% BSA (DNA free)	144 µl	60 µl	-20°C
5x ChIP Buffer C1	2.2 ml	950 µl	4°C
Buffer C	1.6 ml	700 µl	4°C
Carrier	64 µl	28 µl	-20°C
ChIP-seq grade antibody H3K4me3	8 µg	8 µg	-20°C
ChIP-seq grade GAPDH TSS primer pair	96 µl	42 µl	-20°C
ChIP-seq grade Myoglobin exon 2 primer pair	96 µl	42 µl	-20°C
ChIP-seq grade water	7 ml	3 ml	4°C
Protein A-coated magnetic beads	480 µl	200 µl	4°C
Elution Buffer E1	3.4 ml	1.5 ml	4°C
Elution Buffer E2	144 µl	64 µl	4°C
Glycine	400 µl	200 µl	4°C
Purification Beads	320 µl	140 µl	4°C
Lysis Buffer L1	40 ml	20 ml	4°C
Lysis Buffer L2	40 ml	20 ml	4°C
Protease inhibitor cocktail	76 µl	35 µl	-20°C
Rabbit IgG	8 µg	8 µg	-20°C
Shearing Buffer S1	7.2 ml	3.6 ml	4°C
Wash Buffer 1 w/o iso-propanol	2 ml	700 µl	4°C

Wash Buffer 2 w/o iso-propanol	2 ml	700 µl	4°C
Wash Buffer W1	8.4 ml	3.5 ml	4°C
Wash Buffer W2	8.4 ml	3.5 ml	4°C
Wash Buffer W3	8.4 ml	3.5 ml	4°C
Wash Buffer W4	8.4 ml	3.5 ml	4°C

3. Materials Required, Not Supplied

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

- Gloves to wear at all steps
- RNase/DNase-free 1.5 ml and 15 ml tubes
- Formaldehyde, 37%, molecular grade
- Phosphate buffered saline (PBS) buffer
- Trypsin-EDTA
- Cell culture medium
- qPCR SYBR® Green Mastermix
- ChIP-seq grade antibodies
- Fluorescence-based assay for DNA concentration measurement
- Dounce homogenizer with loose a pestle (2 ml)
- Scalpel blades
- Petri dishes
- 1.5ml magnetic rack
- Cell counter system
- Sonication device and the associated microtubes
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- Rotating wheel
- Vortex
- Thermomixer
- Fluorometer
- qPCR cycler

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Protocol

- Before proceeding please read the Pre-Chip Assay Preparation Guidlines and Protocol for Chromatin Shearing Analysis documents on the product datasheet
- Protocol for the first step differs for cells (step 5.1) and tissues (step 5.2).
- The protocol below describes the chromatin preparation (formaldehyde cross-linking, cell collection, lysis and chromatin shearing) from a batch of approximately 7 million cells or 30-40 mg of tissue. This will be sufficient for 6 ChIP reactions (using approximately 1 million cells or 5 mg of tissue per IP), 1 input and 1 sample for chromatin shearing assessment.

- Please note that the described protocol uses a prompt direct fixation in a cell culture plate. Fixed and scraped cells cannot be accurately counted. This means that for adherent cells you need to use an approximate estimation of cells number per plate. Alternatively, an additional parallel plate for counting can be prepared. Suspension cells can be counted before the fixation.

5.1 Cell collection and DNA-protein cross-linking.

For adherent cells:

5.1.1 Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.

5.1.2 Remove the medium and rinse the cells with pre-warmed PBS (10ml for a 75 cm^2 culture flask). Gently shake the flask for 2 mins.

5.1.3 Remove the PBS and add sterile trypsin-EDTA to the culture flask to detach adherent cells from the bottom. Add enough trypsin-EDTA to completely cover the cells (e.g. 1 ml for a 75 cm^2 culture flask). Gently shake the culture flask for 1-2 mins or until the cells start to detach.

Δ Note: The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment may damage the cells. Regularly check if the cells start to detach.

5.1.4 Immediately add fresh culture medium to the cells when they are detached. For correct inactivation, the volume of medium must be 2x the volume of trypsin-EDTA (e.g. 2 ml for a 75 cm^2 culture flask). Transfer cell suspension to a 15 ml tube.

5.1.5 Rinse the flask by adding 10 ml of warm PBS. Add this volume to your 15 ml tubes containing cells from above point. Proceed immediately with step 5.1.7.

<u>For suspension cells:</u>

5.1.6 Collect suspension cells in a 50 ml tube and go directly to point 5.1.7 of the protocol.

5.1.7 Centrifuge for 5 mins at 500 *x g* at room temperature and remove the supernatant.

5.1.8 Resuspend the cells in 20 ml of warm PBS and count them. Collect the cells (approximately 7 million) by centrifugation for 5 mins at 500 *x g* at room temperature.

5.1.9 Resuspend the cells (approximately 7 million) in 500 µl of PBS (room temperature) and add 13.5 µl of 37% formaldehyde (under a fume hood) to each tube. Mix by gentle vortexing and incubate 8 mins at room temperature to allow fixation to take place.

Δ Note: The fixation time might require an additional optimization.

5.1.10 Add 57 µl of Glycine to the cells to stop the fixation. Incubate for 5 mins at room temperature with gentle shaking. Keep everything at 4°C or on ice from now on.

5.1.11 Collect the cells by centrifugation at 500 *x g* for 5 mins and 4°C. Discard the supernatant without disturbing the cell pellet.

5.1.12 Wash the cells twice with 1 ml of cold PBS: resuspend the cell pellet in 1 ml of cold PBS. Collect the cells by centrifugation at 500 *x g* for 5 mins and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

Δ Note: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.

5.2 Tissue disaggregation and DNA-protein cross-linking.

5.2.1 Weigh 30-40 mg of fresh or frozen tissue in a petri dish. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.

5.2.2 Chop tissue into small pieces (between 1-3 mm^3) using a scalpel blade.

5.2.3 Add 5 µl of protease inhibitors cocktail to 1 ml of ice-cold PBS and disaggregate the tissue using a dounce homogenizer (loose pestle) to get a homogeneous suspension.

5.2.4 Transfer the tissue suspension into a 1.5 ml tube and centrifuge at 850 *x g* for 5 mins at 4°C. Gently discard the supernatant and keep the pellet.

5.2.5 Add 27 µl of 37% formaldehyde (under a fume hood) to 1 ml of PBS (room temperature) and resuspend the tissue suspension in diluted formaldehyde.

5.2.6 Mix by vortexing and incubate for 8 mins at room temperature with gentle rotation on a rotator to allow fixation to take place.

Δ Note: The fixation time might require an additional optimization.

5.2.7 Add 100 µl of Glycine to the tissue suspension to stop the fixation. Incubate for 5 mins at room temperature with gentle mixing on a rotator.

5.2.8 Centrifuge samples at 850 *x g* for 5 mins at 4°C. Discard the supernatant without disturbing the pellet. Keep everything at 4°C or on ice from now on.

5.2.9 Wash the pellet twice with 1 ml of cold PBS: resuspend the cell pellet in 1 ml of cold PBS. Collect the cells by centrifugation at 850 *x g* for 5 mins and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

Δ Note: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.

5.3 Cell lysis and chromatin shearing for cells and tissues.

5.3.1 Add 1 ml of ice-cold Lysis Buffer L1 to the 1.5 ml tube containing cells or tissue suspension. Resuspend the samples by pipetting up and down several times and transfer them to a 15 ml tube. Add 6 ml of Lysis Buffer L1 and incubate for 10 mins at 4°C with gentle mixing.

5.3.2 Pellet samples by centrifugation at *500 x g* (cells) or 850 *x g* (tissue suspension) at 4°C for 5 mins and discard the supernatant.

5.3.3 Resuspend the cell pellet in 1 ml of ice-cold Lysis Buffer L2 by pipetting up and down several times. Add another 6 ml of Lysis Buffer L2 and incubate for 10 mins at 4°C with gentle mixing.

5.3.4 Pellet the cells again by centrifugation at 500 *x g* (cells) or 850 *x g* (tissue suspension) at 4°C for 5 mins and discard the supernatant.

5.3.5 Prepare complete Shearing Buffer by adding 3.5 µl of 200x protease inhibitor cocktail to 700 µl of Shearing Buffer S1. Keep on ice.

5.3.6 Add 700 µl of complete Shearing Buffer S1 to the cells (7 million cells) or tissue sample (30-40 mg of tissue). The final cell concentration in the Shearing Buffer should be 1 million of cells or ~5 mg tissue per 100 µl of S1. Resuspend by pipetting up and down several times. Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate sonication microtubes:

5.3.7 Incubate on ice for 10 mins.
Δ Note: The maximum volume for shearing is 300 µl per 1.5 ml Microtube. The use of correct type of microtubes is essential for the efficiency of sonication.

5.3.8 Shear the chromatin by sonication using a sonicator. Choose the protocol which is adapted to your device:
Δ Note: We recommend performing pilot experiments for each new sample type.

5.3.9 Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 *x g* at 4°C for 10 mins. Pool the supernatants which contain the sheared chromatin. Use the chromatin for

immunoprecipitation (Step 6.4 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months.

5.3.10 Take an aliquot of 50 µl of sheared chromatin for the shearing assessment. The protocol is described on our datasheet.
Δ Note: We recommend analyzing the shearing for each batch of chromatin. This step can be omitted when sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously. Store the chromatin aliquot at -20°C until analysis.

5.4 Magnetic immunoprecipitation for cells and tissues.

5.4.1 Determine the number of IP reactions to be run including the negative and positive control IPs. Take the required amount of Protein A-coated magnetic beads and transfer it to a clean 1.5 ml tube. 20 µl of beads are required per IP.

5.4.2 Dilute 300 µl of 5x ChIP Buffer C1 with 1.2 ml of ChIP-seq grade water to obtain 1x ChIP Buffer C1. Place the diluted ChIP Buffer C1 on ice.

5.4.3 Wash the beads 4 times with 320 µl of ice-cold 1x ChIP Buffer C1. To wash the beads, add 1x ChIP buffer C1, resuspend the beads by pipetting up and down several times and place the tubes in a magnetic rack. Wait for 1 minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times.

5.4.4 After the last wash, resuspend the beads in 1x ChIP Buffer C1 adding the original volume of beads (this means 20 µl per IP).

5.4.5 Set aside 1 µl of the sheared chromatin to use as INPUT sample starting from the step 6.5.1.45.

5.4.6 Prepare the ChIP reaction mix as described below for 1 IP. Scale accordingly to the number of IPs including a small excess (0.5 extra reaction):

- 6 µl of BSA
- 1.5 µl of 200x protease inhibitor cocktail
- 56 µl of 5x ChIP buffer C1
- 100 µl of sheared chromatin
- 20 µl of Protein A-coated magnetic beads
- (116.5 µl –x µl) ChIP-seq grade water
- Add x µl of ChIP-seq grade antibody x - amount of ChIP grade antibody. The total volume of the ChIP reaction mix per IP is 300 µl.

Δ Note: The required amount of antibody per IP varies. Check the supplier's recommendation or perform a titration curve using different amounts of antibody. Use 1 µg of IgG

(negative control antibody) for the negative control IP. If a positive control IP is included, use 1 µg of the H3K4me positive control antibody. If required, NaBu (20 mM final concentration) or other inhibitors can be added.

5.4.7 Incubate overnight at 4°C on a rotator.

5.4.8 Perform the washes as follows: briefly spin the tubes and place them in the magnetic rack. Wait for 1 minute and remove the supernatant. Add 350 µl of Wash Buffer W1: gently shake the tubes to resuspend the beads and incubate for 5 mins on the rotator at 4°C.

5.4.9 Repeat the washing step as described above once with Wash Buffer W2, W3 and W4, respectively.

5.5 Elution, decross-linking and DNA purification for cells and tissues.

- Before the first use of the kit, prepare Wash Buffer 1 and Wash Buffer 2 by adding an equal volume of isopropanol. Wash Buffers 1 and 2 should be stored at 4°C. Never leave the bottle open during storage to avoid evaporation.

5.5.1 After removing the last wash buffer, add 100 µl of Elution Buffer E1 to the beads, resuspend the beads pellet and incubate for 30 mins on the rotator at room temperature.
Δ Note: If a precipitation is observed in Elution Buffer E1, warm it at 37°C until it becomes clear. This will not impair the reaction.

5.5.2 Briefly spin the tubes and place them into the magnetic rack. Wait for 1 minute, transfer the supernatant to a new 1.5 ml tube and add 4 µl of Elution Buffer E2. At the same time, add 99 µl of Elution Buffer E1 and 4 µl of Elution Buffer E2 to the 1 µl INPUT sample. Incubate for 4 hours or overnight at 65°C with shaking.
Δ Note: If desired, include a chromatin sample for shearing assessment at this step. Perform decross-linking and DNA purification in parallel with the IP and input samples. Please follow the instructions described in our datasheet

5.5.3 Add 2 µl of carrier to each IP and INPUT sample.

5.5.4 Add 100 µl of 100% isopropanol to each IP and INPUT sample.
Δ Note: Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.

5.5.5 Resuspend the Purification Beads by vortexing and transfer 10 µl to each IP and INPUT sample.

5.5.6 Incubate the IP and INPUT samples for 10 mins at room temperature on the rotator.

5.5.7 Briefly spin the tubes, place in the magnetic rack, wait 1 minute and discard the buffer.

5.5.8 Add 100 µl of Wash Buffer 1 per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes, place in the magnetic rack, wait 1 minute and discard the buffer.

5.5.9 Add 100 µl of Wash Buffer 2 per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes and place them into the magnetic rack, wait 1 minute and discard the buffer.

5.5.10 Spin the tubes again and place them on the magnetic rack. Discard the remaining Wash Buffer 2 if necessary. Resuspend the beads pellet in 25 µl of Buffer C. Incubate at room temperature for 15 mins on the rotator.

5.5.11 Spin the tubes and place them into the magnetic rack, wait 1 minute and transfer the supernatant containing the immunoprecipitated DNA into a new 1.5 ml tubes. Discard the beads.

5.5.12 Determine the total number of regions to be analyzed by qPCR for each sample. Take the required volume of Input and immunoprecipitated samples for qPCR analysis. Take into account that these samples will be diluted 1/10 and 5 µl will be used per PCR.

Δ Note: The dilution of samples and the volume per PCR may vary depending on a sensitivity of a master mix and qPCR cycler used.

5.5.13 Store the remaining DNA at -20°C until further use.

5.6 Quantitative PCR analysis.

- For each primer pair, run the Input DNA alongside the immunoprecipitated samples and negative IgG control.

5.6.1 Take an aliquot of immunoprecipitated DNA and a corresponding INPUT and dilute them 1/10 using ChIP-seq grade water.

5.6.2 Prepare the qPCR mix as follows (20 µl reaction volume using the provided control primer pairs):

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer pair
- 4 µl of water
- 5 µl of diluted immunoprecipitated or INPUT DNA

Use the following PCR program:

Step	Time/cycles		Temperature
Denaturation	3-10 mins*		95°C
Amplification	30 secs	40 cycles	95°C
	30 secs		60°C
	30 secs		72°C (acquire fluorescent data)
Melting Curve**	Follow qPCR instrument manufacturer recommendations		

*Carefully check supplier's recommendations about Taq polymerase activation time

**Include and inspect the melting curves based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single specific product

Δ Note: These conditions may require optimization depending on the type of master mix, qPCR system used and user provided primer pair.

5.6.3 Record the threshold cycles (Ct values) from the exponential phase of the qPCR for the immunoprecipitated DNA sample and input for each primer pair.

5.6.4 Calculate the relative amount of immunoprecipitated DNA compared to INPUT DNA for the control regions (% of recovery) using the following formula:

% recovery = 2^((Ctinput – 6.64) - Ctsample)x100%

• Ctsample and Ctinput are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and INPUT, respectively.
• 2 is the amplification efficiency
• 6.64 is a compensatory factor to correct the input dilution
Δ Note: This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results, the amplification efficiency with given primer pair has to be close to 100% meaning that for each cycle the amount of product is doubles (E=2). The real amplification efficiency, if known, should be used.
The formula takes into account that 1% of INPUT was used as suggested in the protocol (1 µl INPUT vs 100 µl of chromatin per IP). If the amount of INPUT used is different from 1%, an introduction of a compensatory factor in the formula is required to correct the input dilution (x) as follows:

%recovery = 2^[(Ctinput-log2(X%)) - Ctsample]x100%

Where: log2(x) accounts for the INPUT dilution
Example: If you use an INPUT of 5 µl from 250 µl of chromatin used per IP, it corresponds to 50 X dilution. The compensatory factor is equal to log2(50)=5.64 and the formula to calculate the recovery will be as follows:

%recovery = 2^[(Ctinput-5.64) - Ctsample]x100%

5.6.5 If an expected enrichment over positive loci with a low background is observed, proceed to the library preparation using an appropriate protocol (not supplied with this kit).

6. Notes

Technical Support

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